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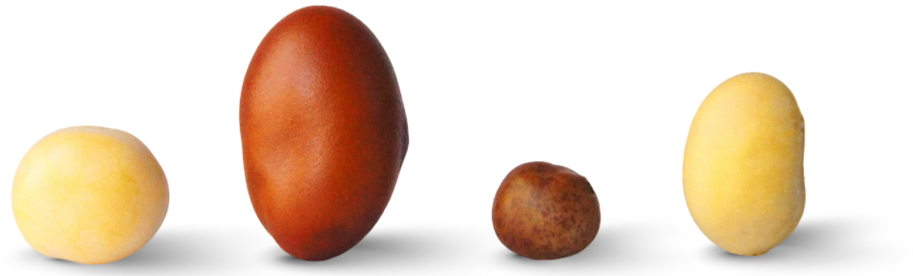
Beyond the Label

The effect of processing on the structure and digestibility of plant-derived foods

Jaqueline Auer



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The effect of processing on the structure and digestibility of plant-derived foods

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Cover: Photograph showing four pulses: yellow pea (*Pisum sativum* L.), faba bean (*Vicia faba* L.), grey pea (*Pisum sativum* L., variety), and soy (*Glycine max* (L.) Merr.). Photograph and image processing by Jaqueline Auer, 2025.

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Beyond the Label – The effect of processing on the structure and digestibility of plant-derived foods

Abstract

The increasing demand for plant-based food reflects growing concerns about environmental sustainability and public health. Compared to animal-based products, foods made from plant proteins typically have a lower protein quality and reduced bioavailability of minerals (e.g., iron and zinc). This is due to the presence of antinutritional factors (e.g., phytate) and structural features that affect nutrient release and uptake in the body.

This thesis investigates how various processing methods influence the structure, breakdown, and nutrient release of plant-based protein sources, focusing on soy, faba bean, yellow pea, and grey pea. Static *in vitro* digestion was used to simulate the nutrient release in the gastrointestinal tract, combined with cell uptake studies to evaluate protein quality and mineral bioavailability.

The results showed that processing methods such as fermentation, protein coagulation, and enzymatic crosslinking significantly influence protein breakdown and nutrient uptake by altering the food structure and matrix. Fermentation reduced the phytate content in tempeh, thereby enhancing mineral bioavailability. Protein coagulation markedly increased protein hydrolysis, likely due to the lower fibre content and porous gel structure. In contrast, emulsion gels made from pea protein, which contain higher fibre and possess a stronger gel network, limit enzymatic access and reduce protein breakdown.

The findings indicate a risk of reduced nutritional value of the protein sources evaluated in the thesis due to the presence of phytate and the overall structural complexity. However, the results also highlight the potential of targeted food processing strategies to enhance the nutritional quality of plant-based foods.

Keywords: Mineral bioavailability; Food structure; Phytate; Faba bean; Pea; Soy; Iron uptake

Beyond the Label – Effekter av processer på struktur och digererbarhet hos växtbaserade livsmedel

Abstract

Den ökade efterfrågan på växtbaserad mat speglar ett växande intresse för en hållbar miljö och folkhälsa. Jämfört med animaliska produkter har livsmedel baserade på växtproteiner inte samma proteinkvalitet och oftast en lägre biotillgänglighet av viktiga mineraler som järn och zink. Detta beror på förekomsten av antinutrienter och strukturella faktorer som påverkar upptaget i kroppen. I avhandlingen studerades hur olika bearbetningsmetoder av växtbaserade proteinkällor påverkar struktur, nedbrytning och näringsfrisättning, med fokus på soja, åkerböna, gulärt och gråärt.

En statisk *in vitro*-metod användes för simulering av nedbrytning i mag-tarmkanalen och kombinerades med studier av cellulärt upptag för utvärdering av proteinkvalitet och biotillgänglighet. Resultaten visade att bearbetningsmetoder som fermentering, proteinkoagulering och enzymatisk tvärbindning kan påverka proteinnedbrytning och näringsupptag avsevärt. Fermentering minskade fytatinnehållet i tempeh och förbättrade därmed mineralbiotillgängligheten. Protein koagulering vid tillverkning av tofu-liknande produkter ökade proteinhydrolysen markant, troligen p.g.a. mindre mängd fiber och mer porös struktur.

Emulsionsgeler baserade på ärtproteinkoncentrat visade att starkare gelnätverk och högre fiberinnehåll begränsade enzymatisk åtkomst och därigenom minskade proteinnedbrytningen. Denna trend observerades även i traditionellt bearbetade livsmedel som tofu och tempeh. Förekomsten av kostfibrer och kolhydrater begränsade enzymaktiviteten och näringsfrisättningen, vilket tyder på att de fungerar som fysiska och kemiska barriärer under matspjälkningen.

Sammanfattningsvis visar resultaten att närvaro av fytat och den strukturella komplexiteten reducerar näringsupptaget. Resultaten belyser dock en möjlig förbättringspotential genom riktade strategier under livsmedelsbearbetningen, för att förbättra den näringsmässiga kvaliteten hos växtbaserade livsmedel. Denna avhandling stöder behovet av ökad kunskap för fortsatt utveckling av hälsosamma och hållbara växtbaserade produkter.

Nyckelord: Mineralbiotillgänglighet; Livsmedelsstruktur; Fytat; Åkerböna; Ärta; Soja; Järnupptag

Dedication

To my family – the one I was born into, and the one I found along the way.

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Jaqueline Auer; Marie Alminger, Marina Marinea, Mathias Johansson, Galia Zamaratskaia, Anders Högberg, Maud Langton (2024). Assessing the digestibility and estimated bioavailability/ bioaccessibility of plant-based proteins and minerals from soy, pea, and faba bean ingredients. LWT 197 (115893).

II. Jaqueline Auer, Hanna Eriksson Röhnisch, Sarah Heupl, Marina Marinea, Mathias Johansson, Marie Alminger, Galia Zamaratskaia, Anders Högberg, Maud Langton (2025). The effect of transglutaminase and ultrasound pre-treatment on the structure and digestibility of pea protein emulsion gels. Food Hydrocolloids 169 (111620)

III. Jaqueline Auer, Loes Duivenvoorde, Meike van der Zande, Marie Alminger, Laura Alejandra Fernandez Castañeda, Jing Lu, Giovanni Tizzanini, Galia Zamaratskaia; Anders Högberg, Maud Langton. Effect of processing on the protein digestibility and mineral bioavailability of legumes using *in vitro* digestion and a Caco-2/HT29-MTX co-culture model. (Submitted)

The contribution of Jaqueline Auer to the papers included in this thesis was as follows:

I. Performed the majority of the experiments (excluding starch, fat, and fibre analysis), evaluated the results, and wrote the manuscript.

II. Designed the study together with the co-authors, performed the majority of the experiments (excluding fibre analysis, CT measurements/ image analysis, and NMR analysis, including related statistical evaluation), evaluated the results, and wrote the manuscript.

III. Designed the study together with the co-authors, performed the majority of the experiments (excluding lipid analysis and sugar analysis), evaluated the results, and wrote the manuscript.

The following papers were submitted or published during the timeframe of the doctoral project, but are not part of this thesis.

I. Jaqueline Auer, Johanna Östlund, Klara Nilsson, Mathias Johansson, Anja Herneke, Maud Langton (2023). Nordic crops as alternatives to soy—an overview of nutritional, sensory, and functional properties. *Foods* 12 (2607)

II. Laura Alejandra Fernandez Castañeda, Jaqueline Auer, Su-lin L. Leong, William R. Newson, Volkmar Passoth, Maud Langton, Galia Zamaratskaia (2024). Optimizing soaking and boiling time in the development of tempeh-like products from faba bean. *Fermentation* 10 (0407)

III. Ansung Kim, Jaqueline Auer Sarah Heupl, Mihaela Mihnea, Åsa Öström, Jun Niimi, Maud Langton. Consumer preferences of plant-based mince meat analogs: linking physico-chemical properties, structural features, and sensory attributes. (submitted).

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Abbreviations

GHG	Greenhouse gas
EPA	Eicosapentaenoic acid
DHA	Docosahexaenoic acid
EU	European Union
HO-1	Heme oxygenase
Dcytb	Duodenal cytochrome b
DMT1	Divalent metal transporter 1
NSPs	Non-starch polysaccharides
IP	Inositol phosphates
G'	Storage modulus
G"	Loss modulus
OPA	o-phthaldialdehyde
Phy:Fe	Molar ratio of phytate to iron
Phy:Zn	Molar ratio of phytate to zinc
Caco-2	Human epithelial colon adenocarcinoma cell line
HT29	Mucus-producing goblet cells
ANOVA	Analysis of variance
EAAs	Essential amino acids
HIS	Histidine
ILE	Isoleucine
LEU	Leucine
LYS	Lysine
PHE	Phenylalanine
THR	Threonine
VAL	Valine
ALA	Alanine
ARG	Arginine
ASP	Aspartic acid
GLU	Glutamic acid
GLY	Glycine
PRO	Proline
SER	Serine
TYR	Tyrosine

DH	Degree of hydrolysis
PI	Pea isolate
PC	Pea concentrate
US	Ultrasound
TG	Transglutaminase
NT	No treatment (Control)
PCA	Principal Component Analysis
NMR	Nuclear Magnetic Resonance
SY	Soy bean
GP	Grey pea
YP	Yellow pea
FB	Faba bean
Phe	Phytate (IP6)
DM	Dry matter

1. Introduction

Plant-derived proteins are often a more sustainable alternative to animal proteins due to their lower environmental impact. Indeed, livestock production accounts for approximately 57% of food-related greenhouse gas (GHG) emissions (including livestock feed), whereas plant protein sources produce significantly less GHG emissions (29%) and require less land and water usage (Crippa et al., 2021; Kustar & Patino-Echeverri, 2021; Xu et al., 2021).

Beyond sustainability, shifting toward a more plant-based diet also offers various notable health benefits (Hertzler et al., 2020). In general, individuals that follow a plant-based diet tend to receive higher intakes of polyunsaturated fatty acids and fibre, along with other nutrients such as α-linolenic acid, folate, vitamin E, and magnesium, nutrients that are frequently found to be at risk of suboptimal supply among omnivores (Neufingerl & Eilander, 2023). Moreover, unlike red meat, plant-derived proteins are low in saturated fat and are cholesterol-free. Thus, replacing animal-derived proteins with plant-based options has been linked to a reduced risk of cardiovascular disease, diabetes, and certain cancers (Manickavasagan & Amanat, 2022; Naghshi et al., 2020; Sun et al., 2021). In line with this, the Nordic Nutrition Recommendations advocate for greater consumption of plant-based foods, notably fruits, vegetables, legumes, whole grains, nuts, and seeds (Nordic Council of Ministers, 2023).

However, despite their overall benefits, plant-based diets are often associated with an increased risk of deficiencies in vitamin B12, vitamin D, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), calcium, iodine, iron, and zinc. This is particularly concerning given that iron deficiency is the most common nutritional deficiency worldwide, affecting 42% of children under five, 40% of pregnant women, and 33% of non-pregnant women (Pasricha et al., 2021; WHO, 2025). Zinc deficiency is also a global issue, impacting an estimated 17% of the population, including vulnerable groups such as infants, pregnant women, and the elderly (Wessells & Brown, 2012; Yokokawa et al., 2024).

In addition, total protein intake is generally lower among vegetarian and vegan diets compared to omnivores, although it usually remains within the lower threshold of the acceptable macronutrient distribution range (Neufingerl & Eilander, 2023). However, beyond quantity, plant proteins

differ from animal proteins in overall structure and amino acid composition, which often leads to lower protein quality (Herreman et al., 2020; Hertzler et al., 2020).

However, these limitations in protein quality and mineral bioavailability are often not evident to consumers. Ingredient lists and nutrition labels typically lack information about amino acid profiles or the bioavailability of key nutrients such as iron and zinc. As a result, consumers may assume that all protein sources and foods naturally high in iron or zinc offer the same nutritional value, which can lead to unintended deficiencies, especially among those who are heavily reliant on plant-based diets. Bridging this knowledge gap is essential to support consumers in making informed dietary choices and to guide the development of plant-based products that simultaneously promote health and sustainability.

Therefore, this thesis aims to explore the nutritional and functional properties of proteins derived from soy, faba bean, yellow pea, and grey pea, including their commercial extracts such as isolates, concentrates, and flours. A particular focus is placed on understanding how food structure and the overall food matrix affect protein digestibility and mineral bioavailability.

2. Background

Within the European Union (EU), soy (*Glycine max* (L.) Merr.), faba bean (*Vicia faba* L.), and pea (*Pisum sativum* L.) are among the most widely cultivated legumes (van Loon et al., 2023) with domestic soybean production reaching approximately 2.9 million tonnes in 2023 (Eurostat, 2024). However, the EU remains heavily reliant on imports, with around 14 million tonnes of soy received annually, of which over 95% is used for animal feed (van Loon et al., 2023).

Although soybean cultivation in Europe is limited to warmer regions due to the longer growing season, faba beans and peas are cool-season crops well-suited to colder climates, including Scandinavia (Sepngang et al., 2020). Nevertheless, pea and faba bean production together occupy about only 1% of EU cropland. This highlights a significant opportunity for the expansion of domestic legume cultivation, both for animal feed and, more importantly, human consumption (van Loon et al., 2023; Zander et al., 2016).

Across the EU, the majority of dietary protein intake comes from animal sources, making up approximately 60% (EPRS, 2024). To promote a shift toward more plant-based foods, the development of nutritious products with appealing textures and flavours is crucial (He et al., 2020). The plant-based food market continues to expand beyond traditional options such as tofu and tempeh, typically made from whole soybeans, and now offers alternatives such as plant-based milk, yogurt, cheese, and meat analogues derived from a variety of plant sources (McClements & Grossmann, 2021).

These more modern plant-based foods often rely on protein extracts such as concentrates and isolates, combined with various processing methods, to achieve a desirable texture and an enhanced protein content (Liu et al., 2020; Nishinari et al., 2018). Concentrates, typically produced through air classification, result in a protein content > 65%, whilst isolates, commonly obtained via wet chemical extraction, yield a protein content > 90% (Boye et al., 2010).

To optimise the applicability of plant-derived proteins in various food applications and improve their nutritional value, it is crucial to gain insight into how different plant-derived proteins and their commercial extracts behave during processing and digestion.

2.1 Digestion and uptake of proteins and minerals

Proteins are essential macronutrients involved in numerous body functions, including growth, tissue repair, maintenance of cells, and the synthesis of hormones and neurotransmitters (Day et al., 2022). Proteins are composed of 20 different amino acids, nine of which cannot be synthesised by the human body and therefore must be obtained through the diet (Manickavasagan & Amanat, 2022). The amino acid composition and the digestibility of proteins can vary significantly between sources, and plant-based proteins are often considered to be lower in quality than most animal-derived proteins (Damodaran, 2017). Depending on the physiological requirements, tryptophan, lysine, and phenylalanine are considered to be limiting essential amino acids (EAAs) in plant-derived proteins for infants. For children (aged > 3 years), adolescents, and adults the most limiting EAAs in plant protein sources are lysine in cereals, methionine and cysteine in legumes, and histidine in potatoes. Therefore, aside from soy, which is considered a complete protein, most plant-derived proteins, including faba bean and pea protein, must be combined with other protein sources to achieve a complete amino acid profile (Herreman et al., 2020; Mariotti, 2017).

In addition to proteins, *minerals* such as iron and zinc are vital for human health. They play essential roles in oxygen and electron transport, cell division and differentiation, and the regulation of gene expression (Piskin et al., 2022). The bioaccessibility and bioavailability of both iron and zinc depend on numerous factors but they are generally higher in animal-derived foods compared to plant-based sources (Lim et al., 2013). Thereby, *bioavailability* refers to the proportion of a compound that is absorbed by intestinal cells and reaches the target tissues in an intact or metabolised form, whereas *bioaccessibility* refers to the proportion of a compound that is released from the food matrix during digestion and is accessible for absorption (Rodrigues et al., 2022).

2.1.1 Protein digestion and intestinal uptake

According to the Nordic Nutritional Recommendation, the recommended daily protein intake for adults is 0.83 grams per kilogram of body weight (Nordic Council of Ministers, 2023). Once consumed, protein digestion commences in the stomach, where the food bolus is mixed with gastric juice and pepsin. Pepsin breaks down proteins into smaller peptides by cleaving peptide bonds between amino acids (Fuller & Tomé, 2005; Loveday, 2022;

Sensoy, 2021). Thus, pepsin can partially digest 10–15% of the dietary protein (Goodman, 2010).

From the stomach, partially digested proteins move into the small intestine, where they are further broken down by pancreatic enzymes such as trypsin, chymotrypsin, and elastase (endopeptidases). The resulting oligopeptides are then additionally cleaved by carboxypeptidase A and B and aminopeptidases (exopeptidases), resulting in approximately 30% free amino acids and 70% oligopeptides (Fuller & Tomé, 2005; Goodman, 2010; Sensoy, 2021).

The uptake of amino acids and peptides is facilitated by specific transporters located on the apical sides of enterocytes (Ganapathy, 2012; Goodman, 2010). Overall, around 80% of all amino acids are taken up into the enterocyte as di- and tripeptides through a proton-dependent transporter, whilst individual amino acids are taken up through a variety of amino acid transporters (Ganapathy, 2012; Goodman, 2010).

Upon entering the enterocyte amino acids can either be temporarily stored or transported across the basolateral membrane into the bloodstream, where they can then be distributed to various tissues. Dipeptides and oligopeptides that are absorbed into enterocytes are typically further hydrolysed by intracellular peptidases into individual amino acids before being transported into the bloodstream (Ganapathy, 2012).

2.1.2 Iron bioaccessibility and intestinal uptake

The recommended daily intake of iron is 15 mg for women and 9 mg for men (Nordic Council of Ministers, 2023). Dietary iron is present in two primary forms: heme iron, which is found in animal-based sources, and non-heme iron, which is found in both plant-based and animal-based foods. The bioavailability of heme iron is generally around 25-35%, a significantly higher amount compared to non-heme iron, which typically has a bioavailability of around 2-9% (Ems et al., 2024; Hurrell & Egli, 2010; Piskin et al., 2022).

During digestion, iron is released from the food matrix. The acidic environment (pH ~1.5–3.5) in the stomach, along with the presence of ascorbic acid (vitamin C), promotes the solubilisation and conversion of Fe^{3+} to Fe^{2+}, enhancing its bioavailability (Piskin et al., 2022).

Once reaching the small intestine, brush border enzymes and/ or ascorbic acid continue the reduction of Fe^{3+} to Fe^{2+}, before it can be transported into

enterocytes. Within the enterocytes, iron can either be stored as ferritin or transported into the bloodstream, where it binds to transferrin for systemic distribution to tissues (Correnti et al., 2024; Zimmermann & Hurrell, 2007).

Dietary heme iron, on the other hand, is absorbed directly into intestinal cells, where it is degraded to Fe^{2+}, which enters the same intracellular pathway as non-heme iron (Correnti et al., 2024). An overview of the different uptake processes is presented in Figure 1.

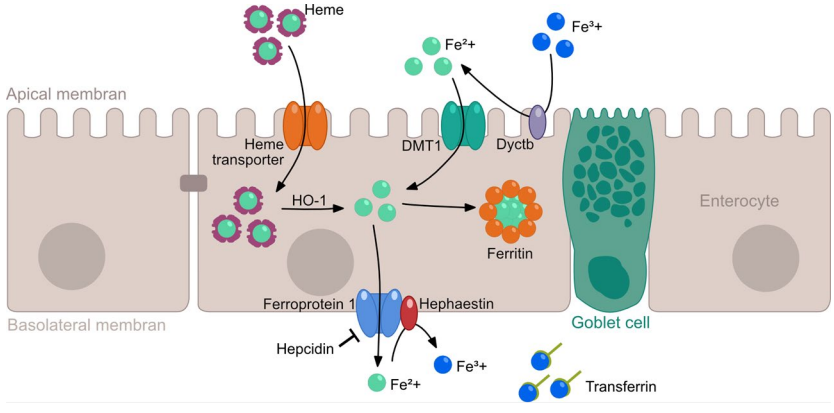

Figure 1. Mechanisms of intestinal iron uptake and transport adapted from Correnti et al., (2024). Heme iron is transported into enterocytes via a heme transporter and degraded by heme oxygenase (HO-1), releasing Fe^{2+}. Non-heme iron (Fe^{3+}) is reduced to Fe^{2+} by duodenal cytochrome b (Dcytb) and transported into the enterocyte via divalent metal transporter 1 (DMT1). Fe^{2+} may be stored in ferritin or exported by ferroportin 1. Hephaestin facilitates the oxidation of Fe^{2+} to Fe^{3+} before binding to transferrin in the bloodstream. Hepcidin can bind to ferroportin 1, inhibiting iron export. Goblet cells, involved in mucus secretion, are also shown.

Iron absorption is therefore tightly regulated by the body's internal iron status (Piskin et al., 2022). This regulation is primarily controlled by hepcidin, a liver-produced hormone that regulates iron homeostasis. When iron levels in the body are sufficient or high, hepcidin levels increase, reducing dietary iron absorption by inhibiting ferroportin 1. Conversely, when the body is low in iron, hepcidin levels decrease, allowing for greater iron absorption (Correnti et al., 2024; Zimmermann & Hurrell, 2007).

Additionally, physiological and individual factors, including age, metabolic demands, iron stores (ferritin levels), inflammation, or the presence of antinutrients (e.g., phytate), can influence iron absorption

(Correnti et al., 2024; FAO/WHO/UNU, 2007; Hurrell & Egli, 2010; Zimmermann & Hurrell, 2007)

2.1.3 Zinc bioaccessibility and intestinal uptake

The recommended daily intake of zinc is 9.7 mg for women and 12.7 mg for men (Nordic Council of Ministers, 2023). Similar to iron, dietary zinc primarily exists in two main forms: as organic complexes bound to proteins in meats and as inorganic salts in plant-based foods. Whilst the differences in bioavailability between these forms are not yet fully understood, it is widely accepted that as for iron, zinc from animal-derived sources is more bioavailable than zinc from plant-based foods (Lim et al., 2013).

During digestion, zinc is partially released from the food matrix, predominantly due to the stomach's acidic environment (Efsa, 2014; Reddy et al., 1982).

Once reaching the small intestine, zinc is taken up on the enterocyte membranes, where it enters the cells as Zn^{2+}. Inside the enterocyte, zinc can bind to proteins, be stored in vesicles, remain free in the cytoplasm, or be exported into the bloodstream. In addition, zinc transporters at the surface of apical and basolateral side cells can transport zinc into the cell or back out into the gut/ blood stream, which helps to maintain internal balance and respond to changes in zinc availability (Maares & Haase, 2020).

Similar to iron, zinc absorption is tightly regulated by the body's zinc and physiologic states, the amount of zinc present in the intestinal lumen as well as the presence of dietary promoters or inhibitors (Krebs, 2000).

2.2 Dietary factors influencing nutrient digestion and uptake

Beyond physiological regulation, the digestibility and absorption of proteins largely depend on the extent to which proteases in the gastric and intestinal environments can access proteins and efficiently hydrolyse peptide bonds. This enzymatic hydrolysis is significantly influenced by the intrinsic structure of plant proteins as well as the structural organisation of plant tissues. Moreover, the presence of antinutritional compounds can further reduce protein digestibility and mineral bioaccessibility, ultimately compromising the nutritional quality of plant-based foods (Herreman et al., 2020; Loveday, 2022; Nyemb et al., 2016; Sá et al., 2019).

2.2.1 Plant-protein structure

In plants, particularly legumes, proteins are predominantly stored in the cotyledon, as illustrated in Figure 2. The cotyledon consists of a continuous, interconnected network of cell walls that surround and protect individual plant cells. These cell walls are composed of polysaccharides that are unable to be hydrolysed by human digestive enzymes and therefore function as dietary fibre, promoting regular bowel movements and supporting a healthy gut microbiota. Starch and lipids, which are also stored within the cotyledon, serve as an important energy source and can provide essential fatty acids (Lunn & Theobald, 2006; Martineau-Côté et al., 2022; Muzquiz et al., 2012).

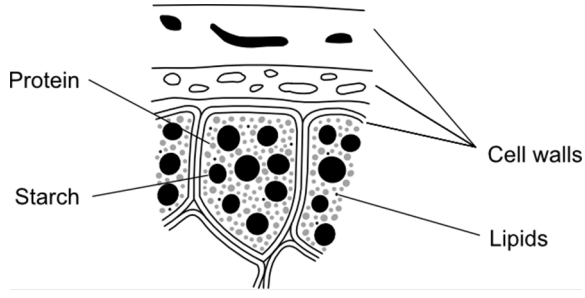

Figure 2. Schematic organisation and distribution of major nutrients in the cotyledon of legume seeds, adapted from Bach Knudsen, (2001); Kornet et al., (2020).

The structural integrity of plant cells plays a crucial role in overall nutrient digestion. When cellular integrity is preserved, macronutrients remain encapsulated within the cells, limiting their exposure to digestive enzymes and bile acids, consequently reducing their digestibility (Capuano & Janssen, 2021; Ezeogu et al., 2008; Rovalino-Córdova et al., 2019; Wong et al., 2009). As a result, plant-derived protein tends to have higher digestibility in more refined products, such as flour, protein concentrates, and isolates, compared to whole legumes, where the plant cell walls are intact (FAO/WHO/UNU, 2007).

Alongside physical barriers, the molecular structure of plant proteins further contributes to their limited digestibility. Many plant proteins exhibit compact, tightly folded conformations predominantly stabilised by hydrophobic interactions, and, in some cases, by disulfide bonds. These structural features, along with the presence of antinutritional factors, can hinder enzymatic hydrolysis, making plant proteins generally more resistant to digestion than their animal-derived counterparts (Santos-Hernández et al.,

2020; Yu, 2005). Thus, both the localisation of proteins within plant tissues and their intrinsic structural properties play vital roles in determining their nutritional availability.

2.2.2 Antinutritional factors

The nutritional quality of plant proteins is further influenced by the presence of antinutrients. Compounds such as protease inhibitors (e.g., trypsin inhibitors in legumes), polyphenols, haemagglutinins (lectins), and saponins have been shown to adversely affect protein and amino acid digestibility, reducing overall nutritional value (Rahate et al., 2021; Sá et al., 2019).

Moreover, dietary fibres can further limit protein digestion through several mechanisms (Karim et al., 2024; Zhang et al., 2024). Insoluble dietary fibres primarily act by physically encapsulating proteins within the plant cell wall matrix, thus hindering enzyme access. In contrast, different soluble dietary fibres can modify the physicochemical properties of the digestive environment by increasing bolus viscosity, slowing the diffusion of digestive enzymes, and delaying gastric emptying, all of which reduce protein hydrolysis and amino acid absorption (Grundy et al., 2016; Karim et al., 2024; Zhang et al., 2024).

In addition, the presence of other antinutritional factors, such as phytates, can further reduce nutritional quality by impairing both protein digestibility and the bioavailability of essential minerals such as iron and zinc, thereby contributing to micronutrient deficiencies (Dahdouh et al., 2019; Hunt et al., 2008)

2.2.3 Role of phytate on mineral bioavailability

Phytate naturally occurs in many plant-based foods, particularly grains, legumes, nuts, and seeds, where it serves as the primary phosphorus and energy storage compound for the plant (Angel et al., 2002; Reddy et al., 1982; Zhang et al., 2022).

Phytate (IP6) consists of an inositol ring fully phosphorylated with six phosphate groups (Figure 3), providing 12 reactive (proton-releasing) sites (Sarkhel & Roy, 2022). These sites vary in acidity: six are strongly acidic (pK 1.5–2.0), two are weakly acidic (pK ~6.0), and four are very weakly acidic (pK 9.0–11.0) (Angel et al., 2002; Reddy et al., 1982). Therefore, as phytate loses protons, its phosphate groups become negatively charged, enabling them to bind positively charged minerals such as iron or zinc.

Binding can commence in the stomach, where the low pH activates the strongly acidic sites, but the most significant mineral complexation occurs in the small intestine (pH ~6–7.5), where additional sites become deprotonated. This leads to the formation of insoluble phytate-mineral complexes at neutral pH in the small intestine that hinder mineral absorption.

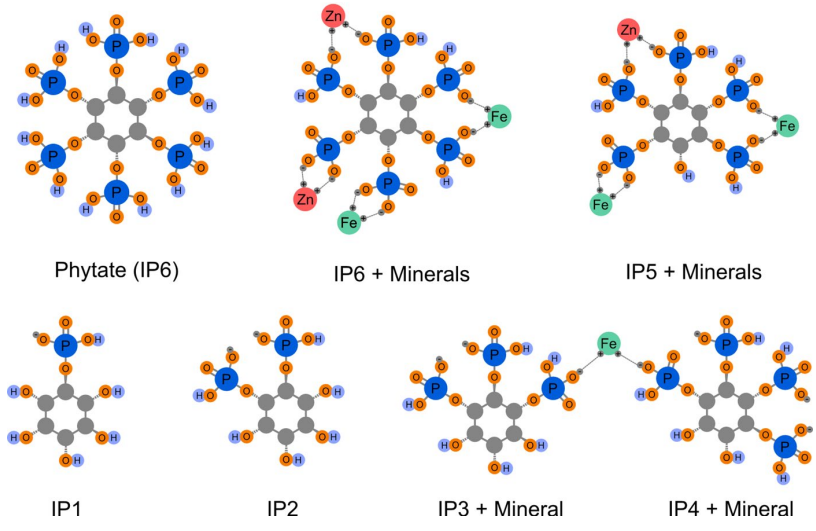

Figure 3. Phytate (IP6) structure and its mineral binding capacity, illustrating IP6 and IP5 which can directly bind minerals, IP4 and IP3 which can bind minerals between different isomers (Sandberg et al., 1999) as well as the isomers IP2 and IP1 (Reddy et al., 1982; Sarkhel & Roy, 2022)

When two or more phosphate groups are removed from IP6, the mineral-binding capacity is significantly reduced. This reduction has been shown to enhance the absorption of minerals such as iron and zinc, thereby improving overall nutrient availability (Lönnerdal et al., 1989; Sandberg et al., 1999)

In addition to minerals, IP6 can also form complexes with other food components, such as proteins and starches. Protein binding occurs either through electrostatic interactions at low pH (pH 3) or via salt bridge formation at higher pH levels. Starch binding, on the other hand, takes place through the formation of hydrogen bonds at pH <1.5. These interactions can also influence the digestibility and nutritional availability of proteins and carbohydrates (Angel et al., 2002; Prattley et al., 1982; Sarkhel & Roy, 2022). However, their impact on nutrient absorption is generally less

pronounced compared to their more substantial effects on iron and zinc bioavailability.

2.2.4 Processing to enhance nutritional properties

To improve the nutritional properties of plant-derived foods, various processing methods can be applied. The most commonly used techniques are dehulling, soaking, germination, fermentation, and various forms of heat treatment (Chitra et al., 1996; Nosworthy et al., 2018; Sarkhel & Roy, 2022).

To improve *mineral bioavailability*, phytate must be broken down into lower inositol phosphate forms. This can be achieved with the addition of exogenous phytase (Fredrikson, Biot, et al., 2001; Wang & Guo, 2021) or through the activation of endogenous phytase (Eklund-Jonsson et al., 2008; Fredrikson, Alminger, et al., 2001; Rousseau et al., 2020), often during soaking, germination, and fermentation. Among these, fermentation, such as that used in traditional tempeh production, has demonstrated considerable potential for reducing phytate content. This is due to the production of extracellular proteases and phytases, which effectively lower phytate levels and, in turn, enhance both mineral bioavailability and protein digestibility in legumes and grains (Eklund-Jonsson et al., 2008; Erkan et al., 2020; Suparmo & Markakis, 1987).

Cooking, dehulling, soaking, and germination can also enhance *protein digestibility* (Chitra et al., 1996; Sá et al., 2019). Cooking and other forms of heat treatments are particularly effective in deactivating antinutritional factors, including protease inhibitors and lectins, which interfere with protein digestion (Muzquiz et al., 2012). Moreover, thermal treatments can denature proteins, thereby increasing enzymatic hydrolysis (Sá et al., 2019). Dehulling, however, reduces the content of insoluble dietary fibre which enhances protein digestibility by minimising the physical barriers to enzyme access (Chitra et al., 1996; Mattila et al., 2018). Similarly, soaking and germination improve digestibility by altering the structural integrity of the food matrix, facilitating a more efficient enzymatic breakdown during digestion (Ibarruri & Hernández, 2018; Purwandari et al., 2024).

2.3 Structural and functional aspects of plant-derived proteins

Whilst the nutritional properties of proteins determine their impact on human health, the functional properties of proteins influence their applicability within diverse food processing, contributing to desirable textures in many food products. These functional properties are largely determined by the *protein's structure*. Soy, faba bean, and pea proteins primarily consist of globulins (see Table 1). These proteins generally contain lower levels of sulphur-containing amino acids, such as cysteine and methionine, which reduces the formation of disulfide bonds during heating and affects protein structure, stability, and gel formation properties (Baune et al., 2021; Martineau-Côté et al., 2022).

Table 1. Overview of the different storage proteins present in soy, faba bean, and yellow pea (El Fiel et al., 2002; Gu et al., 2020; Lam, Karaca, et al., 2018; Nicolai & Chassenieux, 2019).

Protein fraction	Soy bean	Faba bean	Yellow pea
Globulins	~70–90%	~65–80%	~70–80%
7S (vicilin-type)	β-conglycinin	Vicilin	Vicilin
11S (legumin-type)	Glycinin	Legumin	Legumin
Albumins	-	~2%	~10–20%
Prolamins	-	<5%	<5%
Glutelins	-	~10–15%	~5–10%

Additionally, plant globulins typically denature at relatively high temperatures (80 °C to 95 °C), which can further reduce their functional properties (Liu et al., 2022; Manickavasagan & Amanat, 2022; Shand et al., 2007; Sun & Arntfield, 2012).

In regard to protein extracts, e.g., protein isolates and concentrates derived from different plants, the *extraction method* can significantly influence their composition and functional properties (Chigwedere et al., 2023; Cui et al., 2020; Langton et al., 2020; Shand et al., 2007). Considerable variation exists between isolates derived from different extraction protocols (Stone et al., 2015; Vogelsang-O'Dwyer et al., 2020) as well as those produced on an industrial scale (Taherian et al., 2011). Whilst flours and concentrates are often obtained through milling and dry fractionation

(Pelgrom et al., 2013), wet fractionation is commonly used for the production of isolates with a high protein content (Cui et al., 2020; Lam, Can Karaca, et al., 2018). This difference in processing not only affects the final protein content but can also lead to alterations in the overall protein structure. In contrast, isolates are often denatured during their commercial preparation (Osen et al., 2014), and air-classified proteins usually retain their native conformation, which in turn influences their functional properties (Arntfield & Murray, 1981).

2.3.1 Role of pH and salt on protein functionality

Aside from the extraction method, changes in pH, as well as the presence of minor food constituents such as salts, can further influence protein structure and functionality (Aryee et al., 2018; Damodaran, 2017; Li-Chan & Lacroix, 2018; Shen et al., 2022).

In general, plant globulins demonstrate a tendency to aggregate during extraction, which reduces solubility, particularly near their isoelectric point (Nicolai & Chassenieux, 2019). Thus, plant protein extracts commonly exhibit a U-shaped solubility curve, with minimal solubility near their isoelectric point (around pH 4-5), where proteins carry no net charge and frequently aggregate. This reduced solubility impacts gel formation, emulsification, and foaming properties compared to animal proteins, which generally maintain higher solubility across a broader pH range (Day et al., 2022). In both more acidic and alkaline conditions, the solubility of plant proteins increase due to a rise in net charge. This leads to electrostatic repulsion between protein molecules which prevents clumping and enhances water dispersion. Consequently, proteins swell and bind more water above and below their isoelectric point (Buxbaum, 2015; Damodaran, 2017).

Besides pH the presence of salts can significantly affect protein solubility depending on their concentration and type. At low salt concentrations (below 0.2 M), salts can enhance protein solubility and water-binding capacity (salting-in). Hydrated salt ions, notably anions, bind weakly to charged groups on proteins without disrupting their hydration shell, and the additional water associated with the bound ions contributes to increased hydration. However, at higher salt concentrations, much of the available water becomes bound to the salt ions, leading to protein dehydration, aggregation, and precipitation (salting-out). Lastly, the overall effect of a salt also depends on

the specific cation–anion pair and how these ions interact directly with the protein (Damodaran, 2017; Lo Nostro & Ninham, 2012).

2.3.2 Processing to enhance protein structure and texture

To improve the functional properties of plant-derived proteins, various thermal, physical, chemical, and biological treatments can be applied (Sá et al., 2019). These modifications in functionality influence the protein structure, resulting in food products with varying textures and structural characteristics (Wilkinson et al., 2000).

In general, *thermal treatments* can disrupt protein structures, causing denaturation or aggregation (Li-Chan & Lacroix, 2018). Thermal denaturation is commonly used in protein gel formation, a process that imparts texture to many food products. During gel formation, proteins undergo structural changes, including the unfolding of their molecular structure, which exposes hydrophobic and hydrophilic regions. These exposed regions interact to form bonds such as hydrogen bonds, disulfide linkages, and ionic interactions. The resulting network of interconnected protein molecules traps water and other components, ultimately forming a gel (Nath et al., 2023). This process is applied in tofu production, where soy proteins are denatured and subsequently coagulated, typically with salts (e.g., calcium sulphate) or acids. The resulting curds are then pressed into solid blocks with varying textures. The final gel structure of tofu is influenced by the nature of the protein-protein interactions and the type of coagulant used, which together determine its texture, firmness, and water-holding capacity (van der Riet et al., 1989).

Physical processing techniques such as high-pressure processing and ultrasound treatment can be used to induce changes in protein conformation and functionality without significantly altering their native characteristics (Mulla et al., 2022).

For example, ultrasound treatment can expose hidden functional groups, such as sulfhydryl (-SH) groups, and increase the accessibility of enzyme-active sites which enhances enzymatic catalytic efficiency (Su & Cavaco-Paulo, 2021). Additionally, high-pressure processing, when combined with thermal treatment, such as in extrusion, can significantly alter food texture. The combination of pressure, heat, and mechanical shear during extrusion causes the starch and protein matrix to gelatinise and reorganise, resulting in

a moist, fibrous, and tender structure that closely mimics the texture of meat (Guyony et al., 2022; Pasqualone et al., 2020).

Chemical modifications, including acylation, esterification, oxidation, glycosylation, and phosphorylation, can alter protein charge, polarity, hydrophobicity, and size, potentially improving functionality. However, most chemical modifications are not approved for food use due to possible nutritional loss or the formation of toxic by-products (Damodaran, 2017; Li-Chan & Lacroix, 2018).

Biological modifications primarily include microorganism-driven processes, such as fermentation, and enzymatic treatments involving the direct application of purified enzymes. During fermentation, microorganisms such as bacteria, yeasts, and fungi produce enzymes *in situ* that break down complex compounds into more bioavailable forms (Sá et al., 2019). A classic example of this is tempeh production, in which whole soybeans, or, in more recent studies, other legumes and cereals, are used as substrates and are inoculated with the fungus *Rhizopus oligosporus* (Eklund-Jonsson et al., 2008; Erkan et al., 2020; Mei Feng et al., 2007). This results in a firm, compact product with a characteristic nutty flavour and improved nutritional quality (Purwandari et al., 2024; Suparmo & Markakis, 1987).

Enzymatic modification, on the other hand, is usually more specific and easier to control. The most common form is proteolysis, which improves functional properties such as emulsification, foaming, and solubility, and can produce protein hydrolysates for specialised nutritional applications (Li-Chan & Lacroix, 2018; Sá et al., 2019). Additionally, enzymes such as transglutaminase catalyse covalent crosslinking between glutamine and lysine residues, forming a more stable and elastic protein network. This enhances gel strength, water-holding capacity, and overall texture in protein-based foods (Schäfer et al., 2007; Sun & Arntfield, 2011, 2012).

3. Aims and Objectives

The overall aim of this thesis was to investigate the protein quality (e.g., functional and nutritional properties) of four plant-derived proteins, faba bean, yellow pea, grey pea, and soy, and to evaluate the impact of processing on protein structure and nutrient release. In addition, the thesis aimed to assess how processing affects the bioavailability of iron and zinc in legume-based products, leading to the following objectives:

- Determine the protein quality of four plant-derived proteins (soy, faba bean, yellow pea, and grey pea) by analysing their amino acid composition, overall digestibility, and structural properties (Papers I-III).

- Evaluate the effect of food processing (e.g., heat treatment, fermentation, and enzymatic modification) and associated changes in protein structure on protein digestibility and the release of nutrients (Papers II, III).

- Estimate the bioavailability of iron and zinc in both processed and unprocessed legume-based products (Papers I, III).

- Assess iron uptake in the gastrointestinal tract using an *in vitro* digestion method combined with a Caco-2/HT29 co-culture model that simulates intestinal absorption (Paper III).

4. Materials and Methods

This chapter provides an overview of the materials and methods used to characterise the composition, structure, and nutritional properties of the raw materials and model food systems included in Papers I–III. The methodology is described in detail in the respective papers.

4.1 Preparation of food model systems

In Paper I, commercial protein extracts from soy, faba bean, and yellow pea were analysed (Table 2) to estimate overall protein quality and to assess the mineral bioaccessibility of various ingredient types, including flours, concentrates, isolates, and texturised products. As all raw materials were commercial products, only limited information on the milling, extraction, or texturising processes was available.

Table 2. Overview of the commercial raw materials analysed and the corresponding supplier, adapted from Paper I.

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

However, given that these ingredients are being increasingly consumed as part of plant-based diets and are widely used in the formulation of meat and dairy alternatives, it is important to characterise their nutritional and functional properties. This will provide valuable insights into their potential to meet dietary requirements and supports the development of more nutritionally balanced plant-based foods.

From the commercial extracts listed in Table 2, pea protein isolate (Pisane C9) and concentrate (F55x) were selected to develop a more complex model system in the form of emulsion gels, as described in Paper II. The purpose of this was to investigate how food structure influences pea protein digestibility and nutrient release. To create emulsion gels with distinct structural characteristics, ultrasound treatment and transglutaminase-induced crosslinking were applied prior to heat-induced gelation.

Whilst the model system in Paper II allowed for controlled modification of structure, Paper III focused on real food systems obtained from whole legumes using traditional processing methods such as boiling, fermentation, and protein coagulation. Four different crops were included: faba bean (Sampo, Sweden 2019), grey pea (Rättviksärt, Sweden 2022), yellow pea (Ingrid, Sweden 2022), and soy (ES Pallador, France 2023). These crops were processed through soaking and cooking or further transformed into tofu or tempeh.

4.2 Compositional characterisation

To characterise the different raw materials and food products described in Papers I–III, the macronutrient content, amino acid composition, fibre and sugar content, as well as levels of minerals and phytate, were determined.

4.2.1 Macronutrients

The macronutrients included general nutritional properties such as total protein content, total fat, starch, dietary fibre, ash, and moisture content.

Therefore, the crude protein content was determined using Kjeldahl method with a conversion factor of 6.25 in Paper I and 5.4 in Papers II and III (FAO/WHO, 2011). A protein conversion factor of 6.25 was used to ensure better comparability with other studies (Krul, 2019; Sousa et al., 2020, 2023). However, because a conversion factor of 5.4 is generally considered to be more accurate for legumes, the factor of 5.4 was used for Papers II and III (Tomé et al., 2019).

The total fat content of the different commercial ingredients (Paper I) was determined as described by the Official Journal of the European Communities, Commission Directive 152/2009 EC (2009), whilst in Paper III, lipid extraction was performed following the method described by (Hara & Radin, 1978). Whilst both methods are reliable for determining total lipid

content, the method described by Hara and Radin (1978) may underestimate total fat content if lipids are bound or embedded in complex matrices.

The starch content presented in Paper I was determined using a method described by Larsson and Bengtsson (1983), whilst the total dietary fibre content presented in Paper II was determined according to the Uppsala method (Theander et al., 1995), using gas chromatography. Although this is a well-established and reliable method for measuring dietary fibre, notably non-starch polysaccharides (NSPs) and lignin through the analysis of neutral sugars and uronic acids, it does not aim to quantify all carbohydrates.

The monosaccharide content (Paper III) was characterised using a modified version of the existing method from Sluiter et al. (2008) using high-performance anion exchange chromatography (HPAEC) with pulsed amperometric detection using an ICS 3000 system (Dionex, Sunnyvale, USA) and an AEC column (CarboPac PA 1 analytical 4 × 250 mm). This method provides information on dietary carbohydrates; however, it cannot distinguish between sugars derived from digestible and indigestible carbohydrates, nor does it quantify uronic acids. The ash content in Paper I-III was measured according to AOAC official method 942.05 using a muffle furnace (Model 62700, Barnstead Thermolyne Corporation, Ramsey, Minnesota, United States) and the dry matter content was determined according to AOAC official method 934.01, using a convection oven (Model 2000655, J:P: Selecta, Barcelona, Spain).

4.2.2 Amino acid composition

In Papers I and III, the amino acid composition was determined following the method described by Özcan & Şenyuva (2006), using an LC-MS system equipped with a Phenomenex C18 (2) column, coupled to an Agilent 6120 single quadrupole MS operated in SIM-positive mode (Agilent Inc., Santa Clara, CA, USA). In both papers, acid was used to hydrolyse the proteins, tryptophan could not be detected, and the quantification of cysteine and methionine was also limited (Ozols, 1990). Since proteins from grain legumes are relatively low in sulphur-containing amino acids (methionine and cysteine) as well as tryptophan, this limitation in detection did not significantly affect the interpretation of amino acid quality. However, it should be considered when comparing percentage distributions (Boye et al., 2010).

4.2.3 Minerals

The concentrations of iron and zinc presented in Papers I and III were determined by microwave digestion (Milestone Microwave Laboratory System, EthosPlus, Sorisole, Italy) under acidic conditions, as described by Fredrikson, Carlsson, Almigen & Sandberg (2002) followed by atomic absorption spectrometry (240/280 Series AA Systems; Agilent, Santa Clara, USA). This method is well-established and reliable for measuring mineral content in food matrices. Microwave digestion ensures an efficient breakdown of organic material, allowing for accurate quantification of minerals. Atomic absorption spectrometry offers high sensitivity and specificity for trace elements such as iron and zinc. However, it requires careful calibration and matrix matching, and it typically measures one element at a time, which may limit throughput compared to multi-element methods such as inductively coupled plasma mass spectrometry (Fredrikson et al., 2002).

4.2.4 Antinutrients

Phytate (inositol hexakisphosphate, IP6) concentrations were measured using high-performance ion chromatography coupled with a UV-vis detector (UV-4075; Jasco, Oklahoma City, OK, USA) as described previously by Carlsson, Bergman, Skoglund, Hasselblad & Sandberg, (2001). The characterisation of lower inositol phosphates was carried out following the method described by Skoglund et al., (1997) using the same system. This method enables the separation and quantification of individual inositol phosphate forms, which is essential because the degree of phosphorylation of myo-inositol phosphates indicates the extent to which mineral absorption is inhibited.

4.3 Characterisation and visualisation of food structures

Besides the general composition, rheological characterisations were used to analyse the structure of the different emulsion gels. This was essential for determining the effects of structural changes on protein digestion and nutrient release.

To study the rheological properties of the different emulsion gels a Discovery HR-3 rheometer (TA Instruments, New Castle, DE, USA) equipped with a 40 mm aluminium plate (112471) was used with a gap of

1 mm. During this, measurements of the storage modulus (G') and loss modulus (G") were recorded. Based on these measurements, the viscoelastic properties described as $tan(\delta)$ were calculated as the ratio of the G" to the G'. Whilst the G' measures the energy stored in a material during deformation, reflecting its elastic (solid-like) behaviour, the tan δ (delta) measures the balance between viscous and elastic behaviour (G"/G'), showing whether a material behaves more similarly to a liquid (high tan δ) or a solid (low tan δ).

To visualise the gel structure, a microscope (Nikon, Eclipse Ni–U microscope, Tokyo, Japan) equipped with a 40× (0.75 NA) apochromatic objective was used. Prior to this, the gels were fixed in 2.5% glutaraldehyde and 0.1% ruthenium red solution, followed by post-fixation with 1% osmium tetroxide (Langton et al., 2020). The samples were subsequently dehydrated using ethanol with increasing concentrations. Lastly, the samples were infiltrated and embedded using Technovit 7100. Thin sections of 1 µm thickness were cut and stained with light green to visualise the protein network, and iodine to show the starch granules.

4.4 Estimation of nutritional properties

To estimate the nutritional properties, overall compositional data was first used as an initial indication. However, to obtain a more detailed understanding of the nutritional changes that occur during digestion, *in vitro* digestion was performed following thorough characterisation of the digested ingredients (Paper I), model systems (Paper II), and foods (Paper III). Across all studies, the primary focus was on overall protein digestion (Papers I–III), with an additional emphasis on the release of other metabolites (Paper II), individual amino acids (Paper III), and minerals (Papers I and III).

4.4.1 *In vitro* digestion

To investigate the digestibility and bioavailability of proteins and minerals, a variety of approaches can be employed, such as *in vitro* methods, as well as animal and human studies (Dias et al., 2018; Fuller & Tomé, 2005). Whilst human studies are considered as the gold standard, static *in vitro* digestion models have been shown to effectively predict *in vivo* digestion outcomes (Bohn et al., 2018; Santos-Sánchez et al., 2024). In Papers I–III, *in vitro* digestion was performed according to the standardised INFOGEST protocol

(Brodkorb et al., 2019), with minor modifications as outlined by (Sousa et al., 2023).

To assess overall protein digestibility (Papers I–III), the concentration of free amino groups following *in vitro* digestion was measured using the o-phthaldialdehyde (OPA) method (Nielsen, Petersen & Dambmann, 2001). To gain further insight into the nutritional properties, the amino acid profile of the soluble protein fraction e.g., free amino acids, were characterised (Paper III) following the procedure described by Sousa et al. (2023). This allowed for a more detailed understanding of the release of individual amino acids, rather than relying solely on overall protein digestibility.

To investigate the release of different metabolites during gastric and intestinal digestion, nuclear magnetic resonance (NMR)-based metabolomics was applied (Paper II). This untargeted approach allowed for a broader characterisation of major metabolic changes and nutrient release throughout the digestive process.

4.4.2 Iron and zinc bioavailability

To estimate the relative bioavailability of iron and zinc in the different ingredients (Paper I) and food products (Paper III), the molar ratios of phytate to minerals (Phy:Fe and Phy:Zn) were calculated. These ratios provide indicators of mineral bioavailability and are commonly used to compare and classify foods based on their potential to deliver absorbable nutrients (Efsa, 2014; Hurrell & Egli, 2010). For iron, a Phy:Fe molar ratio below 1, preferably below 0.4, is considered beneficial for enhancing non-haem iron absorption from plant-based meals. Phy:Fe = 6 can be considered adequate in composite meals high in ascorbic acid and meat (Hurrell & Egli, 2010). According to the European Food Safety Authority (EFSA), a Phy:Zn ratio below 5 indicates high zinc absorption, a ratio of 5–15 corresponds to moderate absorption, and ratios above 15 are associated with low bioavailability (Efsa, 2014). This approach offers a practical and cost-effective way to estimate mineral bioavailability, which is particularly valuable for comparative analyses and initial screening of food ingredients. However, it does not consider the influence of other dietary promoters (e.g., ascorbic acid) or inhibitors (e.g., polyphenols and dietary fibre). Additionally, it does not reflect physiological variables, including baseline mineral status, gut integrity, or regulatory mechanisms involved in mineral homeostasis. Therefore, whilst useful as a preliminary indicator, phytate-to-

mineral ratios cannot act as substitutes for more physiologically relevant models such as standardised *in vitro* digestion protocols, cellular uptake assays, or human intervention studies.

4.4.3 Cell uptake measurements

Cell uptake studies are valuable for understanding how nutrients, particularly minerals, are absorbed at the cellular level, offering insights into their bioavailability in the body. Therefore, *in vitro* digestion, followed by cell uptake studies, was used to investigate iron uptake into human intestinal cells. To ensure an adequate iron content in the digested food products, minor modifications to the INFOGEST protocol were applied, as described in Paper III.

Lastly, iron uptake was assessed using a co-culture model consisting of Caco-2 cells (Caco-2; HTB37; ATCC, Manassas, VA, USA) and mucus-producing goblet cells (HT29-MTX-E12; ATCC, VA, USA). Caco-2 cells synthesise ferritin in response to increased intracellular iron concentrations, and thus the ratio of ferritin to total protein (expressed as ng ferritin/mg protein) was used to estimate cellular iron uptake (Tako et al., 2011). Ferritin levels were determined using an enzyme-linked immunoassay (Eagle Biosciences, Amherst, NH, Product number FRR31-K01), with minor modifications, as described by Glahn (2022). Total cell protein concentrations were quantified using the Bio-Rad DC™ Protein Assay Kit (500-0116, Bio-Rad Laboratories Inc., Hercules, CA, USA).

The Caco-2 cell line is a well-established *in vitro* model for studying human intestinal iron uptake. Derived from a colon carcinoma, these cells spontaneously differentiate into enterocyte-like cells that closely resemble mature duodenal enterocytes. Despite their widespread use and strong correlations between data from Caco-2 studies and human trials (Au & Reddy, 2000; Glahn, 2022), there are, as with all *in vitro* methods, certain limitations such as the absence of a mucus layer.

To address this, Caco-2 cells are often co-cultured with the HT-29 cell line treated with methotrexate (HT-29 MTX), which produces mucus. This co-culture system adds a mucus layer to the polarised monolayer, which more closely mimics *in vivo* intestinal conditions and provides a protective barrier (Birch et al., 2018; Hevia et al., 2023; Wuyts et al., 2015).

4.5 Statistical analyses

The results of the compositional analysis were evaluated using one-way analysis of variance (ANOVA, Type I), followed by Tukey's post-hoc test (Papers I and III). The degree of hydrolysis measurements ($n = 3$, $n = 2$) were analysed using ANOVA (Type III) for unbalanced population sizes (Paper I) and ANOVA (Type I) for balanced population sizes (Papers II and III), followed by Tukey's post-hoc test (Papers I and III) or Fisher's least significant difference (LSD) test (Paper II). The dynamic rheological measurements presented in Paper II were log-transformed prior to statistical analysis (except Tan δ) and analysed using one-way ANOVA, followed by Fisher's LSD test. All statistical analyses were conducted using R (Version 4.3.0, RStudio Inc., Boston, USA), whilst multivariate data analysis (Paper II) was performed using SIMCA (Version 17.0, Umetrics, Sweden).

5. Results and Discussion

This thesis investigates the protein quality of four crop species: soy, faba bean, yellow pea, and grey pea, the latter representing a more novel protein source. The research focuses on both the nutritional and functional properties of commercial protein extracts (Papers I and II), as well as whole and processed legumes (Paper III). In addition, the work explores how food structure (Paper II) and the surrounding food matrix (Papers II and III) influence protein digestion and nutrient release. Here, *food structure* primarily refers to the texture of the product (Paper II), whilst the *food matrix* encompasses the overall composition and nutrient interactions within the food system (Papers II and III). Lastly, the thesis examines the role of phytate in mineral bioavailability and evaluates how different processing methods may reduce phytate levels, thereby potentially improving mineral absorption (Paper III).

5.1 Plant protein quality

The quality of plant-derived proteins is determined by both their functional and nutritional properties. Functional properties, such as gel formation properties, influence their suitability for various food applications. Meanwhile, nutritional quality predominantly refers to the composition and bioavailability of essential macro- and micronutrients, which affect their capacity to meet human dietary requirements.

5.1.1 Nutritional properties

The nutritional quality of plant-derived proteins is largely influenced by their amino acid composition, overall protein digestibility, and the bioavailability of essential nutrients, the latter of which is discussed in section 5.2.

In terms of *amino acid composition*, both the total content of essential amino acids (EAAs) and their specific balance are critical for assessing protein quality. When comparing total EAA content (Figure 4), no significant difference was observed between soy and yellow pea protein ($p = 0.925$), suggesting that there is a comparable protein quality between the two. In contrast, faba bean showed a significantly lower EAA content ($p < 0.001$), indicating a reduced nutritional quality.

Regarding the overall amino acid balance, all three crops contained high levels of leucine and lysine, whilst cysteine and methionine were consistently present in lower amounts. When amino acid concentrations were compared to the FAO/WHO, (2011) recommended reference values (in mg/g protein), most of the tested proteins failed to meet the suggested level for valine, and several also fell short for isoleucine, with faba bean proteins showing the lowest concentrations of both (Paper I). This further supports the conclusion that, among the studied proteins, faba bean has the lowest overall amino acid quality.

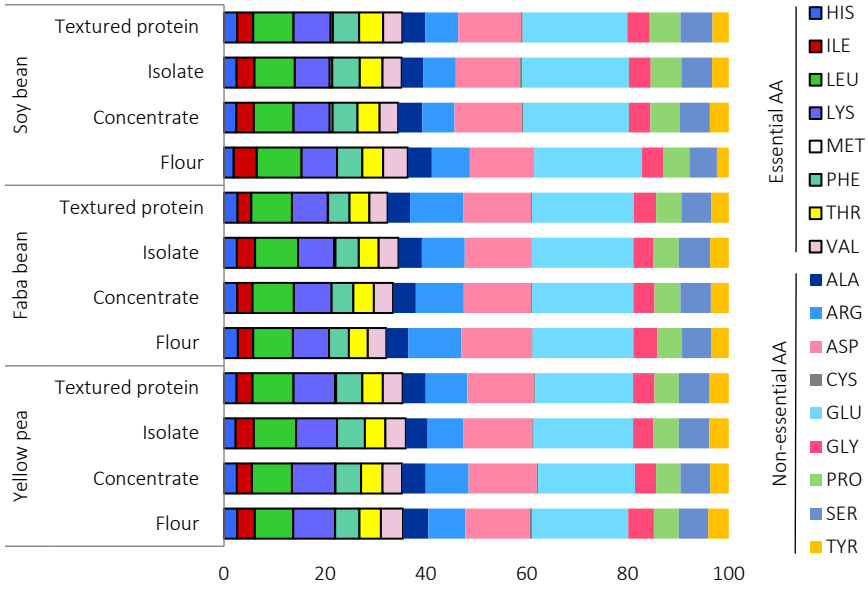

Figure 4. Amino acid composition (%) of commercially available soy, faba bean and yellow pea protein ingredients. The data illustrates the relative distribution of essential and non-essential amino acids, highlighting key differences in protein quality among these plant-based sources (adapted from Papers I and III).

These findings align with the well-documented limitation of plant-derived proteins, which tend to be low in sulphur-containing amino acids (Ciurescu et al., 2018; Herreman et al., 2020). To improve nutritional quality, blending different protein sources has proven to be an effective strategy to both compensate for amino acid limitations and achieve a more balanced nutritional profile (Herreman et al., 2020). This can be achieved not only through strategic product formulation but also through a balanced diet. It is

important to note that dietary proteins do not need to provide all indispensable amino acids in optimal proportions within a single meal, as the body can utilise amino acids consumed at different times throughout the day to meet metabolic requirements (Mariotti, 2017). Thus, despite its limitations, faba bean can still meaningfully contribute to protein intake when it is included as part of a varied and balanced diet.

Beyond amino acid composition, overall digestibility and the release of individual amino acids are critical factors in determining protein quality. When comparing protein extracts and texturised proteins from soy, faba bean, and yellow pea, shown in Figure 5, no significant differences were observed in digestibility between the sources ($p = 0.342$). Depending on the product type, the degree of hydrolysis (DH) on average ranged from 61% to 83%. These findings are consistent with previous studies indicating that plant-derived protein sources, particularly refined products such as flours, concentrates, and isolates, can exhibit high digestibility, typically ranging from 80% to 90% (FAO/WHO/UNU, 2007; Sousa et al., 2023).

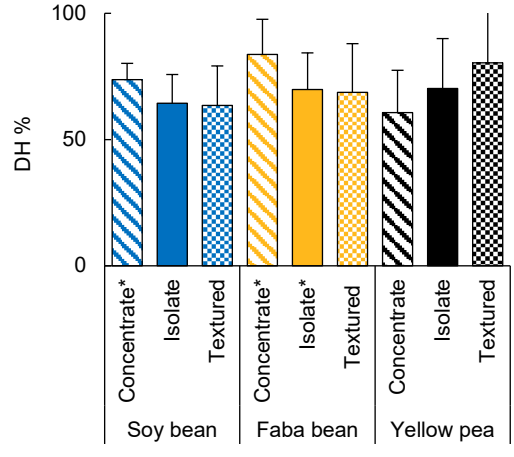

Figure 5. Degree of protein hydrolysis in % based on the number of bonds hydrolysed in the digesta and the total number of peptide bonds per protein equivalent for different protein extracts and texturised proteins (n = 3, n* = 2), adapted from Paper I.

However, depending on the applied processing method, the digestibility of protein extracts can vary (Luo et al., 2017; Singh et al., 2015). Previous studies on pea protein isolate have reported a wide range of digestibility, with degrees of hydrolysis (DH) ranging from 25% to 85%, depending on the overall food structure and surrounding matrix. Indeed, pea protein

incorporated into an emulsion was found to be more readily hydrolysed than the protein isolate alone. According to Reynaud et al. (2020), this enhanced hydrolysis in emulsions can be attributed to the high-pressure processing applied during emulsification (Reynaud et al., 2020). This highlights that protein digestibility is not only influenced by the source but can also be significantly shaped by the structure and overall food matrix.

5.1.2 The effect of food structure on protein digestion

To study the effect of food structure on protein digestibility and nutrient release during digestion (Paper II), emulsion gels with varying structural characteristics were prepared using commercial pea protein isolate (PI) and pea protein concentrate (PC). These two protein sources were selected because of their differences in overall composition (Figure 6) and protein structure, both of which are influenced by the applied extraction methods (Arntfield & Murray, 1981; Osen et al., 2014).

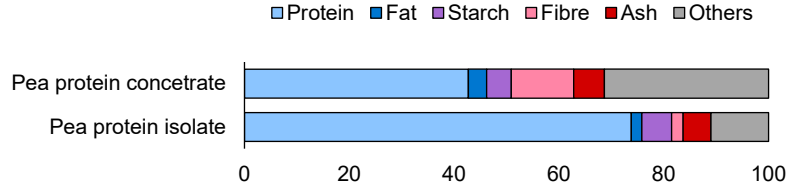

Figure 6. Compositional differences between pea protein isolate and pea protein concentrate, including protein, fat, starch, fibre, and ash content (Papers I and II). Others include unquantified components (Theander et al., 1995).

Protein concentrates, which are usually produced through milling and air fractionation, contain fewer protein aggregates, whereas protein isolates undergo more extensive processing, often leading to protein denaturation and aggregation (Capuano & Janssen, 2021; Taherian et al., 2011). Since protein aggregates do not necessarily undergo major structural changes during heat treatment, as shown in Figure 7, they hinder protein–protein interactions and compromise effective gel formation (Paper II).

To address this limitation, ultrasound (US) pre-treatment was applied to disrupt protein aggregates (Figure 7). This led to enhanced rheological properties in gels made from PI, as reflected by an increased storage modulus (G') and a reduced tan δ (Figure 8), compared to gels formed with untreated PI where aggregates remained largely intact.

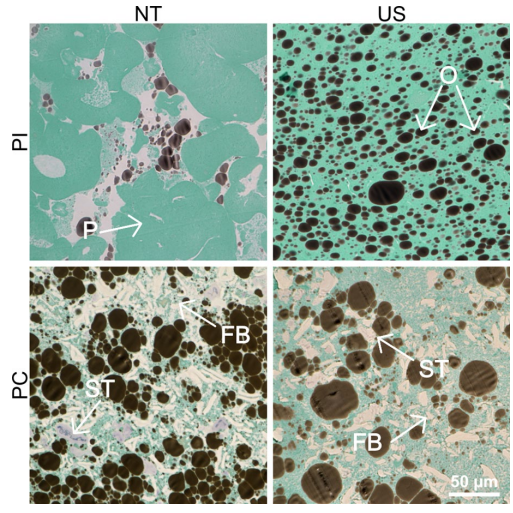

Figure 7. Microstructure of the pea protein gels made from pea isolate (PI) and pea concentrate (PC), before (NT) and after ultrasound pre-treatment (US), using light microscopy. Proteins (P) are stained blue/green, oil droplets (O) appear black, and starch granules (ST) are stained purple/red. The different structural features are indicated with arrows (adapted from Paper II).

To further improve the structural and textural properties of PI-based gels, transglutaminase (TG) was incorporated into the gel system. When TG was added, a modest increase in G' and a moderate reduction in tan δ were observed, whereas the combination of US and TG treatments resulted in a significant increase in G' and a marked decrease in tan δ, indicating synergistic effects on gel network formation and elasticity.

In contrast, US treatment led to a significant reduction in G´ for the gels made from PC. Similarly, adding TG or combining TG with US resulted in a weaker gel structure. However, TG treatments both with and without US pre-treatment increased gel elasticity (lower tan δ), suggesting that TG modified the protein network by reducing rigidity whilst enhancing flexibility.

The reducing rigidity could possibly be attributed to TG-induced crosslinking interfering with native non-covalent interactions, such as hydrogen bonding and hydrophobic interactions, which are essential for the formation of a cohesive gel network. Such interference can occur when TG alters protein conformation or competes with existing interactions during network formation (Jong & Koppelman, 2002; Yu et al., 2022). Moreover, thermal processing parameters e.g. heating and cooling rates, can modulate

TG activity, further influencing the extent and uniformity of crosslinking and, consequently, the structural properties of the final gel (Sun & Arntfield, 2011).

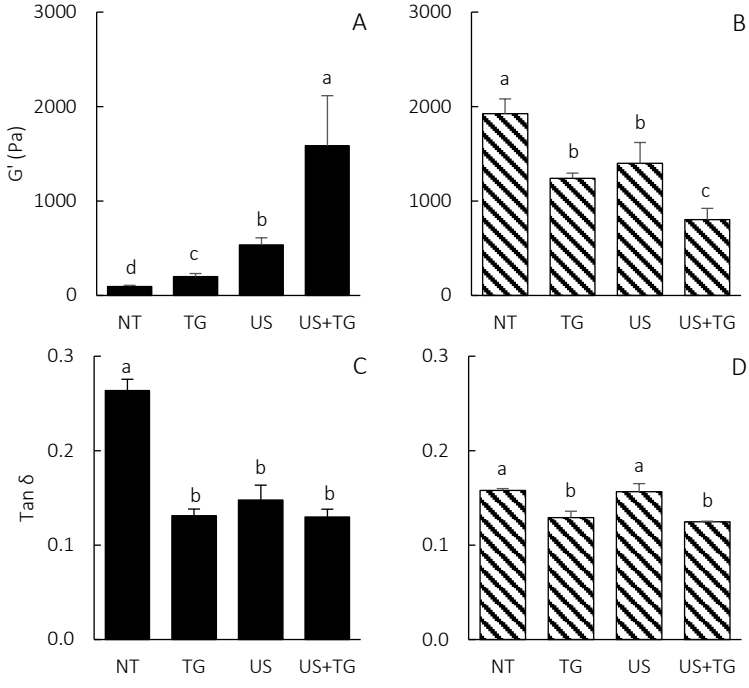

Figure 8. Effect of ultrasound and/or transglutaminase treatment on the storage modulus (G') and tan δ of pea protein emulsion gels made from pea isolate (A, C) and pea concentrate (B, D), adapted from Paper II.

Aside from the overall effect of different pre-treatments on the gel structure, it should also be noted that PC gels exhibit an overall higher G' than gels made from PI. This can be attributed to the overall higher fibre content in PC, as dietary fibres can increase the gel strength of protein gels (Johansson et al., 2022; Nath et al., 2023).

Insoluble dietary fibre, present in large amounts in PC (Paper II), has been shown to improve water-holding capacity and water distribution, resultingly increasing gel stability (Xu et al., 2023; Zhuang et al., 2020). Moreover, polysaccharides can enhance the gel properties of protein-based hydrogels either by swelling during heating (e.g. starch), which compacts the protein network, or by acting as fillers that form dense networks which improves the texture. Interactions between polysaccharides can also result in hydrogen

bonding, further strengthening gel structure and elasticity (Dille et al., 2015; Nath et al., 2023).

To evaluate how these differences in gel structure and possible differences in composition (e.g., fibre content) influence protein digestibility, the degree of protein hydrolysis (DH) after *in vitro* digestion was measured (Figure 9).

When characterising the effect of different treatments on the DH, significant differences were found between the PI gels treated with US + TG (DH 69%) and PI gels treated with TG (DH 90%) as well as between the PC gels treated with US+TG (DH 64%), and the samples treated with TG and NT (DH 37% and 41%). Moreover, when comparing the overall average between gels made from PI (77%) and PC (48%) a significant difference in protein digestibility was observed, indicating that the type of raw material influences protein digestion. This highlights a possible effect of the overall gel structure on protein digestibility, after which differences in gel strength, elasticity, and composition, particularly fibre and polysaccharide content, may influence enzyme accessibility and hydrolysis efficiency.

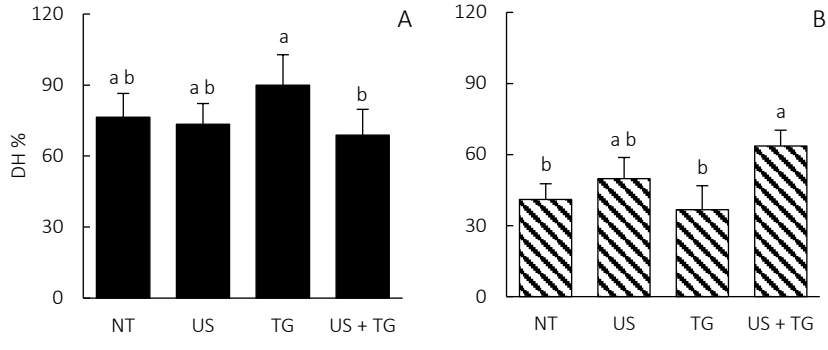

Figure 9. Degree of protein hydrolysis (DH) of the different gels made from pea isolate (A) or pea concentrate (B) at the end of the intestinal phase (Paper II)

To further illustrate the relationship between gel structural properties, specifically G′, tan δ, fibre content, and DH during the gastric and intestinal digestion, Principal Component Analysis (PCA) was performed (Figure 10). Gels with higher G′ values tended to exhibit lower DH, suggesting that stronger, more rigid networks may hinder enzymatic access and reduce protein breakdown. In contrast, higher tan δ values, indicative of more viscous and less elastic gels, were associated with increased gastric DH,

implying that less cohesive structures may facilitate protein hydrolysis. Additionally, a higher fibre content appeared to correlate with lower DH, suggesting that compositional factors such as fibre may also contribute to reduced protein digestibility.

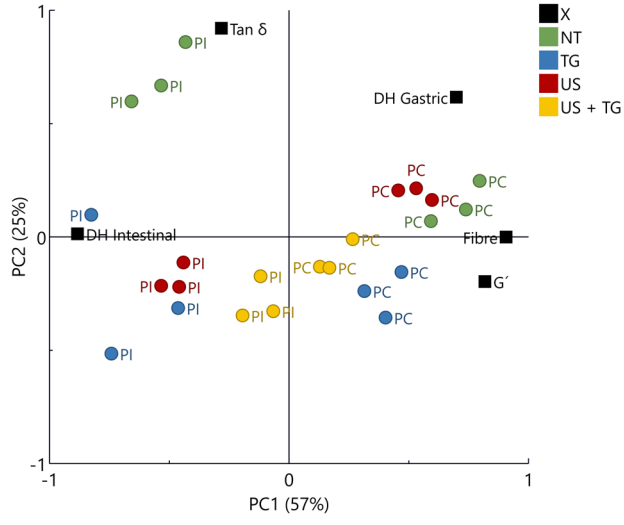

Figure 10. Principal Component Analysis (PCA) biplot illustrating the relationships between compositional and structural variables and protein digestibility (x) in gels made from pea isolate (PI) and pea concentrate (PC) subjected to different treatments (NT = no treatment, TG = transglutaminase, US = ultrasound, US + TG = combined ultrasound and transglutaminase). PC1 and PC2 account for 57% and 25% of the total variance, respectively.

When further evaluating metabolite release during gastric and intestinal digestion (Paper II), NMR-based metabolomics revealed that PI gels treated with US+TG released significantly less glycine compared to other PI gels. This finding supports the observation that firmer food structures can restrict nutrient release (Singh et al., 2015), particularly during gastric digestion.

In terms of PC gels, untreated and US-treated gels showed a higher glucose release compared to TG and US+TG treated PC gels during the gastric digestion. This suggests an effect of TG treatment on glucose release, which could be attributed to the encapsulation of starch granules within the protein network, thereby limiting enzymatic accessibility (Lang et al., 2020; Mei Wee & Henry, 2019).

Overall, these findings underscore the importance of both physical structure and matrix composition in modulating protein bioaccessibility (Loveday, 2022; Luo et al., 2017; Nyemb et al., 2016; Parada & Aguilera, 2007; Singh et al., 2015). Changes in food structure can lead to significant variations in digestion efficiency (Singh et al., 2015). For example, cooked egg protein displayed significantly higher digestibility (91%) compared to raw egg protein (51%) despite having the same overall composition. This difference is predominantly due to heat-induced denaturation, which unfolds proteins and makes them more accessible to digestive enzymes, as well as the inactivation of protease inhibitors, both of which enhance protein digestibility (Evenepoel et al., 1998). Further, differences in the food matrix can influence protein digestion. Thus, the presence of polysaccharides, including those found in legumes, have been reported to limit protein digestion by reducing the diffusion of digestive enzymes to their substrates (Karim et al., 2024). The extent of this inhibitory effect depends on factors such as the concentration, viscosity, and molecular structure of the polysaccharides, as well as the physicochemical properties of the protein substrate (Bach Knudsen, 2001; Gilani et al., 2005; Karim et al., 2024; Kaur et al., 2022; Lu et al., 2024), rendering the overall impact challenging to predict.

5.1.3 Effect of the food matrix on protein digestion

To further investigate how the overall food matrix influences digestibility and nutrient release, the effects of processing on soy, faba bean, yellow pea, and grey pea were examined (Paper III). Three traditional processing methods, soaking/cooking, fermentation, and protein coagulation, were applied to produce tempeh- and tofu-like products with varying food structures and compositions (Figure 11).

Based on the compositional analysis, protein coagulation (tofu) increased protein concentration whilst reducing sugar content compared to both boiled and, to a certain extent, fermented beans (tempeh). The higher ash content observed in tofu also suggests an increased concentration of minerals and potential antinutrients, which could be further verified through mineral and phytate quantification (Paper III).

Fermentation also reduced the sugar fraction and increased protein levels compared to cooked beans, likely due to microbial metabolism and structural modifications during fermentation. In addition to overall compositional

changes, both fermentation and protein coagulation altered the amino acid profile, carbohydrate composition, and mineral content of the final products (Paper III).

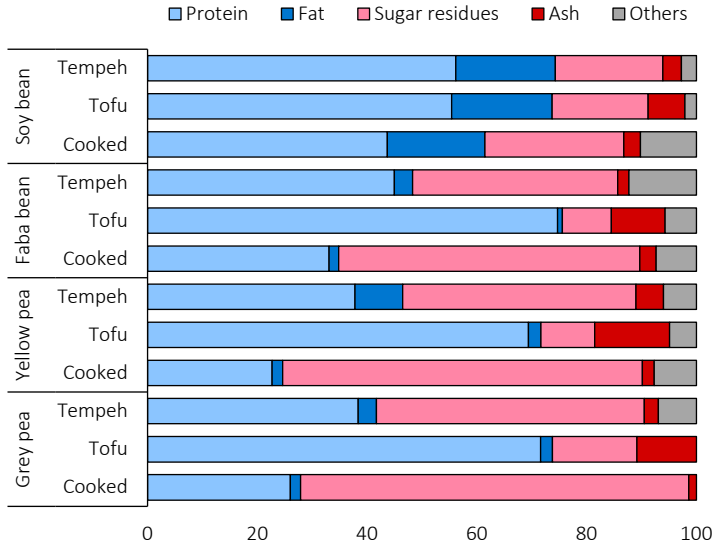

Figure 11. Compositional changes in soy, faba bean, yellow pea, and grey pea after fermentation (tempeh) and protein coagulation (tofu) compared to the soaked and boiled crop (adapted from Paper III)

To study the effect of processing and the resulting changes in the food matrix on protein digestibility, *in vitro* digestion followed by the OPA assay was used. Based on the results presented in Figure 12, cooked pulses showed the lowest degree of hydrolysis (DH), with an average of 37%. Fermented products exhibited a higher average DH (47%); however, a significant difference between cooked and fermented products was only observed for grey peas. Tofu showed the highest DH, averaging 75%, which significantly exceeded that of the corresponding boiled pulses (Paper III).

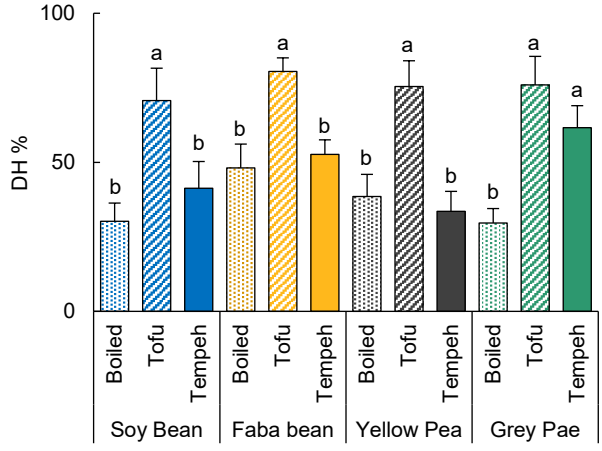

Figure 12. Degree of protein hydrolysis (DH) in % comparing the effect of processing. Lowercase letters indicate significant differences between the different processing methods ($p < 0.001$), adapted from Paper III.

To investigate the relationship between processing methods and raw material composition on protein hydrolysis, PCA was performed to examine the associations among protein source, processing type, and nutrient composition (Figure 13). The PCA biplot revealed distinct clustering based on both legume type and processing method. DH clustered closely with tofu samples, which were characterised by high protein and ash content and low sugar and fat levels. This suggests that tofu processing enhances protein digestibility, as reflected by higher DH values. In contrast, boiled samples, particularly those from faba bean and yellow pea, clustered near the sugar vector and farther from DH, indicating lower protein hydrolysis. These findings suggest that both the intrinsic composition of the legumes and the applied processing technique significantly influence DH, with protein-rich, low-sugar matrices such as tofu promoting greater protein breakdown.

These differences in digestibility can be further attributed to variations in food structure (Singh et al., 2015). Tofu generally exhibits a more open and homogeneous protein network, which likely facilitates enzyme accessibility during digestion. In contrast, boiled and fermented beans retain a more complex and compact matrix that may hinder enzyme penetration and reduce protein breakdown.

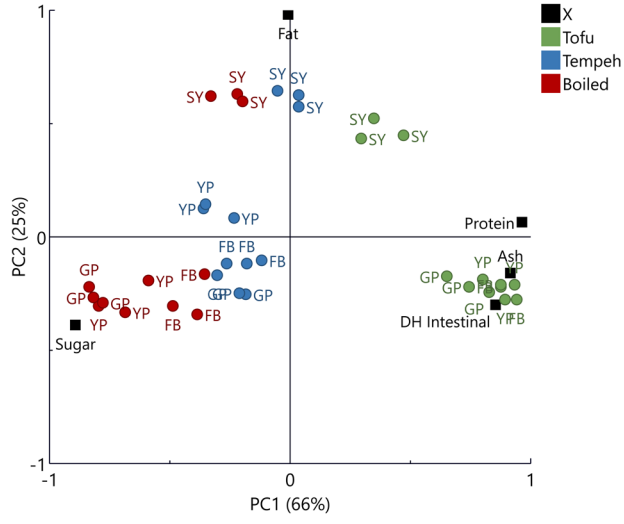

Figure 13. Principal Component Analysis (PCA) biplot showing tofu, tempeh, and boiled legumes prepared from soy (SY), grey pea (GP), yellow pea (YP), and faba bean (FB) in relation to compositional variables and protein digestibility (x). PC1 and PC2 explain 66% and 25% of the total variance, respectively.

Along with overall protein digestibility, the release of individual amino acids from the food matrix following *in vitro* digestion was evaluated (Paper III). Comparing the amino acid profiles of the undigested products to those in the soluble fraction after digestion revealed a high release of leucine, isoleucine, and lysine, as well as glutamic acid and aspartic acid across all samples. However, the release of threonine, alanine, and arginine remained consistently low, despite their considerable presence in the undigested material. A similar pattern has been observed for garden peas, grass peas, soybeans, and lentils, where leucine, lysine, and phenylalanine were identified as the most abundant essential free amino acids released after intestinal digestion (Santos-Hernández et al., 2020). In contrast, threonine was released in relatively lower amounts, comparable to that of methionine (Santos-Hernández et al., 2020). However, no limitation in threonine release was observed in black beans, pigeon peas, and wheat bran. Nonetheless, the digestibility of threonine was generally lower than that of other amino acids (Hodgkinson et al., 2022; Sousa et al., 2023) which may be explained by the

localisation of these amino acids within less accessible regions of the protein matrix or by their lower susceptibility to enzymatic hydrolysis (Paper III).

Overall, these findings further support the idea that alterations in the food matrix affect protein digestibility and the release of individual amino acids, ultimately influencing nutritional quality. Thus, both food structure and the presence of other food components play a significant role in determining how effectively proteins are broken down and absorbed during digestion.

5.2 Mineral bioavailability

Mineral bioavailability is strongly influenced by interactions with phytate, which can inhibit mineral absorption. Therefore, the amount of phytate, especially in its fully phosphorylated form (IP6), relative to the amount of individual minerals can serve as an indicator of their estimated bioavailability (Zhang et al., 2022).

When comparing the iron, zinc, and phytate content of various protein sources (Table 3), soy, faba bean, and yellow pea protein ingredients displayed clear differences in both mineral and phytate levels, depending on the type of processing applied. Isolates generally had the highest iron and zinc concentrations, notably faba bean and yellow pea isolate. However, phytate levels and phytate-to-mineral molar ratios varied, influencing potential mineral bioavailability.

To estimate the bioavailability of iron and zinc in the raw materials, the molar ratios of phytate to iron (Phy:Fe) and phytate to zinc (Phy:Zn) were calculated. Phy:Fe below 1 (ideally below 0.4) is recommended to support better non-haem iron absorption from plant-based foods. However, a Phy:Fe ratio of up to 6 may be considered as adequate in composite meals that are high in non-heme iron enhancers, e.g. ascorbic acid and meat (Zhang et al., 2022). Regarding zinc, a Phy:Zn ratio below 5 indicates good absorption, whilst values above 15 are associated with low bioavailability (Efsa, 2014).

Based on these reference values, both faba bean and pea isolates showed the lowest Phy:Fe and Phy:Zn ratios, suggesting that they may serve as good sources of bioavailable iron and zinc, particularly when consumed alongside absorption-enhancing foods. In contrast, all other ingredients exceeded the recommended thresholds, indicating they are less likely to provide sufficient bioavailable minerals when consumed alone (Paper I).

Table 3. Iron and zinc content (mg/kg dry weight), phytate content (g/kg dry weight), and molar ratio of phytate to iron/zinc, adapted from Papers I and III.

Category		Composition				
		Iron	**Zinc**	**Phytate**	**Phe:Fe**	**Phe:Zn**
Soy bean	**Flour**	99	50	10.9	9	22
	Concentrate	104	31	14.6	12	47
	Isolate	139	51	10.9	7	21
	Texturised	114	26	16.3	12	61
Faba bean	**Flour**	63	45	13.8	18	30
	Concentrate	85	111	28.9	29	26
	Isolate	389	114	18.4	4	16
	Texturised	70	76	23.4	28	31
Yellow Pea	**Flour**	54	34	9.4	15	27
	Concentrate	117	74	23.2	17	31
	Isolate	200	88	13.1	6	15
	Texturised	194	86	17.2	7	20
Pooled standard deviation		6	4	0.8	1.7	3.3

Although iron and zinc recommendations refer to total dietary intake rather than individual ingredients, given the relatively high phytate levels in the analysed samples, reducing the phytate content remains a crucial step for improving mineral bioavailability. This is especially important as fortification alone has limited effectiveness, particularly when phytate levels are high (Gupta et al., 2020; Koréissi-Dembélé et al., 2013).

5.2.1 Effect of processing on phytate

Whilst the amount of phytate present in the individual legume varies between crops, cultivars, and growing conditions (Mayer Labba et al., 2021; Reddy et al., 1982; Zhang et al., 2022), its reduction into lower inositol phosphates mainly depends on the activity of phytase during processing. Therefore, the impact of soaking and cooking, fermentation, and protein coagulation on phytate degradation was assessed by quantifying both total phytate (IP6) and lower inositol phosphates (IPs) in the different products (Figure 14).

The results showed that fermentation not only reduced IP6 but also, to a certain extent, IP5, and increased levels of IP2, which potentially improved mineral bioavailability compared to the boiled products. In contrast, tofu production led to an increase in total phytate content and altered the phytate

profile. This increase in phytate is likely due to protein concentration during coagulation and the strong binding affinity of IP6 to proteins (Ishiguro et al., 2008). Noteworthily, the formation of IP3 and IP4 was observed in yellow pea tofu. This suggests that modifying parameters such as temperature and soaking or boiling times during tofu production could reduce IP6 levels and improve nutritional quality.

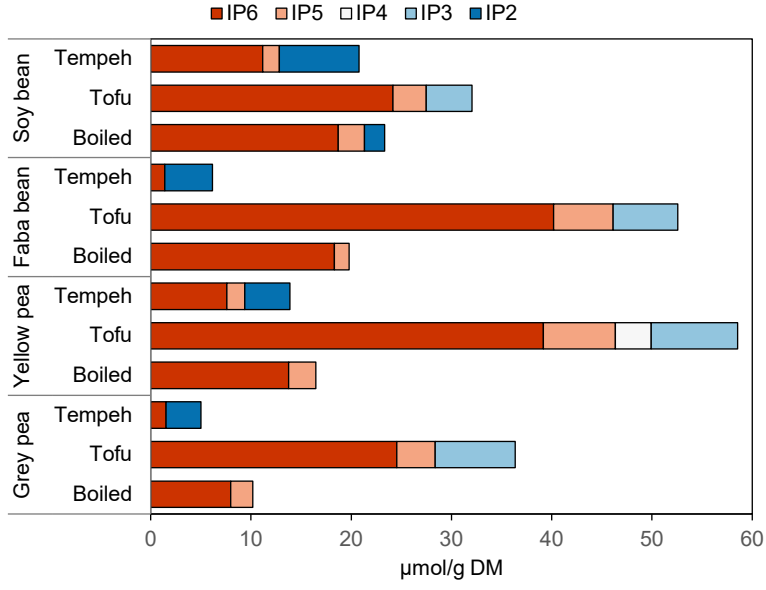

Figure 14. Changes in phytate concentration and overall composition in processed and unprocessed soy, faba bean and pea products.

When comparing the effect of different processing methods on mineral and phytate content (Table 4), tempeh consistently demonstrated the lowest phytate levels and therefore the most favourable molar ratios for both iron and zinc across all legumes. Tofu products had the highest iron and zinc contents, particularly from faba bean and both yellow and grey pea, although they also exhibited higher phytate levels. Therefore, the overall molar ratios of tofu remained relatively high and comparable to those of cooked legumes. In addition to processing effects, crop type also played a key role in mineral accessibility. Among the different legumes, grey pea products consistently exhibited lower Phy:Fe ratios across all processing methods compared to yellow pea and faba bean products. This is likely due to the naturally lower phytate content in grey peas (Paper III), which contributes to their more

favourable mineral profile. These findings underscore the potential of grey peas as a valuable plant-based protein source with improved mineral bioavailability.

Table 4. Iron and zinc content (mg/kg DM), phytate content (g/kg DM), and molar ratio of phytate to iron/zinc, adapted from Paper III.

Product		Composition				
		Iron	**Zinc**	**Phytate**	**Phe:Fe**	**Phe:Zn**
Soy bean	**Boiled**	55	41	13.0	20	32
	Tofu	81	56	15.5	16	27
	Tempeh	63	44	3.0	4	7
Faba bean	**Boiled**	67	53	11.4	14	21
	Tofu	171	142	30.8	15	21
	Tempeh	91	61	-	-	-
Yellow pea	**Boiled**	36	29	7.8	18	26
	Tofu	164	104	27.5	14	26
	Tempeh	51	38	3.6	6	9
Grey pea	**Boiled**	47	37	4.6	8	12
	Tofu	160	119	15.0	8	13
	Tempeh	59	39	-	-	-
Pooled standard deviation		6	4	0.4	0.8	0.9

5.2.2 Effect of phytate reduction on mineral bioavailability

Based on the low molar ratios of phytate to iron/zinc in yellow pea and soy tempeh, as well as the complete reduction of phytate in faba bean and grey pea tempeh, fermentation demonstrates a strong potential for enhancing the bioavailability of iron and zinc.

To further assess the effect of processing on iron bioavailability, tofu and tempeh made from faba bean, grey pea, and soy were subjected to *in vitro* digestion, followed by iron uptake measurements using a Caco-2/HT29 co-culture model. Thereby, the fully differentiated cell lines were exposed to digested food products at different dilutions (2×, 5×, and 10×) to evaluate concentration-dependent uptake, and ferritin formation in the cells was measured as an indicator of iron uptake.

When comparing ferritin formation across the different products and dilutions (see Figure 15), significantly higher ferritin levels were observed in cells exposed to the 5× and 10× diluted digested tempeh samples. In contrast, no significant difference in ferritin formation was found between

the control and tofu samples. These results support the previously estimated lower iron bioavailability of tofu compared to tempeh, as discussed in Section 5.2.1.

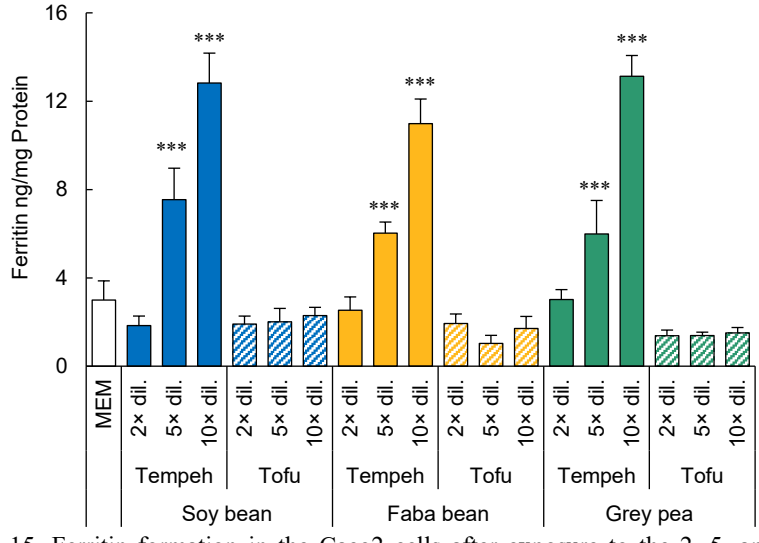

Figure 15. Ferritin formation in the Caco2 cells after exposure to the 2, 5, and 10× dilutions of digesta to evaluate concentration-dependent uptake. *** indicate significant differences $p<0.001$ from the control (MEM), adapted from Paper III.

Although the 2× diluted digested tempeh samples contained the highest iron concentration, they did not promote ferritin formation, likely due to limitations in the *in vitro* method, as cell damage and/or death (indicated by LDH release and TEER reduction; refer to Paper III) may have interfered with cellular iron metabolism. Similarly, the general trend of higher ferritin formation in the 10× diluted samples compared to the 5× dilutions may be due to reduced cellular stress in the higher diluted samples, suggesting that higher concentrations of certain potentially toxic compounds in the less diluted samples could negatively affect cell viability. These findings emphasise the need for a standardised uptake protocol to avoid the need for testing multiple concentrations in future studies and to ensure comparability among different results (Glahn, 2022; Hevia et al., 2023).

Despite the variations across dilutions, a consistent and significantly higher ferritin formation was observed from the tempeh samples compared to tofu, highlighting the beneficial impact of fermentation on the iron bioavailability of legumes. These findings indicate that different processing

methods can enhance distinct nutritional aspects: whilst fermentation (tempeh) may increase mineral absorption, coagulation (tofu) may favour protein digestibility. This underscores the importance of selecting processing techniques based on targeted nutritional outcomes when designing legume-based foods.

6. Conclusion

By evaluating protein composition, digestibility, and mineral bioavailability across a range of ingredients and food (model) systems, this work provides insights into how food processing can be used to enhance the functionality and nutritional quality of legume-based foods.

The findings from Papers I, II, and III indicate an overall high protein quality across the studied crops (soy, faba bean, yellow pea, and grey pea). Amino acid analysis revealed no major limitations in their nutritional profiles, and protein digestibility did not vary significantly between the crops. However, a strong correlation was observed between digestibility and the food's structure and physicochemical properties, particularly fibre content.

When proteins were digested in their isolated forms, digestibility was relatively high and consistent across all sources (Paper I). In contrast, when these proteins were incorporated into more complex food systems, both protein digestibility and micronutrient release were influenced by the structure and overall food matrix (Papers II and III).

When estimating the bioavailability of iron and zinc in relation to phytate content, mineral bioavailability emerged as a key nutritional factor, as high levels of phytate were identified as a major barrier to the absorption of both iron and zinc (Papers I and III). Among the tested processing methods, fermentation demonstrated strong potential in reducing phytate concentrations, leading to more favourable phytate-to-mineral molar ratios and resultingly enhanced the potential for mineral uptake. This finding was further supported by cellular uptake studies using a combined *in vitro* digestion Caco-2/HT29 co-culture model which confirmed increased iron uptake from samples with reduced phytate levels (Paper III).

Thus, although the limitations in mineral bioavailability can be addressed through strategic processing methods, the bioavailability of minerals is not readily apparent to the consumer. This is particularly concerning when considering that legumes are often promoted as good dietary sources of essential minerals. However, in their unprocessed form, only a small fraction of the total mineral content is bioavailable. Consequently, consumers may be misled into believing that they are adequately meeting their nutritional needs through legume consumption, potentially leading to widespread mineral

deficiencies, especially among vulnerable populations that rely on plant-based diets.

From a societal perspective, this underscores the urgent need to address hidden micronutrient deficiencies that can affect public health outcomes. For the food industry, it presents both a challenge and an opportunity: to innovate and implement processing technologies that enhance mineral bioavailability whilst maintaining desirable sensory and functional properties. Transparency is also critical, and clearer labelling and consumer education about mineral bioavailability could empower individuals to make informed dietary choices, bridging the gap between nutrient content and actual nutritional value. Ultimately, coordinated efforts across research, industry, and public health sectors are essential to maximise the nutritional benefits of plant-based foods and support healthier and more sustainable diets.

7. Outlook

This thesis demonstrates an overall high protein quality among the different analysed crops. However, it also highlights the significant influence of food structure and matrix composition on protein digestibility. Whilst targeted processing strategies can enhance both the functional and nutritional properties of plant-based foods, key challenges remain in optimising these products for a wide range of dietary needs and population groups.

The overall findings reinforce the current scientific consensus that plant-derived proteins can adequately support the nutritional requirements of healthy adults (Larsson & Johansson, 2002; Mariotti, 2017; Messina et al., 2018; Neufingerl & Eilander, 2023). However, the shift toward more plant-based diets may present specific challenges for older individuals. Research suggests that aging is associated with reduced protein digestion and amino acid absorption, as well as a general decline in protein intake. In particular, older adults may require higher intakes of leucine, a branched-chain amino acid essential for stimulating muscle protein synthesis (Baum et al., 2016; Szwiega et al., 2021). Although the current Nordic Nutrition Recommendations do not call for increased protein intake in individuals < 70, this may need reconsideration, especially when a significant proportion of protein is derived from whole, minimally processed plant foods.

Similarly, protein digestibility within traditional diets in many low- and middle-income countries is often lower due to the reliance on minimally processed plant proteins and the presence of higher levels of antinutritional compounds (Gilani et al., 2012). Thus, whilst protein adequacy may not be a major issue among western populations, tailored dietary strategies and processing solutions will be crucial to meet the specific needs of older individuals and populations in less industrialised regions.

Beyond nutritional considerations, attention must also be given to the sensory qualities of plant-based foods. Off-flavours, undesirable textures, and lower palatability remain significant barriers to consumer acceptance (Giacalone et al., 2022; Vatansever et al., 2020). Future research should explore how processing techniques, such as fermentation, can improve both taste and texture whilst maintaining or enhancing nutritional quality.

Moreover, although this thesis focuses on protein digestion occurring in the stomach and small intestine, it is also important to consider the effects of undigested proteins that reach the large intestine.

Proteins and peptides that escape digestion may undergo microbial fermentation in the colon, leading to the formation of both beneficial and potentially harmful metabolites (Peled & Livney, 2021; Rodríguez-Romero et al., 2022).

Looking ahead, a holistic approach that integrates nutritional adequacy, sensory quality, gut health, and sustainability will be vital for the successful development of next-generation plant-based foods. Future studies should include human studies, detailed investigations of protein–microbiota interactions, and consumer-focused product development, ensuring that scientific advances are translated into accessible, enjoyable, and health-promoting dietary solutions.

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Popular Science Summary

Plant-based diets are gaining popularity, not only for health reasons, but also because of the lower environmental impact. Whilst plant-based foods such as peas, beans, and soy are rich sources of proteins and minerals, the uptake of these nutrients in the body is often lower compared to animal-based foods. This is due to a combination of these nutrients being embedded in complex food structures and the presence of certain compounds (known as antinutrients) that can inhibit absorption.

In this thesis, the effect of different food processing methods on the structure, digestibility, and nutrient release in plant-based protein sources was investigated. The focus was on four legumes: soy, faba bean, yellow pea, and grey pea. By simulating human digestion and uptake in lab models, the degradation of protein and absorption of iron and zinc were evaluated.

The results showed that processing methods such as fermentation (as in tempeh), coagulation (as in tofu), and enzymatic treatment can significantly influence how nutrients are released during digestion. For instance, chemical analyses showed a more efficient protein degradation in tofu than in boiled beans. Fermentation reduced phytate levels, a compound that inhibits mineral absorption, and improved iron bioavailability from tempeh.

The structure of the food also proved to be important. In pea-based gels, stronger gel structures and gels with a higher fibre content made it more difficult for digestive enzymes to access and break down the proteins. The same effect was observed in tofu and tempeh, demonstrating that both structure and composition are crucial for how nutrients are absorbed.

In conclusion, this work shows that greater knowledge and optimisation of conditions and techniques for the processing of plant-based foods can improve their nutritional value and increase the uptake of key nutrients. These findings can support the development of healthier and more sustainable plant-based products, benefiting both people and the planet.

Populärvetenskaplig Sammanfattning

Allt fler människor väljer en växtbaserad kost, inte bara av hälsoskäl, utan också för att den har en lägre miljöpåverkan. Även om livsmedel baserade på ärtor, bönor och soja är rika på protein och mineraler, kan det vara svårare för kroppen att tillgodogöra sig dessa näringsämnen, jämfört med upptaget från animaliska livsmedel. Detta beror på en kombination av att näringsämnena är inbäddade i komplexa livsmedelsstrukturer, och förekomsten av vissa (så kallade antinutritionella) ämnen som kan hämma upptaget.

I avhandlingen undersöktes hur olika behandlingstekniker och tillagningsmetoder påverkar livsmedlets struktur, hur växtbaserade proteinkällor bryts ned i mag-tarmkanalen, och hur tillgängliga aminosyror (proteinets byggstenar) och mineraler är för upptag i kroppen. Fokus låg på fyra baljväxter: soja, åkerböna, gulärt och gråärt. Genom laboratoriemodeller av människans matspjälkningssystem, som efterliknar hur maten bryts ner och hur näringsämnen tas upp i tarmen, undersöktes hur väl kroppen kan ta upp proteiner och viktiga mineraler som järn och zink.

Resultaten visade att bearbetningsmetoder som fermentering (som i tempeh), koagulering (som i tofu) och enzymatisk behandling har stor påverkan på hur näringsämnen frisätts vid matspjälkning. Till exempel visade analyserna att proteinet i tofu bröts ner lättare än proteinet i kokta bönor. Fermentering minskade halten av antinutrienten fytat, vilket förbättrade tillgängligheten av järn från tempeh avsevärt.

Livsmedlets struktur visade sig också vara viktig. I ärtbaserade geler gjorde starkare gelstrukturer och högre fiberinnehåll det svårare för matsmältningsenzymerna att komma åt och bryta ner proteinerna. Samma effekt sågs i tofu och tempeh, vilket visar att både mikrostruktur och sammansättning är avgörande för hur näringsämnen blir tillgängliga för upptag i kroppen.

Sammanfattningsvis visar detta arbete att vi, genom ökad kunskap och optimering av tekniker för hur växtbaserade livsmedel bearbetas/tillagas, kan förbättra deras näringsvärde och underlätta kroppens upptag av viktiga näringsämnen. Det kan stödja utvecklingen av hälsosammare och mer hållbara växtbaserade produkter, något som gynnar både människan och planeten.

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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}	36^{de}	45^{abcde}	4	<0.001	25*	30*
Threonine	39^{abcd}	33^{e}	40^{abc}	34^{cde}	44^{a}	40^{abc}	33^{de}	39^{abcde}	38^{bcde}	33^{de}	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.

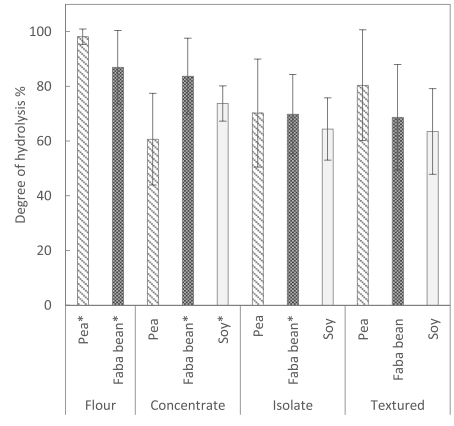

Fig. 2. Degree of hydrolysis (DH) in % for the different raw materials (n = 3 or n* = 2).

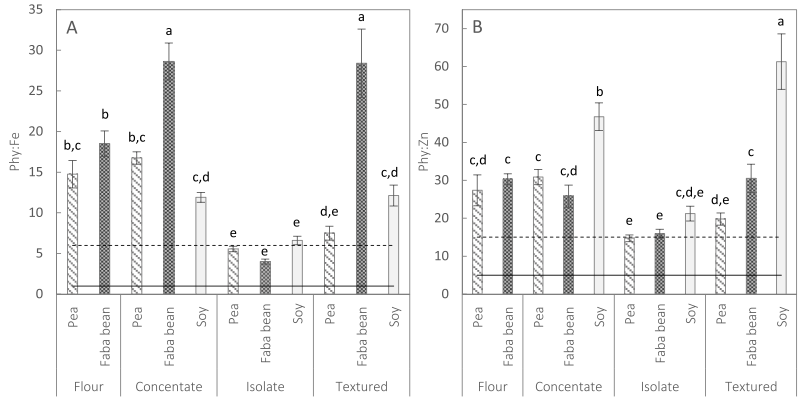

Fig. 1. Estimated mineral bioavailability based on the molar ratio of phytate to iron/zinc. (A) Molar ratio of phytate to iron (Phy:Fe), where Phy:Fe < 1 (solid line), or preferably <0.4, is needed for adequate iron absorption from plain cereal or legume-based meals without absorption enhancers. Phy:Fe = 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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The effect of transglutaminase and ultrasound pre-treatment on the structure and digestibility of pea protein emulsion gels

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ABSTRACT

This study examines the effects of ultrasound and transglutaminase pre-treatments on the structure, rheological properties, and digestibility of emulsion gels made from pea protein isolate and concentrate. Pre-treatments enhanced the elasticity and deformation resistance of gels made from pea protein isolate, with the combination of both treatments yielding the highest storage modulus. In contrast, emulsion gels from pea protein concentrate showed a more complex response, with untreated samples exhibiting higher storage modulus. These differences reflect variations in gelation behaviour between isolates and concentrates, likely due to differences in composition and extraction processes. Protein digestibility, assessed using the o-phthalaldehyde assay, showed significant differences between pre-treatments, but the impact was less pronounced compared to the difference between gels made from isolate and concentrate. Gels made from pea protein isolate had a hydrolysis degree of 77 %, while those from pea protein concentrate had 48 %, with this difference mainly attributed to the higher amounts of starch and fiber in the concentrate, which affected both the gel structure and digestibility. Nuclear magnetic resonance-based metabolomics revealed lower glucose release in transglutaminase-treated gels made from pea protein concentrate and lower glycine release from ultrasound and transglutaminase-treated gels made from pea protein isolate during gastric digestion. However, no significant differences were observed after intestinal digestion, indicating no major limitations in nutrient release due to processing. Overall, these findings highlight the role of protein source and processing methods in influencing rheological properties and nutrient bioavailability in protein systems.

1. Introduction

The consumption of more plant-based foods has a positive impact on environmental sustainability (Crippa et al., 2021; Kustar & Patino-Echeverri, 2021) and human health (Ahnen et al., 2019; Stilling, 2020). Among the various plant protein sources, pea protein (Pisum sativum L.) has garnered significant interest due to its low allergenicity, high nutritional value, widespread availability, and cost-effectiveness (Ge et al., 2020; Zahari et al., 2022). However, like other plant-derived proteins, its application as a food ingredient faces challenges, particularly in relation to functionality, flavour, and colour (García Arteaga et al., 2020; Lam, Karaca, et al., 2018).

The functional properties of proteins, which include water-binding capacity, solubility, and the ability to form network structures such as gels or films (Li-Chan & Lacroix, 2018), are greatly influenced by the extraction methods (Lam, Karaca, et al., 2018; Shand et al., 2007; Taherian et al., 2011). Consequently, considerable variation exists between pea isolates derived from different extraction protocols (Stone

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et al., 2015; Vogelsang-O'Dwyer et al., 2020) and those produced on an industrial scale (Taherian et al., 2011). While flours and concentrates are often obtained through milling and dry fractionation (Pelgrom et al., 2013), wet fractionation is commonly used for the production of isolates with a high protein content (Cui et al., 2020; Lam, Can Karaca et al., 2018). This difference in processing not only affects the final protein content but can also lead to alterations in the overall protein structure. Whereas isolates are often denatured during their commercial preparation (Osen et al., 2014), air-classified proteins tend to retain their native conformation, which in turn influences their functional properties (Arntfield & Murray, 1981). Considering that industrial-produced pea protein ingredients are consumed by a growing number of people, it is crucial to focus on improving their quality, functionality, and nutritional value to both meet consumer expectations and support sustainable food production.

To improve the functional properties of commercial pea proteins, a variety of physical, chemical, and enzymatic processes, as well as combinations thereof, have been explored (Eckert et al., 2019; García Arteaga et al., 2020; Klost & Drusch, 2019a; Li-Chan & Lacroix, 2018). Ultrasound pre-treatment is one such method that has been shown to modify proteins by altering their structure (Su & Cavaco-Paulo, 2021) and increasing solubility (Hu et al., 2013), which can expose more enzyme-active sites and enhance enzymatic catalytic efficiency (Su & Cavaco-Paulo, 2021; Tian, Lv, et al., 2024). For instance, combining ultrasound pre-treatment with transglutaminase has been reported to strengthen soy protein hydrogels (Hu et al., 2015).

Transglutaminase catalyses acyl-transfer reactions by transferring γ-carboxamide groups of glutamine residues to free ε-amino groups of lysine, leading to intra- and inter-molecular ε-(γ-glutamyl)-lysine (G-L) cross-links (Djoullah et al., 2015; Jong & Koppelman, 2002; Naqash et al., 2017; Shaabani et al., 2018). These modifications can significantly alter protein gel texture and structure (Schäfer et al., 2007; Sun & Arntfield, 2011, 2012) and affect the location and quantity of G-L isopeptides.

Although a substantial amount of research has focused on the structural effects of ultrasound and transglutaminase pre-treatments, there is limited information about how these modifications impact nutrient digestibility. Fang et al. (2021) demonstrated that cross-linking does not influence protein digestion and absorption, as G-L isopeptides are transported intact across the intestinal epithelium via passive paracellular diffusion. Conversely, other studies suggest that transglutaminase-induced changes in protein conformation and structure can alter digestion and absorption behaviours, ultimately influencing nutritional properties (Fang et al., 2021; Lang et al., 2020; Mei Wee & Henry, 2019; Monogioudi et al., 2011; Rui et al., 2016). Additionally, ultrasound pre-treatment alone has been shown to enhance the release and absorption of bioactive compounds in the gastrointestinal tract (Meena et al., 2024).

Thus, this study aimed to evaluate the effects of ultrasound pre-treatments and/or transglutaminase on protein structure, digestibility, and metabolite release. Pea protein emulsion gels were prepared using two commercially available pea protein powders to further assess how variations in protein structure and overall composition (e.g., protein, fibre, and starch content) affect gel structure and digestibility. Thereby, commercial pea isolate and concentrate were selected due to their differences in composition, widespread use, large-scale commercial availability, and documented functional and nutritional properties (Auer et al., 2024; Baune et al., 2021; Osen et al., 2014, 2015; Rekola et al., 2023).

The first part of the study focuses on evaluating the impact of processing and overall composition on gel structure using rheological measurements and advanced imaging techniques. In the second part, the characterised gels were digested following the standardised INFOGEST protocol. Protein digestibility was assessed using the OPA assay, whilst metabolite release was analysed via nuclear magnetic resonance (NMR) spectroscopy (Vidal et al., 2016). This approach provides comprehensive insights into the effects of ultrasound pre-treatment and transglutaminase on the structure, functionality, and digestibility of pea protein emulsion gels.

2. Material and methods

2.1. Materials

Two raw materials, pea protein isolate (PI) - Pisane C9 from Cosucra groupe Warcoing and pea protein concentrate (PC) - F55x from Vestkorn, were used in this study. Moreover, rapeseed oil (purchased at ICA in Uppsala), NaCl (Merck 1064041000 CAS-No: 7647145), and transglutaminase, Galaya Prime (Novozymes, 200 TGHU-A/g, CAS-No: 80146856) were incorporated into the gel system. Chemicals and enzymes used for the *in vitro* digestions were purchased from Sigma-Aldrich, including α-amylase from human saliva (A1031, CAS 9000-90-2), 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). Lipase (Rabbit Gastric Extract, RGE 15, LOT 1722 and 2504) was purchased from Lipolytech.

2.2. Compositional analysis

The composition analysis of the PI and PC (including protein, starch, fat, and fibre content (neutral detergent fibre (NDF) and acid detergent fibre (ADF), amino acid composition, as well as iron and zinc content) have been presented previously (Auer et al., 2024). However, because a different batch was used for the current study measurements for protein, moisture, and ash content were repeated and are included in this work. In addition, the dietary fibre composition was included as the presence of fibres can influence the structure and digestibility of the emulsion gels.

2.2.1. Protein

The crude protein content was determined through the Kjeldahl method, using a conversion factor of 5.4 (FAO/WHO, 2011). The acidic digestion and protein determination was 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. Dietary fibre

The total dietary fibre of the PI and PC was determined according to the Uppsala method (Theander et al., 1995). Soluble and insoluble dietary fibre were analysed according to Andersson et al. (1999). Briefly, non-resistant starch was removed by α-amylase and amyloglucosidase, and the remaining polysaccharides were precipitated by 80 % ethanol. Polysaccharides were hydrolysed by acid and quantified as alditolacetates by gas chromatography.

2.2.3. Ash and moisture

The 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, Minnesota, United States) at 550 °C for 12 h, cooled in a desiccator for 1 h, and re-weighed. The dry matter content was determined according to AOAC official method 934.01, by drying the samples to a constant weight (>16 h) in a convection oven (Model 2000655, J:P: Selecta, Barcelona, Spain) at 105 °C. Both analyses were performed in duplicate.

2.3. Preparation of the emulsion gels

Dry ingredients (12 % w/w protein isolate or concentrate, 1.5 % w/w NaCl) were dispersed in distilled water and stirred for 30 min, followed by pH adjustment (pH 7) using 1 M NaOH. The protein solution was treated with ultrasound (Sonics VCX – 750 vibra cell, Sonics & Materials, Inc., Newtown, USA) following the method described by Hu et al. (2015)

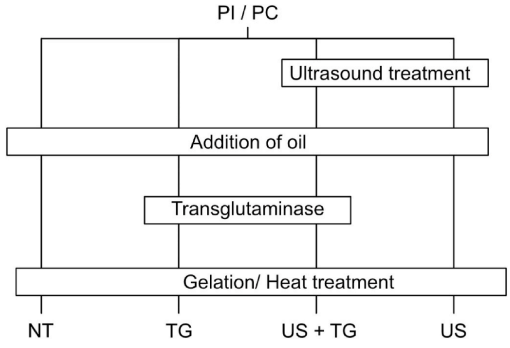

Fig. 1. Schematic overview of the emulsion gels prepared from pea isolate (PI) and pea concentrate (PC), including the different pre-treatments, consisting of ultrasound pre-treatment (US), the addition of oil, the addition of transglutaminase (TG), a combined ultrasound and transglutaminase pre-treatment (US/TG), heat treatment, and a control without any pre-treatment (NT).

and Xiong et al. (2018), with minor modifications to the amplitude settings to shorten the sonication time and effectively disrupt protein aggregates. Briefly, the suspensions were treated at a frequency of 20 kHz with amplitudes corresponding to 300 W (30 s), 525 W (30 s), and 750 W (60 s). To reduce the temperature increase during the sonication process, the solution was kept on ice, and the temperatures of the suspensions did not exceed 40 °C. After the sonication (or after pH adjustment for samples without ultrasound pre-treatment), the oil (15 % w/w) was introduced to the system using an Ultra Turrax T25 (Janke & Kunkel IKA- Labortechnik, Staufen, Germany) at 13,500 rpm for 60 s. To remove entrapped air, the emulsion was placed in a vacuum chamber and degassed for 40 min before the transglutaminase was added (2 TGHU-A/100g of protein). An overview of the emulsion gels prepared from PI and PC, along with the various pre-treatments applied, is presented in Fig. 1.

2.4. Dynamic rheological measurements

To study the gelation processes of the different emulsion gels (described in section 2.3) a Discovery HR-3 rheometer (TA Instruments, New Castle, DE, USA) equipped with a 40 mm aluminium plate (112471) was used with a gap of 1 mm. The emulsion was kept at 50 °C (temperature optimum for transglutaminase) for 60 min before being heated up to 95 °C (gelation temperature). The temperature was then set for 15 min before cooling down to the starting temperature of 25 °C. The temperature increase/decrease was carried out at a ramp rate of 1.5 °C/min. To reduce the evaporation of the sample during the measurement, paraffin oil was used, combined with a solvent trap (Saldanha do Carmo et al., 2020). The storage modulus (G') and loss modulus (G'') were recorded at a frequency of 1 Hz and a strain of 0.5 %. To further characterise the viscoelastic properties, the $tan(\delta)$ was calculated as the ratio of the G'' to the G'. Additionally, an amplitude sweep was performed on each sample after gelation and reaching room temperature (25 °C, 30 min). The amplitude sweep was recorded at a frequency of 1 Hz and a strain of 0.01 %–100 %. The linear viscoelastic region of the gels was determined by observing a 5 % drop in the storage modulus from the average value of the plateau. The obtained fracture point is then described as oscillation strain (%) and oscillation stress (Pa).

2.5. Microstructure analysis

2.5.1. Microscopy

Suspensions were prepared as described in 2.3. Following this, 2.5 mL of each emulsion was placed in a glass vial (⌀12 mm) and heated in the same manner as described in 2.4. using a water bath (DYNEO DD-1000F Refrigerated/heating circulator, Julabo, Seelbach, Germany) and stored at 4 °C overnight. The gels were then cut into approximately $2 \times 2 \times 2\ mm^3$ cubes and fixated overnight in 2.5 % glutaraldehyde (Ted pella Inc., 18427) and 0.1 % ruthenium red solution (Ted pella Inc., 19421) followed by 1 % osmium tetraoxide (Ted pella Inc., 18466) for 2 h (Langton et al., 2020). The samples were dehydrated in a graded ethanol series with increasing concentrations: 30 % for 10 min, 50 % for 20 min, 70 % for 20 min, 90 % for 20 min, 95 % for 20 min, and 100 % for 2 h. For light microscopy (LM), the samples were infiltrated and hardened using Technovit 7100 (Kulzer technik). The embedded samples were then sectioned into 1-μm sections using an ultramicrotome (Leica Microsystems GmbH, Leica EM UC6, Wetzlar, Germany). The sections were stained with light green (Sigma Aldrich, L1886) and iodine (Fluka, 03551). To visualise the structure, a microscope (Nikon, Eclipse Ni–U microscope, Tokyo, Japan) equipped with a 40 × (0.75 NA) apochromatic objective was used. Images were captured with a Nikon Digital Sight DS-Fi2 camera (Nikon, Tokyo, Japan) with 0.12 μm/pixel. For scanning electron microscopy (SEM), samples were dried after the dehydration step with a critical point dryer (Quorum Technologies Ltd, K850 Critical Point Dryer, East Sussex, UK). The dry samples were fractured, sputter-coated with gold (Cressington Scientific Instruments, Sputter coater-108 auto, Watford, UK) and examined at 5 kV (Hitachi, FlexSEM 1000II, Tokyo, Japan). Images were recorded at a magnification of × 1000 (9.9 nm/pixel).

2.5.2. CT and image analysis

For the CT scans samples were prepared in the same manner as for the SEM and scanned using the RX Solutions Easytom 160 (RX Solutions, Franc) equipped with a flat panel detector (1920 * 1536 pixel flat panel detector, minimum voxel size 50 nm). The samples were scanned with 60 kV and a current of 111 μA. The number of projections was 4000 and the voxel size was 0.5 μm. To determine the droplet size distribution, a subvolume of $0.1\ mm^3$ (⌀ 710 μm, h 250 μm) was analysed using AVIZO 3D (2023.2, Thermo Fisher Scientific, Waltham, MA, USA). For the characterisation, the reconstructed sub-volumes were filtered (Anisotropic Diffusion; Threshold 92.6, Iterations 4) before the threshold (Interactive Thresholding) was set at an intensity range between 140 and 255. To separate the oil droplets Chamfer-conservative (neighborhood 6) method was used and the individual droplet volume was used to determine the droplet size distribution.

2.6. In vitro digestion

2.6.1. Sample preparation

Prior to digestion, the emulsion gels were pressed through a perforated sheet (⌀ 1 mm) to simulate the mastication and obtain an even particle size. The amount of gel used for the digestion was normalised according to the protein content of the final gel (0.04g of protein per gram of food) based on the protein content of the PI and PC. For each digestion, 0.25g of a protein-free cookie was added to each digestion to reduce the autolysis of digestive enzymes (Sousa et al., 2023). For the cookie, 40.8 g purified corn starch, 15.7 g sucrose, 4.9 g cellulose, 0.7 g baking powder, 0.5 g ground ginger, and 36.9 g margarine were mixed and baked at 175 °C in portions of ~35 g for 30 min. All cookie ingredients were bought at a local supermarket (ICA, Sweden), except the cellulose (Merck). Lastly, water was added to the gel and cookie to reach an initial weight of 1g of food.

2.6.2. In vitro digestion protocol

To determine the enzymatic activities all enzyme assays were carried out as described in the supplementary information provided by Brodkorb et al. (2019). However, adjustments were made to measure the trypsin activity (Sousa et al., 2023). In short, the pancreatin was suspended in simulated intestinal fluid at a concentration of 100 U trypsin

activity/mL of digest, vortexed for approximately 10 s, followed by ultrasound pre-treatment (Ultrasound bath Elma D-78224, 50/60 Hz, 35W) at room temperature for 5 min. Thereafter, the suspension was centrifuged (Heraeus Pico 17 Centrifuge with 75003424 Fixed Angle Rotor Lab, Thermo Electron Corporation) for 5 min at 2.000×*g* at room temperature. The supernatant was transferred into a new tube, immediately placed on ice, and used for trypsin activity measurements. The same preparation method was used during the digestion experiment. The concentration of bile salts within the bile was determined using a Bile Acid Assay Kit (Sigma-Aldrich MAK309).

The *in vitro* digestion was carried out as described by (Brodkorb et al., 2019) with minor modifications (Sousa et al., 2023). All digestion experiments were performed in triplicates, including one blank (water + cookie) for each digestion cycle, with the same batch of enzymes (amylase activity 79.3 U/mg, pepsin activity of 2677.2 U/mg, trypsin activity in pancreatin of 6.33 U/mg, lipase activity 15.4 U/mg and bile acid concentration of 1.84 mmol/g). To maintain a constant temperature during the digestion an overhead rotor (Tube Revolver Rotator, Thermo Scientific™, Waltham, USA) set to 40 rpm was placed in an incubator (9010-0313 Binder, Tuttlingen, Germany) set to 37 °C.

In the oral phase (2 min at 37 °C), 1g of food (4 % protein w/w, cookie, water) was mixed with 0.8 ml simulated salivary fluid (pH 7), 5 µl $CaCl_2$, 0.1 ml salivary amylase (75 U/ml), and 0.095 ml of Milli-Q water. For the gastric phase (120 min at 37 °C), 1.6 ml of simulated gastric fluid (SGF) and 1 µl $CaCl_2$ were added before the pH was adjusted to 3 using 1M HCl. Following this, 0.1 ml of pepsin with a corresponding 2000 U/ml digesta and 0.1 ml RGE with a corresponding 60 U/ml digesta were added to the mixture. Further, Milli-Q water was added to the mixture to reach a total volume of 4 ml. For the intestinal phase (120 min, 37 °C) 1.7 ml simulated intestinal juice (SIF) and 8 µl $CaCl_2$ were added. Afterwards, the pH was adjusted to 7 using 1M NaOH. The pancreatin was prepared as described earlier and 1 ml pancreatin/SIF mix (100 U trypsin activity/mL of total digesta) and 0.5 ml bile/SIF mix (10 mM of total digesta) were added. Lastly, Milli-Q water was added to the mixture to reach a total volume of 8 ml. The weight and pH of the digesta were monitored through the different digestion steps and the final pH after digestion was <7.29 for both the blanks and samples. For all digestion experiments, samples were collected at the end of both the gastric and intestinal phases. The digestion process was terminated by heating the digesta to 100 °C for 5 min, followed by rapid freezing using liquid nitrogen. The samples were then stored at −20 °C until further analysis.

2.7. Degree of hydrolysis

The digestibilities of the *in vitro* digested emulsion gels were assessed by measuring free amino groups in the gastric and intestinal digests (degree of protein hydrolysis, DH). DH was determined in triplicate, using the o-phthaldialdehyde (OPA) method (Nielsen et al., 2001). For the OPA reagent, 7.62 g sodium tetraborate decahydrate (Merck, 1063080500, 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 Benzene-1,2-dicarboxaldehyde 98 % (OPA, BLDpharm, CAS 643-79-8), were dissolved in 4 mL ethanol, and 176 mg DL-dithiothtreitol (DTT, Sigma-Aldrich, CAS 3483-12-3) were added to the reagent. Further the solution was made up to a total volume of 200 mL. For the serine standard, a concentration range of 0.19–0.95 mmol/L (DL-Serine, Alfa Aesar, A11179, CAS 56-45-1) was prepared. For the calibration curve, 400 µL of standard solution was 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 the degree of protein hydrolysis in the digesta, the samples were centrifuged at room temperature for 10 min at 10,000×*g* (Heraeus Pico and Fresco 17, Thermo Fisher Scientific, Waltham, USA) and absorbance was then 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 the concentration of free amino groups in each digested sample after blank correction, expressed as serine equivalents/g protein. Total NH2 (acid hydrolysate) is the total amount of free amino groups after acid hydrolysis. Acid hydrolysis was conducted at 100 °C for 18 h using 6 mol/L HCl. For the pea isolate, total free amino acid concentration was 7.03 ± 0.17 mmol/g protein, whilst for the pea concentrate it was 7.87 ± 0.14 mmol/g protein, respectively. These values are in agreement with previously presented values that are based on the amino acid composition (Auer et al., 2024).

2.8. NMR-based metabolomics

Emulsion gels were analysed using NMR-based metabolomics to further characterise the effect of the pre-treatments on the digestibility of different metabolites. Digested samples from both the gastric and intestinal phases (see Section 2.5.2), along with four blank digestions from each phase, were included in the analysis, resulting in a total of 64 samples.

2.8.1. Sample preparation for NMR analysis

Each digesta was centrifuged for 30 min, at 10,000×*g* at 4 °C (Eppendorf centrifuge 5430R, Eppendorf Zentrifugen GmbH, Leipzig, Germany). Each aqueous supernatant (500 µL) was subjected to ultrafiltration (≥7 h, 10,000×*g*, 4 °C) to remove macromolecules (Tiziani et al., 2008). Ultrafiltration was carried out after each filter unit (Nanosep 3K omega, Pall Life Sciences) had been washed eight times by centrifugation (8 min, 4000×*g*, 36 °C) of 0.5 mL MilliQ-H_2O (MilliPore Synergy® UV ultrapure type 1 water purification system). The filtrate (100 µL) was mixed with MilliQ-H2O (380 µL), D_2O (60 µL; 99.8 atom % deuterated, Cortecnet), and 60 µL internal standard (TSP) consisting of 0.001 % (w/w) 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt (98 atom % deuterated, Cambridge Isotope Laboratories). The sample (600 µL) was transferred to a 5 mm NMR tube. Sample preparation was performed on ice.

2.8.2. NMR analysis

A one-dimensional (1D) 1H NMR spectrum was acquired for each sample on a Bruker Avance III 600 MHz spectrometer with a 5 mm $^{1}H/^{13}C/^{15}N/^{31}P$ inverse detection cryoprobe and a z gradient. Data was recorded at 25 °C employing Bruker's *zgesgp* pulse sequence (to suppress the water signal) at 4 s relaxation delay, 64 transients, 30 ppm spectral width, and 65,536 collected data points, similar to a previous study (Wagner et al., 2014).

2.8.3. Data processing

The Chenomx NMR Suite Professional Software (version 8.3, Chenomx Inc., Edmonton, Canada) was used to process data, including zero-filling (at least 128K), line broadening (0.3 Hz), manual phase correction, and setting the TSP signal (δ = 0.0 ppm). Each processed spectrum was imported to MATLAB (version 8.0.0.783 - R2012b, MathWorks Inc., Natick, Massachusetts, United States) for automated baseline correction (airPLS; Zhang et al., 2010), alignment (icoshift; Savorani et al., 2010), and binning with internal standard normalisation, which reduced each spectrum to 880 data points (0.01 ppm/bucket) in the chemical range of 0.5–8.5 ppm. The water region (4.6–5.1 ppm) was excluded prior to multivariate statistics.

2.8.4. Multivariate statistics

Multivariate statistics were done using MetaboAnalyst 6.0 (Pang et al., 2024). After applying a variance filter (default settings), binned data was Pareto scaled for partial least square discriminant analysis

Table 1
Compositional analyses of the pea isolate and concentrate including total protein and fibre, ash, and moisture content.

Composition	Pea isolate	Pea concentrate	p-value
Protein	73.79 ± 0.57[a]	42.7 ± 0.83[b]	<0.001
Total fibre	2.21 ± 0.11[b]	11.94 ± 0.12[a]	<0.001
Ash	5.33 ± 0.01[b]	5.93 ± 0.01[a]	<0.001
Moisture[a]	7.6 ± 0.2[b]	8.6 ± 0.2[a]	<0.001

Composition expressed as g/100 g dry weight ± standard deviation.
[a] Expressed as g/100 g raw material.

(PLS-DA) with stratification by raw material (PI, PC) and sample collection phase (gastric and intestinal phase). Each model was evaluated by 5-fold cross validation (CV) and permutation testing (n = 2000; B/W ratio) and was considered significant if Q2>0.5 and p-value<0.05.

2.8.5. Targeted profiling

Using Chenomx, the 1H NMR signals corresponding to the top-ranking features in multivariate statistics were specifically targeted for profiling – i.e. both metabolite identification and quantification (Weljie et al., 2006). Quantitates (μM) were estimated by manually adjusting selected metabolite signals (600 MHz library; version 10) in a pre-determined order. This ensured that the sum of signals matched the corresponding experimental signals (a strategy to reduce overestimated concentrations due to overlapping signals introduced in Röhnisch et al. (2018)). A dilution factor of six was applied to obtain sample concentrations (μM).

2.9. Statistics

The results from the dynamic rheological measurements, droplet size distribution, and compositional analysis are presented as means and standard deviations. One-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) at a confidence interval of 95 % was used to compare the means. All dynamic rheological measurements were log-transformed prior to statistical analysis (except Tan δ) using R (Version 4.3.0, RStudio Inc., Boston, USA).

The results from the OPA and targeted profiling of the NMR spectra were summarised as mean and standard deviation. Data was log-transformed for statistical significance testing with one-way ANOVA followed by Fisher's LSD (post-hoc). The MetaboAnalyst 6.0 framework was used for the NMR data, whereas R (Version 4.3.0, RStudio Inc., Boston, USA) was used to analyse OPA results. ANOVAs with p-values<0.0018 were considered significant, based on Bonferroni correction for multiple testing (n = 28; α = 0.05). Multivariate data analysis was performed using Principal Component Analysis (PCA) in SIMCA (Version 17.0, Umetrics, Sweden) to explore the relationships between pea protein isolates (PI) and concentrates (PC), pre-treatments, structural characteristics, and digestibility. The PCA biplot was used to visualise the clustering of samples and correlations between compositional and structural variables. Data was autoscaled before analysis to ensure equal weighting of all variables. The results were interpreted based on the positioning of samples and feature loadings along the principal components.

3. Results and discussion

3.1. Compositional analysis

The compositional analyses (Table 1) show that the pea isolate (PI) had a significantly ($p < 0.001$) higher protein content than the concentrate. In contrast, the pea concentrate (PC) had a significantly ($p < 0.001$) higher fibre content than the isolate. The ash and moisture content also significantly ($p < 0.001$) differed between raw materials, although the differences were less dominant compared to the protein and fibre content. Based on previous characterisation, further differences in fat and starch content were found between the PI and PC. The PI contained 0.2 g/100 g DM starch and 5.7 g/100 g DM fat, whereas the PC contained 4.7 g/100 g DM starch and 3.5 g/100 g DM fat, respectively (Auer et al., 2024). However, regarding the overall composition, it should be noted that not all carbohydrates were quantified, as the Uppsala method does not account for all monosaccharides (Theander et al., 1995).

Table 2
Determination of dietary fibre (g/kg dry matter).

		Pea isolate	Pea concentrate	p-value
Insoluble sugar residues	Rhamnose	0.9 ± 0.2[b]	2.3 ± 0.0[a]	0.0125
	Fucose	0.2 ± 0.1[b]	0.8 ± 0.1[a]	0.045
	Arabinose	3.9 ± 0.1[b]	47.0 ± 0.5[a]	<0.001
	Xylose	0.6 ± 0.0[b]	5.7 ± 0.1[a]	<0.001
	Mannose	2.4 ± 0.2	2.8 ± 0.1	0.147
	Galactose	3.5 ± 0.0[b]	8.0 ± 0.2[a]	<0.001
	Glucose	2.5 ± 0.0[b]	24.5 ± 0.4[a]	<0.001
	Uronic Acid	3.3 ± 0.1[b]	19.9 ± 0.1[a]	<0.001
Soluble sugar residues	Rhamnose	n.a.	n.a.	
	Fucose	n.a.	n.a.	
	Arabinose	0.1 ± 0.0[b]	1.7 ± 0.1[a]	0.002
	Xylose	0.1 ± 0.0[b]	0.2 ± 0.0[a]	0.0389
	Mannose	0.5 ± 0.0[a]	0.2 ± 0.1[b]	0.0471
	Galactose	0.2 ± 0.0[b]	1.1 ± 0.0[a]	<0.001
	Glucose	0.1 ± 0.0[b]	0.4 ± 0.0[a]	0.0102
	Uronic Acid	1.0 ± 0.1[a]	1.2 ± 0.0[a]	0.0677
	Klason lignin	2.9 ± 0.3	3.4 ± 0.0	

The amount of total fibre found in the PC was in a similar range as previously presented results for milled peas (Martineau-Côté et al., 2022) wherein 14.7g/100g dry weight was reported. However, higher amounts of fibre (18 and 19 g/100g powder) were found in both PI and PC by (Muneer et al., 2018) who simultaneously reported a similar protein content. The differences in total fibre content and composition can be a result of differences in growing conditions, variety, as well as the protein extraction process (Cui et al., 2020). Concentrates are typically produced by dry fractionation (Pelgrom et al., 2013), which retains more fiber and starch, while isolates are made using wet fractionation, resulting in higher protein purity (Cui et al., 2020; Lam, Can Karaca et al., 2018).

The fibre composition of the pea products is presented in Table 2. Thereafter, for both raw materials, arabinose and uronic acid are the most dominant sugar residues in the insoluble fraction, together with galactose in the PI and glucose in the PC.

This is in line with previous results from (Martín-Cabrejas et al., 2003) after which glucose, arabinose, and uronic acid were the most dominant sugars in the insoluble fractions. In the soluble fibre fraction, Arabinose, Galactose, and Uronic acid are dominant in the PC, while for PI, the dominant fractions are Mannose, Galactose, and Uronic acid. However, depending on the conditions of germination, the fibre composition of both the soluble and insoluble fractions can vary (Martín-Cabrejas et al., 2003). Moreover, the fibre composition of the hull and cotyledon can differ, and this can further influence the final composition depending on what fractions remain in the product after the protein extraction (Dalgetty & Baik, 2003).

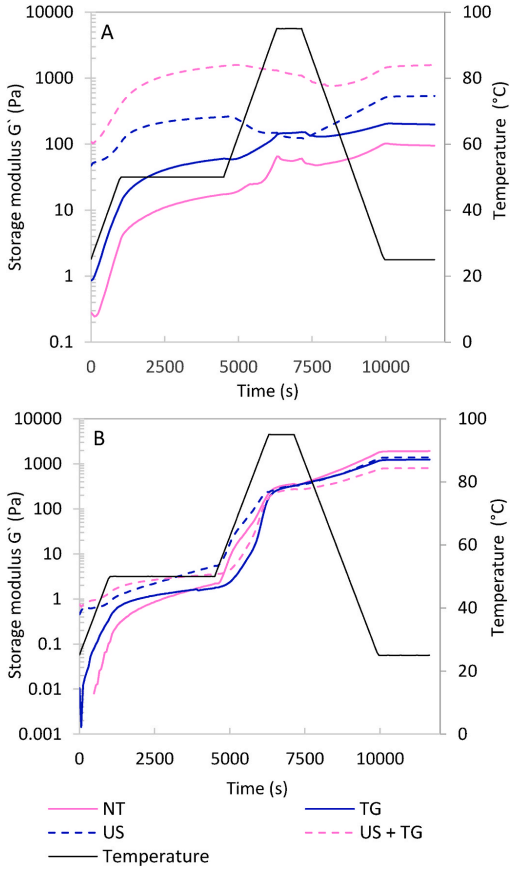

Fig. 2. Recorded changes in storage modulus (G') of pea protein isolate emulsion gels (A) and pea protein concentrate emulsion gels (B) during the heating and cooling period. The figure includes the effects of various pre-treatments: ultrasound pre-treatment (US), transglutaminase treatment (TG), a combination of ultrasound and transglutaminase (US/TG), and a control without any pre-treatment (NT).

3.2. Dynamic rheological measurements

The results from the rheology measurements are displayed in Fig. 2 and Table 3. Based on the recorded changes in the storage modulus (G'), it is evident that the pea isolate emulsion gels (PIEG) without any pre-treatment (NT) result in gels with the lowest G' (Fig. 2A). The addition of transglutaminase (TG) as well as the use of ultrasound (US) as pre-treatment increases the final G', whereas the combination of both pre-treatments results in the highest G'. Thus, the different pre-treatments make the PIEG both more elastic and resistant to deformation compared to the gel without any pre-treatment. However, it must be noted that the initial G' was already higher in the samples treated with US compared to those without any pre-treatment or TG pre-treatment, indicating that the US pre-treatment causes changes in the initial structure. This may be attributed to prior denaturation of the protein during the extraction process, as suggested by previous results on the same type of isolate (Osen et al., 2014).

Table 3
Results from the dynamic rheology measurements including the storage modulus (G') at the beginning of the measurements at 25 °C, after the incubation at 50 °C, and the final G' after cooling to 25 °C as well as the results from the amplitude sweep (Oscillation strain %, Oscillation stress Pa at the fracture point) and Tan δ. The result include gels made from pea isolate and concentrate treated with transglutaminase (TG), ultrasound (US), combined ultrasound and transglutaminase treatment (US + TG), and untreated (NT).

	Pea Isolate					Pea concentrate				
	NT	TG	US	US + TG	p-value[a]	NT	TG	US	US + TG	p − value[a]
G'25 Pa	0.3 ± 0.08^{e}	0.9 ± 0.4^{b}	50.6 ± 5.7^{a}	99.8 ± 44.5^{a}	<0.001	0.03 ± 0.01^{b}	0.02 ± 0.01^{b}	0.5 ± 0.1^{a}	0.6 ± 0.3^{a}	<0.001
G'50 Pa	17.42 ± 5.56^{d}	60.33 ± 4.55^{c}	258.48 ± 28.48^{b}	1532.80 ± 193.66^{a}	<0.001	2.2 ± 0.6^{bc}	1.8 ± 0.8^{c}	5.4 ± 2.2^{a}	3.6 ± 0.4^{ab}	0.017
G' Final Pa	95.0 ± 12.3^{d}	198.6 ± 33.8^{c}	536.4 ± 74.0^{b}	1585.7 ± 529.6^{a}	<0.001	1923.3 ± 157.7^{a}	1241.0 ± 54.3^{b}	1397.8 ± 221.9^{b}	801.8 ± 120.2^{c}	<0.001
Oscillation Strain %	5.4 ± 1.3^{d}	12.1 ± 6.7^{c}	23.3 ± 0.3^{b}	61.3 ± 2.2^{a}	<0.001	29.3 ± 3.5	29.9 ± 9.5	33.5 ± 5.9	33.6 ± 8.0	0.813
Oscillation Stress Pa	2.3 ± 0.5^{d}	14.1 ± 10.3^{c}	94.2 ± 16.8^{b}	1093.9 ± 213.6^{a}	<0.001	294.4 ± 15.2	274.1 ± 19.1	291.2 ± 138.5	167.3 ± 14.8	0.171
Tan δ Final	0.263 ± 0.012^{a}	0.131 ± 0.055^{b}	0.148 ± 0.016^{b}	0.130 ± 0.008^{b}	0.001	0.158 ± 0.002^{a}	0.129 ± 0.007^{b}	0.156 ± 0.009^{a}	0.125 ± 0.001^{b}	<0.001

[a] One-way ANOVA was used to determine differences between pre-treatments. Different superscript letters indicate significant differences according to LSD = least significant difference ($P < 0.05$). Statistical analysis was performed on log transformed data.

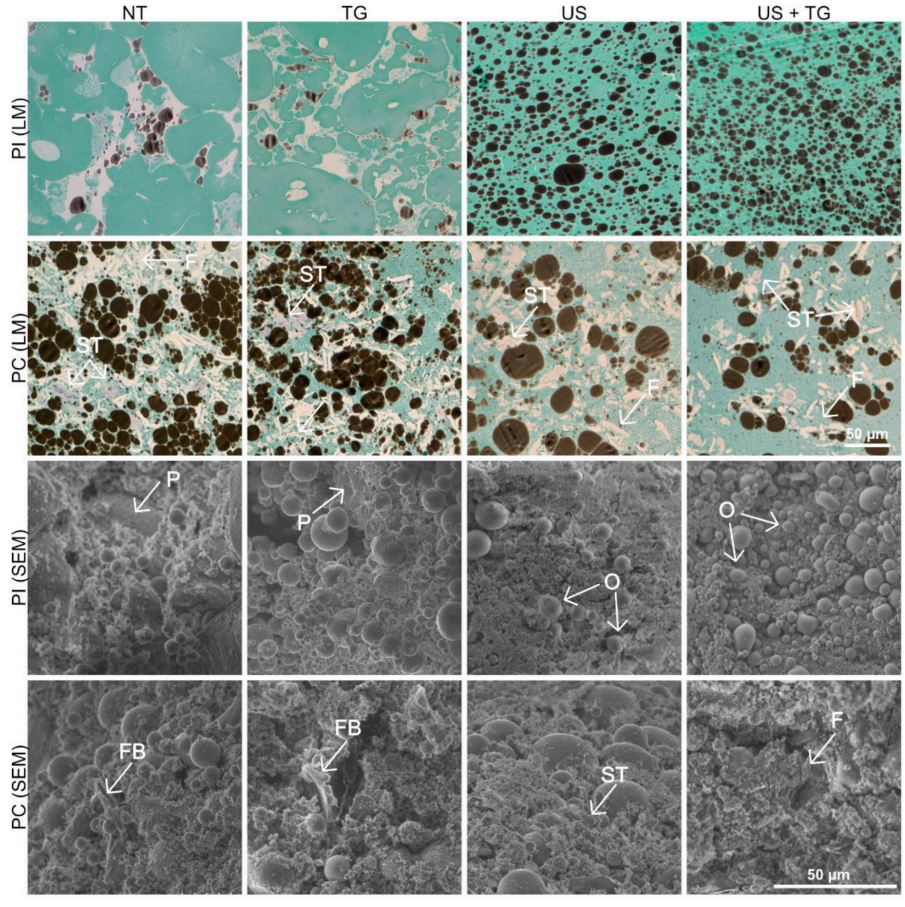

Fig. 3. Microstructure of the pea protein gels made from pea isolate (PI) and pea concentrate (PC), including the effects of ultrasound pre-treatment (US), transglutaminase treatment (TG), a combination of ultrasound and transglutaminase (US/TG), and a control without any pre-treatment (NT), using light microscopy (LM) and electron microscopy (SEM). In the light micrographs, proteins (P) are stained blue/green, oil droplets (O) appear black, and starch granules (ST) are stained purple/red. In both the light and electron micrographs, the different structural features are indicated with arrows.

Contrastingly, the pea concentrate emulsion gels (PCEG) showed a higher increase in the G' during the second heating step to 95 °C, indicating changes in their internal structure at higher temperatures (Fig. 2 B). This may be attributed to differences in protein state. Whereas pea protein isolates are reportedly denatured, air-classified proteins tend to remain in their native form, making them more prone to structural changes during heat treatment (Arntfield & Murray, 1981). Further, the PCEG without any additional pre-treatment resulted in the highest G', whereas the addition of TG or the use of US pre-treatment led to a reduction in the G'. Combining US pre-treatment with TG showed the lowest final G'. Nevertheless, differences in the final G' are less dominant in the PCEG, indicating a lower overall effect of the different pre-treatments than that observed in the PIEG.

The results from the amplitude sweep and tan δ are presented in Table 3. The oscillatory strain and stress were determined at the fracture point, defined as a 5 % drop in the storage modulus from the average plateau value. A high oscillatory strain, therefore, indicates a longer Linear Viscoelastic Region (LVR), whereas a high oscillatory stress suggests a more rigid, stable, and well-structured gel network.

The PIEG treated with both US and TG showed the longest linear viscoelastic region (LVR) and thus the highest oscillation strain as well as the highest oscillation stress, indicating a more stable and structured gel network.

In contrast, samples without any pre-treatment or with only US or TG pre-treatment had a significantly shorter LVR and lower oscillation stress, suggesting that softer gels have a less organised network. For the PCEG, no significant differences in oscillation strain ($p = 0.813$) or oscillation stress ($p = 0.171$) were observed between the pre-treatments. Overall, the PCEG samples were more rigid than the PIEG (except for the US + TG-treated PIEG) but still softer and less structured than the US + TG-treated PIEG, which formed the most rigid gel.

Significant differences ($p < 0.001$) in tan δ were found between the PCEG treated with TG and the samples without TG, indicating that the TG affects the protein structure, resulting in a lower tan δ and a more solid-like behaviour. Further, significant ($p < 0.001$) differences in tan δ were found between the pre-treated and untreated PIEG. Thereafter,

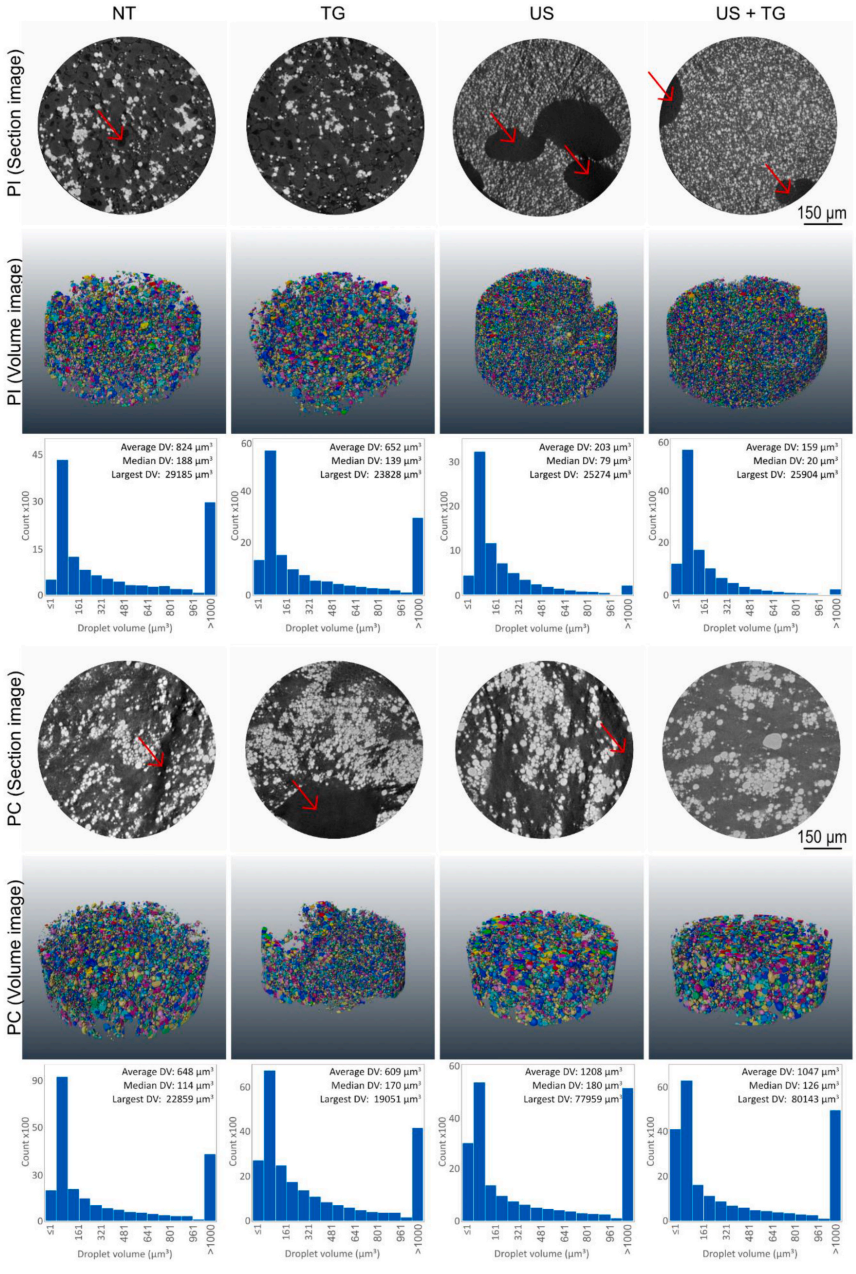

Fig. 4. Sectional images of gels made from pea protein isolate (PI) and pea protein concentrate (PC) after different pre-treatments: ultrasound (US), transglutaminase (TG), a combination of both (US/TG), and a control with no pre-treatment (NT). Computed tomography (CT) reconstructions show oil droplets (light grey) within the protein network (dark grey), and entrapped air (black), marked by red arrows. The volume renderings illustrate the oil droplet network, and the individual droplets are colored arbitrarily for visualization purposes. Droplet size distribution, including maximum, average, and median sizes, was analysed using AVIZO and is displayed below the volume images.

pre-treated emulsion gels resulted in a lower tan δ compared to the gels without pre-treatment. However, no significant differences were found between the pre-treatments. This suggests that all pre-treatments improved the elastic properties of the emulsion gels made from pea isolate, whereas a similar effect was only observed for the PCEG treated with TG and US + TG.

The positive effect of US pre-treatment on the protein structure and functional properties has been presented previously for various proteins (Higuera-Barraza et al., 2016; Su & Cavaco-Paulo, 2021).

US pre-treatment can reduce particle size, induce partial unfolding of the proteins, and increase exposed hydrophobicity, resulting in a reduction of the surface tension at the air−water interface (Xiong et al., 2018). This could improve the solubility and fluid character of soy protein isolate (Hu et al., 2013) and pea isolate (Jiang et al., 2017; Xiong et al., 2018) leading to more physically stable emulsion systems and a tendency of increased oxidative stability (Sha et al., 2021). Although the current study showed that US pre-treatment improved the rheological properties of PIEG, no increase in G′ or tan δ was observed for PCEG as a result of the treatment. This may be due to differences in protein state and particle size (see Fig. 3) between the PI and PC, and also the presence of higher amounts of starch and fibre in the concentrate. Starch granules gelatinize in the presence of water and appropriate temperatures, influencing the overall gel properties (Keskin et al., 2022). In addition, fibres can further influence the gelation properties (Geerts et al., 2017; Johansson et al., 2022; Klost & Drusch, 2019b) resulting in gels with an increased G' and Tan δ (Hou et al., 2022; Johansson et al., 2022).

TG has also been widely used to improve the technological properties of proteins, although globular proteins are often poorly susceptible or unsusceptible to its action (Djoullah et al., 2018). However, when combined with other pre-treatments, such as high-pressure pre-treatment, TG can be used for the techno-functional properties of globular proteins (Neto Queirós et al., 2023). Moreover, the use of US pre-treatment has been successfully used to expose more reactive groups of pea protein, promoting the catalytic efficiency of TG (Mozafarpour & Koocheki, 2023; Wang et al., 2023). Although a significant decrease in tan δ has been observed in the PC gels treated with TG, the addition of TG did not increase the storage modulus, meaning the gels resulted in a lower overall stiffness with still predominantly elastic properties, with minimal viscous dissipation. This has been observed in emulsion gels made from soy protein isolate, where extensive enzymatic crosslinking reduced emulsifying ability and resulted in emulsion destabilization (Luo et al., 2019; Tang et al., 2013). A similar trend was observed in tan δ for the PIEG, although an increase in the G' was also observed if the TG was combined with US pre-treatment. This underlines the importance of using pre-treatment to expose more reactive groups for the TG to catalyse the acyl transfer reaction between glutamine residues and primary amines (Neto Queirós et al., 2023). Furthermore, the presence and distribution of the oil droplets in the gel matrix can further influence the structure and properties of the gels (Zhan et al., 2022).

3.3. Microscopy

The microstructure of the gels was characterised using light microscopy (LM) and scanning electron microscopy (SEM) (Fig. 3). Comparing the PIEG (both LM and SEM micrographs), the gels without US pre-treatment exhibit large protein aggregates with small oil droplets unevenly distributed between them. In contrast, the PIEG with US pre-treatment shows a continuous protein phase with oil droplets more evenly distributed. Additionally, both LM and SEM micrographs of the PIEG reveal a small fraction of unbound and unaggregated protein acting as a filler between the larger aggregates and oil droplets. Overall, PIEG forms more cohesive and homogeneous networks, particularly after US and US + TG treatments, resulting in improved emulsification and gelation.

The PCEG displays a continuous protein phase across all samples, with a significant amount of fibers and starch embedded within the structure. SEM micrographs consistently show that PCEG gels have rougher, more fibrous networks due to the presence of starch and fiber, which limit structural refinement and uniformity. Overall, the combination of ultrasound and TG treatments enhances the structure of PIEG most effectively, whereas their impact on PCEG is less pronounced due to its more complex composition. The oil droplets in PCEG vary in size and tend to cluster together, with these oil clusters and fibers being less prominent in samples treated with US, suggesting that ultrasound has an impact on the gel structure.

The sectional images obtained from the CT reconstruction images (see Fig. 4) support the observations from the LM and SEM micrographs. However, as a larger subvolume was analysed for the CT characterisation, it revealed that the oil droplets in the PCEG appeared predominantly in the form of clusters, which was not evident from the LM or SEM images for all samples. By comparing the size and distribution of the oil droplets of the different gels, a similar distribution pattern was observed, with the large amounts of droplets being between 1 and 161 μm^3. However, due to the voxel size of 0.5 μm, droplets $\leq$1 μm were excluded from further discussion as their detection is limited.

Overall, high amounts of droplets >1000 μm^3 were found in the PIEG without pre-treatment and TG pre-treatments only, as well as in the PCEG with US pre-treatment and US combined with TG pre-treatment, although the differences in the PCEG samples are less dominant.

This may be a result of the pre-treatments, along with differences in overall composition, as well as limitations in the segmentation and separation of droplets when they appear in clusters. The PIEG sample without any pre-treatment showed, on average, the biggest droplets (824 μm^3) followed by the sample treated with TG (652 μm^3), US (203 μm^3), and US combined with TG pre-treatment (159 μm^3). Therefore, the different pre-treatments led to significant differences in droplet size ($p > 0.001$) between the different pre-treatments in the PIEG.

The PCEG samples treated with US showed the biggest average droplet size (1208 μm^3) followed by the sample treated with US and TG (1047 μm^3). No significant difference ($p > 0.05$) was found between the sample without any pre-treatment (648 μm^3) and the TG pre-treatment only (609 μm^3). Thus, the different pre-treatments led to a more homogeneous distribution of the oil droplets in the matrix and a smaller average droplet size in the PIEG. In contrast, the opposite trend was observed in the PCEG, wherein the different pre-treatments increased the droplet size.

The smaller droplet size in PIEG treated with US could be a result of the size reduction of the protein aggregates, and this has been previously reported for pea protein emulsions (McCarthy et al., 2016; Mozafarpour & Koocheki, 2023). The state of the proteins differs between the PC and PI, and this could influence the gelation mechanism (Zhan et al., 2022). Indeed, this would explain why the US pre-treatment does not affect the droplet size of the emulsion gels made from pea concentrate in the same way that the emulsion gels made from isolate do. Moreover, protein solubility (Klost & Drusch, 2019a) and the presence of starch and dietary fibre can influence the distribution of oil droplets in emulsion gels (Zhuang et al., 2019). Polysaccharides can serve as structural components in emulsion gels, either alone or in combination with proteins. When used in mixed gels, this combination often enhances gel performance compared to using either component alone (Yiu et al., 2023). However, polysaccharides can also disrupt the uniformity of the protein network, leading to microphase separation, irregular inclusion shapes, and the breaking or coalescence of oil droplets (Hou et al., 2022).

The impact of polysaccharides depends on several experimental factors, including the type and ratio of polysaccharides to proteins, the pH, ionic strength, and the preparation method (Liu et al., 2022). In the case of dietary fibres, particularly insoluble dietary fibre (IDF), previous studies have shown that increasing IDF levels leads to larger oil droplet sizes. This is mainly because IDF dont interact directly with proteins but are instead physically embedded in the gel matrix, reducing the overall elasticity of the composite, as IDF lacks inherent elasticity (Zhuang et al.,

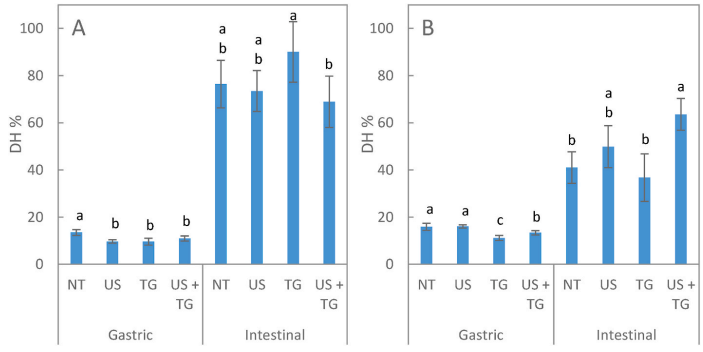

Fig. 5. Degree of protein hydrolysis (DH) of the different gels made from pea isolate (A) or pea concentrate (B) at the end of the gastric and intestinal phase, including gels after different pre-treatments: ultrasound (US), transglutaminase (TG), a combination of both (US/TG), and a control with no pre-treatment (NT). Different superscript letters indicate significant differences according to LSD = least significant difference ($P < 0.05$).

2019). In addition, enzymatic activity and reaction time can further influence droplet size by altering protein interactions and aggregation (Tian, Wang, et al., 2024). Also, the state of the proteins (whether they are denatured) can influence the oil droplet size, since thermal denaturation of proteins can sometimes lead to more droplet clumping (flocculation), as it exposes more hydrophobic areas on the protein surface (Yiu et al., 2023). Finally, the reduced protein content in emulsion gels derived from pea protein concentrate can impair emulsification properties, leading to decreased emulsion stability and consequently influencing the size of oil droplets.

When linking the observed G' and LVR for the different gels with the droplet size and distribution, smaller homogenously distributed oil droplets would lead to more elastic gels with a higher resistance against deformation, whereas larger droplets led to less elasticity and less physically stable gels.

This observation is in agreement with the Van der Poel theory, according to which smaller oil droplets lead to a more solid-like texture due to the increased surface area, and larger droplets result in a more spreadable or soft texture due to fewer interactions and less overall surface area for binding. This has been observed previously for soybean emulsion gels, where the compressive stresses of the gels containing smaller oil droplets were higher than those containing bigger droplets (Kim et al., 2001). However, in gels made from whey protein isolate and different gelling agents (gelatine, κ-carrageenan), the effect of a decrease of the oil droplet size on other fracture parameters and in other gel systems was minor (Sala et al., 2009). Furthermore, the presence of polysaccharides can alter the gel structure in a way that limits the influence of oil droplet size on overall texture (Hou et al., 2022; Yiu et al., 2023).

Aside from the size of the oil droplets, the way in which the droplets are stabilised within the gel system affects the final behaviour of the gels (Kornet et al., 2022). Droplets stabilised by non-ionic surfactants are usually considered inactive, have a limited contribution to the gel's structure, and can ultimately weaken the overall gel structure. However, in protein-stabilised emulsion gels, proteins coat the surface of the droplets, allowing them to interact with the gel's structure, and thus the droplets are an important part of the gel (Yiu et al., 2023).

3.4. Degree of hydrolysis

The effect of gel structure on overall protein digestibility was assessed using the OPA assay. This method quantified the amount of free amino groups in the supernatant after the gastric and intestinal phases of digestion for the different gels, expressed as the degree of hydrolysis (DH). The values presented in Fig. 5 reflect the number of peptide bonds hydrolysed in the digesta relative to the total number of peptide bonds per protein equivalent. A significantly higher ($p = 0.011$) DH was found after the gastric phase for the PIEG without any pre-treatment compared to the gels treated with US and/or TG. At the end of the intestinal phase, the PIEG treated with US and TG showed the lowest DH followed by the gels treated with US and NT. The PIEG treated with TG showed the highest DH, significantly ($p = 0.0171$) differing from the gel treated with US and TG.

The NT and US-treated PCEG resulted in the highest DH at the end of the gastric phase, followed by the gels treated with US + TG and TG only ($p = 0.002$). At the end of the intestinal phase, the PCEG treated with US and US + TG showed the highest DH, followed by the samples treated with TG and NT ($p = 0.0173$).

When comparing the average overall DH between the PIEGs (DH 77 %) and PCEGs (DH 48 %) a significant difference between the gels made from PI and PC ($p = 0.007$) was found. Therefore, in this study, the raw material, e.g., whether PI or PC was used, had a greater effect on digestibility than processing.

Protein digestion primarily relies on the extent to which proteases in the gastric and intestinal environments can access the protein and how efficiently they can carry out the hydrolysis of peptide bonds. Among other factors, hydrolysis can be significantly influenced by the structure of the proteins; aggregates may shield peptide bonds from proteases (Capuano & Janssen, 2021). In addition, the food matrix and overall structure (Loveday, 2022; Nyemb et al., 2016) limit protein digestion, as high cellular integrity often leads to lower protein digestibility in whole plant foods compared to animal-based proteins (Gilani et al., 2005). Furthermore, the presence of food components such as protease inhibitors, dietary fibers, and starch can reduce the overall rate of protein hydrolysis, thereby decreasing protein digestibility (Muzquiz et al., 2012; Sá et al., 2019). Various polysaccharides may hinder nutrient digestion by limiting diffusion and mass transfer, restricting the mixing of digestive enzymes and substrates, blocking enzyme active sites, or inducing conformational changes in proteins. They can also form aggregates or surface interactions that immobilize protein substrates. The extent of this inhibitory effect depends on several factors, including the concentration, viscosity, and molecular structure of the polysaccharides, as well as the physicochemical properties of the protein substrate, such as molecular weight and conformation (Bach Knudsen, 2001; Gilani et al., 2005; Karim et al., 2024; Kaur et al., 2022; Lu et al., 2024). Considering the higher amount of DF and starch in the pea concentrate, this could explain the overall lower DH in the PCEG compared to the PIEGs.

Further, different food structures can induce proteolysis kinetics and the release of specific peptides (Nyemb et al., 2016; Reynaud et al.,

Table 4
PLS-DA models based on binned spectra from NMR-based metabolomics. Including the gels made from pea isolate (PI) and pea concentrate (PC) after the gastric and intestinal digestion.

PLS-DA Model	$PI_{Gastric}$ (n = 16)	$PI_{Intestinal}$ (n = 16)	$PC_{Gastric}$ (n = 16)	$PC_{Intestina}$ (n = 16)
Q2	0.75	0.83	0.75	0.96
p-value	0.0065	0.0015	0.0225	0.0100

2020). Previous studies have demonstrated that the gel properties can affect the digestibility of the proteins, whereas stiffer gels can delay gastric emptying (Barbé et al., 2013) and reduce the overall digestibility (Marinea et al., 2021). The lower degree of hydrolysis (DH) observed in PIEG treated with ultrasound (US) and transglutaminase (TG) could be attributed to the higher storage modulus and oscillatory stress, compared to the less elastic and less physically stable gels, which exhibited a higher DH. A similar trend was observed for the PCEG wherein the less elastic and less physically stable gels showed a higher DH compared to the gels with an increased storage modulus and oscillation stress. However, the observed differences related to the various pre-treatments remain relatively small.

Previous results on soy protein gels also demonstrated that TG limits gastric *in vitro* digestion, as the covalent linking between glutamine and lysine residues prevents enzymatic cleavage. For pepsin, this resistance to digestion is likely an indirect effect, as TG cross-linking restricts protein conformational flexibility, thereby limiting access to its preferred cleavage sites (Phe, Trp, Tyr). However, for trypsin, the effect is direct, as it specifically cleaves after lysine residues, which are no longer available due to isopeptide bond formation.(Rui et al., 2016).

3.5. NMR-based metabolomics

NMR-based metabolomics was done to study water-soluble end-products (e.g. amino acids and mono-sugars) of the different pea protein emulsion gels after *in vitro* digestion. Multivariate statistics (PLS-DA) was used to identify top-ranking features that differentiated between gels produced with or without pre-treatment (e.g., with ultrasound and/or transglutaminase). PLS-DA models obtained after stratification by starting material (PI vs. PC) and sample collection point (gastric vs. intestinal phase) were all significant (Table 4, $p < 0.05$, $Q > 0.5$).

The top-ranking features, which mainly resided in the sugar region, were subsequently targeted for profiling to assign and quantify the corresponding ^{1}H-NMR-signals (Fig. 6, Table 5). Univariate statistics were carried out to reveal discriminating metabolites that remained significant after Bonferroni correction (Table 5; $p < 0.0018$).

The results indicate significant differences ($p < 0.001$) in metabolite concentrations between the different treated emulsion gels during the gastric phase. Overall, a lower glycine release was observed in the PIEG samples treated with US + TG compared to other pre-treatments. For PCEG samples, a higher glucose release was found in the untreated and US-treated gels compared to those treated with TG and US + TG.

The lower glycine release observed in the PIEG samples treated with US + TG may indicate differences in protein digestion during the gastric phase, possibly due to increased gel stability, as discussed in section 3.4. Although the same degrees of hydrolysis (DH) were observed for the US and/or TG-treated samples, the release of individual amino acids can vary. This finding could indicate a difference in protein digestion depending on the pre-treatment. Further, the glycine release was the same in all samples after the intestinal digestion, indicating no effect of the different pre-treatments on the final digestibility.

The higher glucose release in the untreated PCEG and US-treated

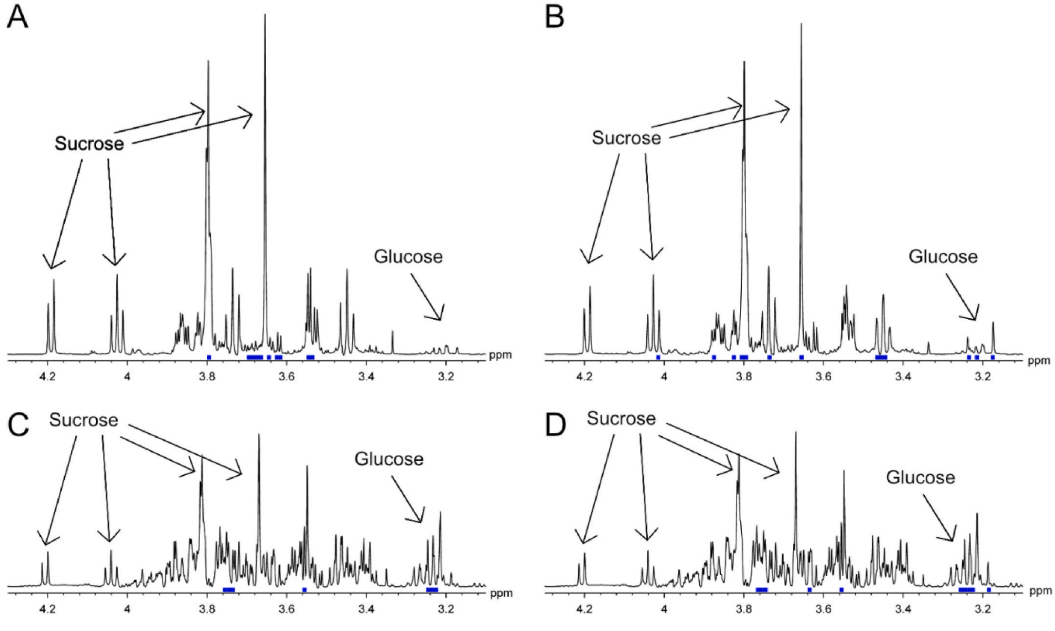

Fig. 6. Sugar region (3.1–4.3 ppm) of the average ^{1}H NMR spectra of each PLS-DA model. Post-acquisition processing included e.g. airPLS-based baseline correction and icoshift-based alignment. The top-ranking features in the sugar region according to each corresponding PLS-DA model are indicated in blue. A Gastric digesta of PIEG ($PI_{Gastric}$; n = 16); B Gastric digesta of PCEG ($PC_{Intestinal}$; n = 16); C Intestinal Phase of PIEG ($PI_{Intestinal}$; n = 16); D Intestinal Phase of PCEG gels ($PC_{Intestinal}$; n = 16).

Table 5
Metabolite concentrations (mean ± standard deviation; μM) from targeted profiling of the sugar region in NMR-based metabolomics spectra, including transglutaminase (TG), ultrasound (US), combined ultrasound and transglutaminase treatment (US + TG), and untreated (NT) emulsion gels after gastric and intestinal digestion, as well as a blank digestion (Blank) containing a protein-free cookie and digestive enzymes.

	Blank (n = 4)	NT (n = 3)	TG (n = 3)	US (n = 3)	US + TG (n = 3)	p-value[a]
$PI_{Gastric}$ (n=16)						
Betaine	49 ± 12	50 ± 2	50 ± 4	45 ± 3	58 ± 8	0.419
Choline	110 ± 11[a]	160 ± 5[b]	170 ± 8[b]	150 ± 12[b]	150 ± 8[b]	<0.001
Glucose	5100 ± 1700	3800 ± 230	3900 ± 270	4000 ± 130	3700 ± 170	0.244
Glycerol	3100 ± 1100	2700 ± 800	8700 ± 95	2100 ± 540	7200 ± 650	NA
Glycine	1200 ± 530[a]	1100 ± 63[a]	980 ± 220[a]	720 ± 21[a]	320 ± 88[b]	<0.001
Methanol	1670 ± 1900	550 ± 220	1100 ± 140	1200 ± 150	1100 ± 380	NA
Sucrose	33000 ± 2800	33000 ± 430	36000 ± 1800	35000 ± 2600	32000 ± 1300	0.177
$PI_{Intestinal}$ (n=16)						
Betaine	370 ± 56	350 ± 38	370 ± 11	384 ± 19	370 ± 31	0.856
Choline	270 ± 20	290 ± 7	300 ± 20	310 ± 14	280 ± 22	0.143
Glucose	24000 ± 4200	27000 ± 1200	24000 ± 840	26300 ± 1090	24000 ± 910	0.293
Glycerol	6200 ± 770	7700 ± 190	8100 ± 290	6400 ± 180	7800 ± 280	NA
Glycine	7500 ± 210	7500 ± 480	7900 ± 390	8100 ± 315	7500 ± 470	0.183
Methanol	790 ± 210	540 ± 240	1200 ± 160	1180 ± 210	1200 ± 50	NA
Sucrose	16000 ± 1100	16000 ± 570	16000 ± 350	17000 ± 1300	16000 ± 660	0.490
$PC_{Gastric}$ (n=16)						
Betaine	41 ± 11[a]	470 ± 19[b]	450 ± 13[b]	450 ± 23[b]	430 ± 6[b]	<0.001
Choline	95 ± 29[a]	850 ± 37[b]	800 ± 17[b]	830 ± 47[b]	760 ± 11[b]	<0.001
Glucose	3300 ± 250[a]	4500 ± 210[b]	3400 ± 210[a]	4400 ± 92[b]	3400 ± 60[a]	<0.001
Glycerol	2100 ± 670	3100 ± 220	14000 ± 240	3400 ± 1100	12000 ± 450	NA
Glycine	–	1800 ± 69	–	1100 ± 230	–	NA
Methanol	430 ± 70	820 ± 49	920 ± 78	1000 ± 150	1100 ± 36	NA
Sucrose	32000 ± 2500	36000 ± 2500	34000 ± 1100	36000 ± 2500	33000 ± 1200	0.076
$PC_{Intestinal}$ (n=16)						
Betaine	370 ± 33[a]	510 ± 48[b]	510 ± 24[b]	490 ± 73[b]	480 ± 12[b]	0.003
Choline	270 ± 13[a]	600 ± 70[b]	590 ± 47[b]	550 ± 79[b]	560 ± 16[b]	<0.001
Glucose	23000 ± 890	26000 ± 2100	29000 ± 1800	24000 ± 3400	25000 ± 680	0.063
Glycerol	6200 ± 1100	6300 ± 400	10900 ± 370	6400 ± 1100	10000 ± 1400	NA
Glycine	7800 ± 290	7800 ± 1100	7600 ± 570	7500 ± 860	7600 ± 640	0.963
Methanol	440 ± 51	710 ± 90	780 ± 51	770 ± 120	830 ± 92	NA
Sucrose	15000 ± 3100	17000 ± 1400	18000 ± 1200	16000 ± 2300	16000 ± 370	0.415

[a] Significant differences between groups (horizontal) according to the post-hoc tests are indicated by different letters. NA: Not assessed because of known sample preservation issues (methanol and glycerol) (Psychogios et al., 2011).

gels, compared to those treated with TG and US + TG, suggests a relationship between transglutaminase pre-treatment and reduced glucose release (Table 5; $p < 1.482 \cdot 10^{-5}$). A similar effect has been previously observed in wheat noodles and rice, in which transglutaminase pre-treatment led to lower glucose release during digestion. A possible explanation for this is that transglutaminase-mediated protein network binding encapsulates starch granules, thereby limiting their digestibility (Lang et al., 2020; Mei Wee & Henry, 2019). This effect was observed in PC but not in PI, which can be attributed to the higher starch content in PC, both in soluble and insoluble glucose residues, compared to PI. Additionally, more PCEG was added during the initial digestion to normalise the protein content of both emulsion gels. The absence of differences in glucose digestion in the intestinal phase may be due to the increased glucose release from the pronounced breakdown of sucrose in the cookie, leading to background digestion across all samples (see Fig. 6). Consequently, the inclusion of the cookie is essential for studying protein digestion, but somewhat limiting when examining carbohydrate digestion.

In general, NMR-based metabolomics enable the measurement of end-products from both starch- and protein digestion (e.g. amino acids and mono-sugars). Interestingly, this study pointed towards a more pronounced impact (of transglutaminase pre-treatment) on the digestibility of starch rather than protein. With the use of a single experiment (1D ^{1}H) and an efficient data-driven approach for hypothesis-generation, this study suggests follow-up studies on starch digestibility (beyond protein digestion), notably in concentrates that contain both protein and starch. The approach used in this study provides a starting point for investigating the effects of *in vitro* digestion on pea-based protein gels using NMR-based metabolomics, a combination of methodology and sample types that is scarce within scientific literature. At the same time, this type of data-driven workflow has limitations. Certain metabolites appear as a singlet in a crowded spectral region (e.g. glycine), rendering them harder to distinguish compared to metabolites with a more characteristic spectral pattern (e.g. sugars). More elaborate identification and quantification efforts (for reference, see analogous efforts over time for serum NMR-based metabolomics (Bansal et al., 2024; Nagana Gowda et al., 2015)) were not considered feasible within the scope of this work. Resultingly, only speculations can be made regarding the impact this would have on the potential to reveal other metabolite changes.

3.6. Principal component analysis

Principal Component Analysis (PCA) was performed to visualise the relationships between pea protein isolates (PI) and concentrates (PC), as well as the effect of pre-treatments on structural characteristics and digestibility (Fig. 7). Therefore, the first two principal components (PC1 and PC2) explained 45.9 % and 34.5 % of the total variance, respectively, accounting for 80.4 % of the variability across the dataset. The PCA biplot (Fig. 7) visualizes both the loadings (grey dots representing measured variables) and the sample scores (colored hexagons, coded by protein type and treatment).

Based on these results, it is evident that both the pre-treatments and the raw materials influence the gel properties, as pea protein emulsion gels made from PI and PC cluster differently. This indicates the significant impact of each treatment and raw material on the gels.

Furthermore, protein content and the degree of hydrolysis after intestinal digestion (DH Intestinal) are strongly positively correlated with

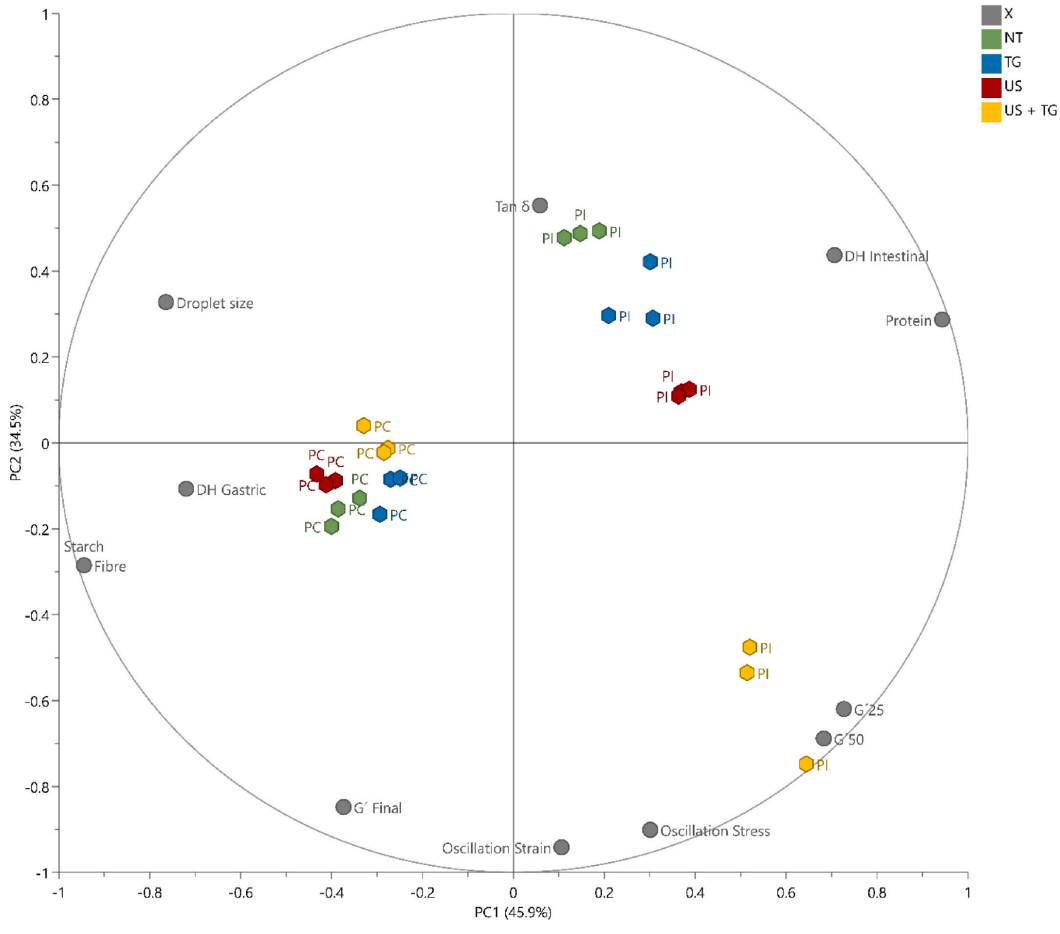

Fig. 7. Principal Component Analysis (PCA) biplot visualising the effect of pre-treatments on the structural features and digestibility of pea protein emulsion gels. The analysis includes gels made from pea protein isolate (PI) and pea protein concentrate (PC) treated with transglutaminase (TG), ultrasound (US), and a combination of ultrasound and transglutaminase (US + TG), as well as untreated samples (NT) compared against compositional and structural variables (X).

PC1, suggesting that gels in this region (e.g., NT- and TG-treated samples made from PI) are associated with a higher protein content and intestinal digestibility. Since the protein content was normalised throughout digestion, the increased digestibility can be attributed to starch and fibre content.

Starch and fibre are negatively associated with intestinal digestibility (DH Intestinal), indicating that their presence may limit protein breakdown in the later stages of digestion. Additionally, droplet size, DH Gastric, starch, and fibre are negatively correlated with PC1, suggesting that PC samples exhibit larger droplet sizes, higher starch/fibre content, and lower gastric digestibility, although this correlation is less dominant.

In terms of structural properties, PCA shows that the elastic modulus (G') and oscillation stress/strain are located in the positive PC1 region, suggesting that firmer gels with higher elasticity correspond to PIEG pre-treated with US + TG. Tan δ, an indicator of gel viscoelasticity, is positively associated with PC2, suggesting that untreated PIEG, along with TG- and US-treated emulsion gels, exhibit more fluid-like properties compared to PCEG and PIEG treated with US + TG.

4. Conclusion

Pea protein isolates (PI) and pea protein concentrates (PC) exhibit distinct behaviours during the gelation process, resulting in emulsion gels with differing rheological properties. These differences can be attributed to variations in overall composition, PC contains less protein and higher levels of starch and fibre compared to PI, as well as differences in protein structure. PI is characterised by larger protein aggregates, commonly observed in commercially extracted protein isolates. Ultrasound pre-treatment effectively solubilises these aggregates in PI, leading to emulsion gels that are more elastic and resistant to deformation compared to gels without pre-treatment. Ultrasound also promotes a more homogeneous distribution of oil droplets within the PIEG, potentially contributing to the enhancement of physical stability. In

contrast, this trend was not observed in pea concentrate emulsion gels (PCEG), likely due to the presence of starch and fibres in PC, which contribute to the overall structure and differing interactions during gelation. These findings underscore that both processing methods and the overall composition of the protein ingredient significantly influence gel structure and the effects of pre-treatment.

After *in vitro* digestion, significant differences in protein digestibility were observed during the gastric phase, with less dominant differences during the intestinal phase. A more pronounced effect, however, was observed between the emulsion gels made from pea protein isolate (PI) and pea protein concentrate (PC), indicating that the type of raw material had a greater influence on digestibility than the processing methods employed.

NMR-based metabolomics suggested differences in glucose and glycine release between the pea concentrate emulsion gels (PCEG) and pea isolate emulsion gels (PIEG) during the gastric phase. These differences may be a result of variations in gel structure. However, it is important to note that no such differences were observed at the end of the intestinal phase, suggesting that the impact of processing on the final digestibility of pea protein emulsion gels may be difficult to detect by NMR-based metabolomics.

CRediT authorship contribution statement

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

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Declaration of competing interest

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

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Data availability

Data will be made available on request.

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This thesis investigates how processing techniques affect the structure, digestibility, and nutrient release of plant-based ingredients and food (model) systems derived from soy, faba bean, yellow pea, and grey pea. Using in vitro digestion and cell uptake models, it shows that fermentation enhances mineral bioavailability by reducing phytate content, whilst protein coagulation improves protein digestibility by altering food structure and composition. The findings underscore the role of processing in enhancing the nutritional quality of plant-based foods for healthier and more sustainable diets.

Jaqueline Auer received her graduate education at the Department of Molecular Sciences, SLU, Uppsala. She attained her B.Sc. and M.Sc. degrees in Food Technology and Nutrition from the University of Applied Sciences, Upper Austria.

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