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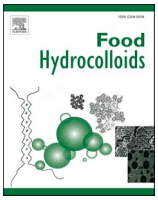

# The effect of transglutaminase and ultrasound pre-treatment on the structure and digestibility of pea protein emulsion gels

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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)

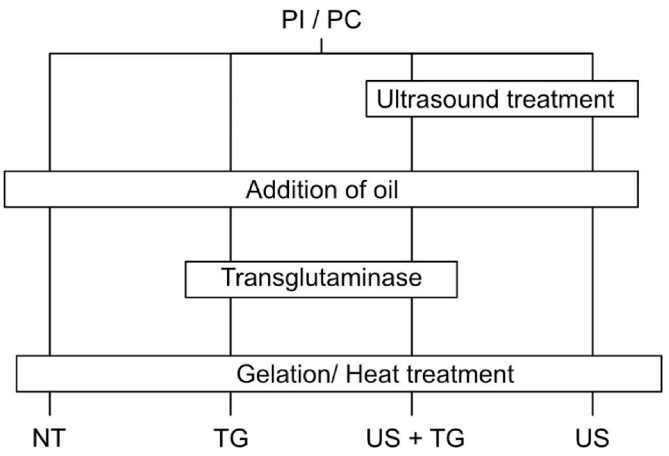


**Fig. 1.** Schematic overview of the emulsion gels prepared from pea isolate (PI) and pea concentrate (PC), including the different pre-treatments, consisting of ultrasound pre-treatment (US), the addition of oil, the addition of transglutaminase (TG), a combined ultrasound and transglutaminase pre-treatment (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-dithiothreitol (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).

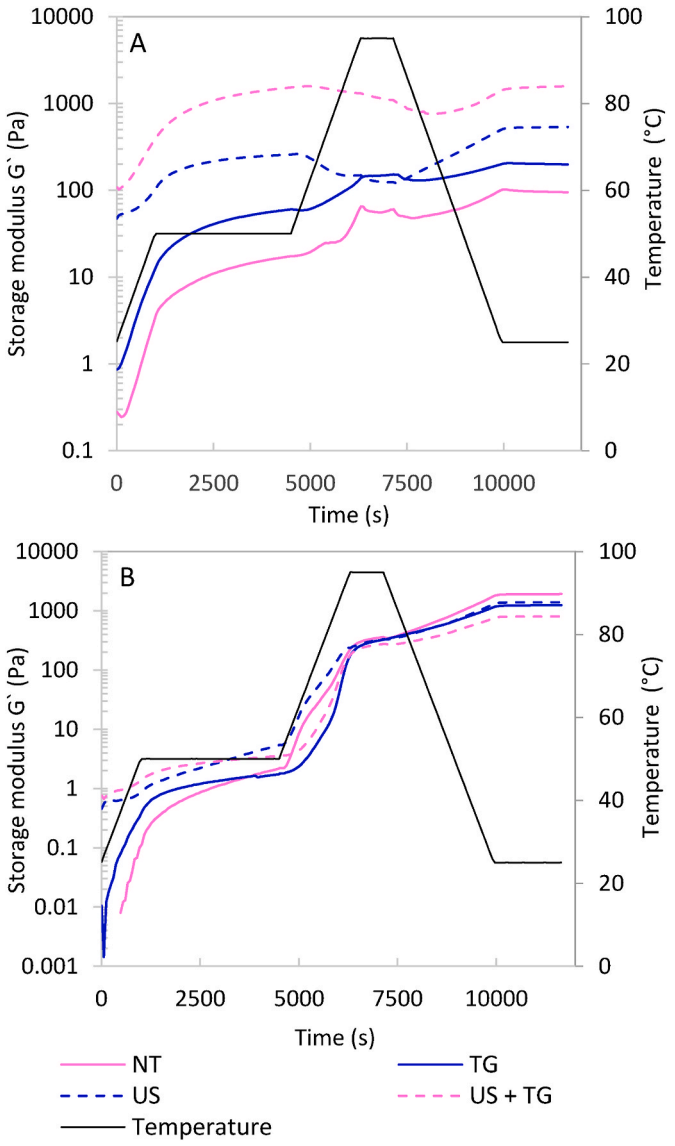


**Fig. 2.** Recorded changes in storage modulus (G′) of pea protein isolate emulsion gels (A) and pea protein concentrate emulsion gels (B) during the heating and cooling period. The figure includes the effects of various pre-treatments: ultrasound pre-treatment (US), transglutaminase treatment (TG), 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$^{c}$ | 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.

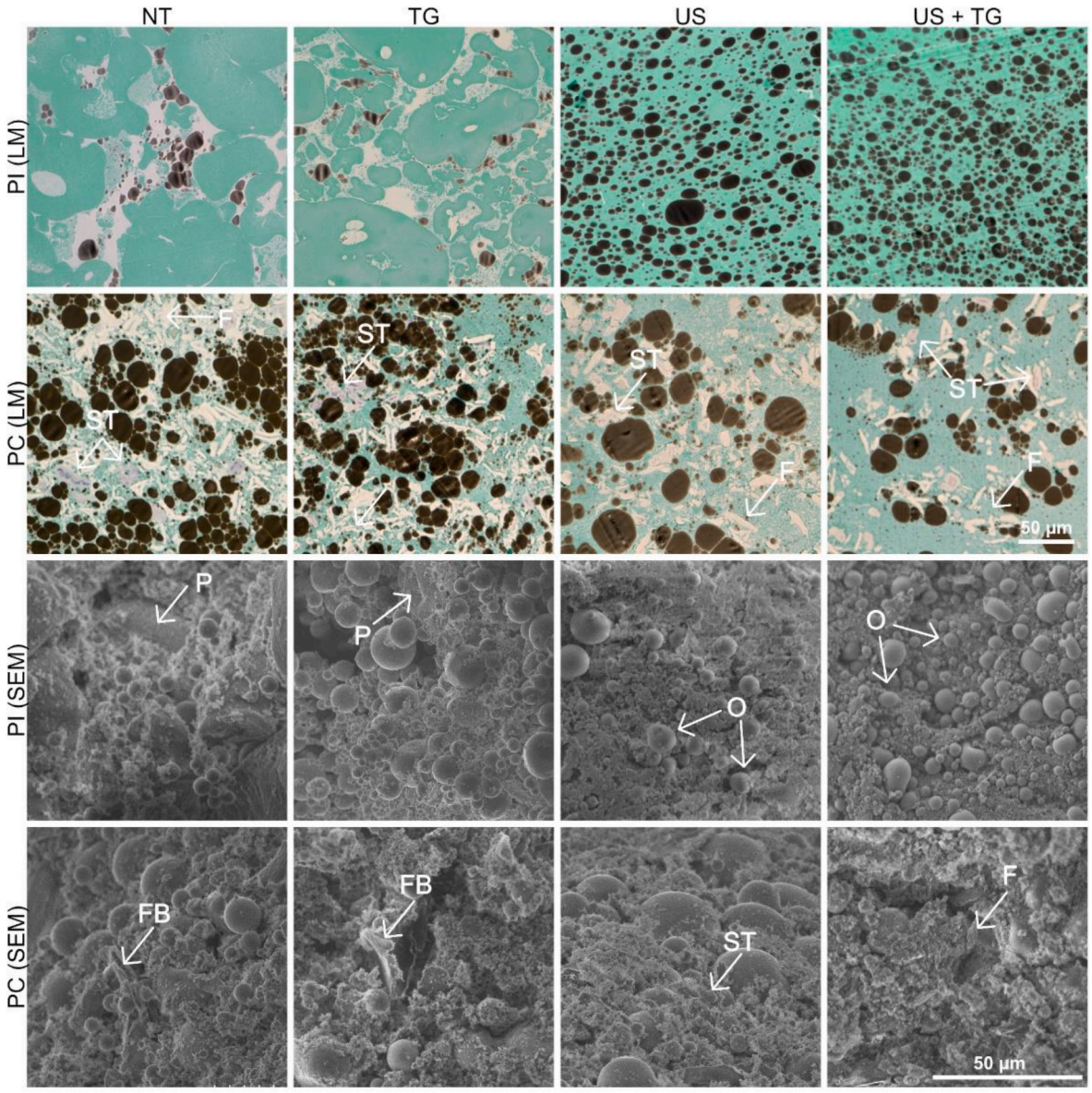


**Fig. 3.** Microstructure of the pea protein gels made from pea isolate (PI) and pea concentrate (PC), including the effects of ultrasound pre-treatment (US), transglutaminase treatment (TG), a combination of ultrasound and transglutaminase (US/TG), and a control without any pre-treatment (NT), using 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,

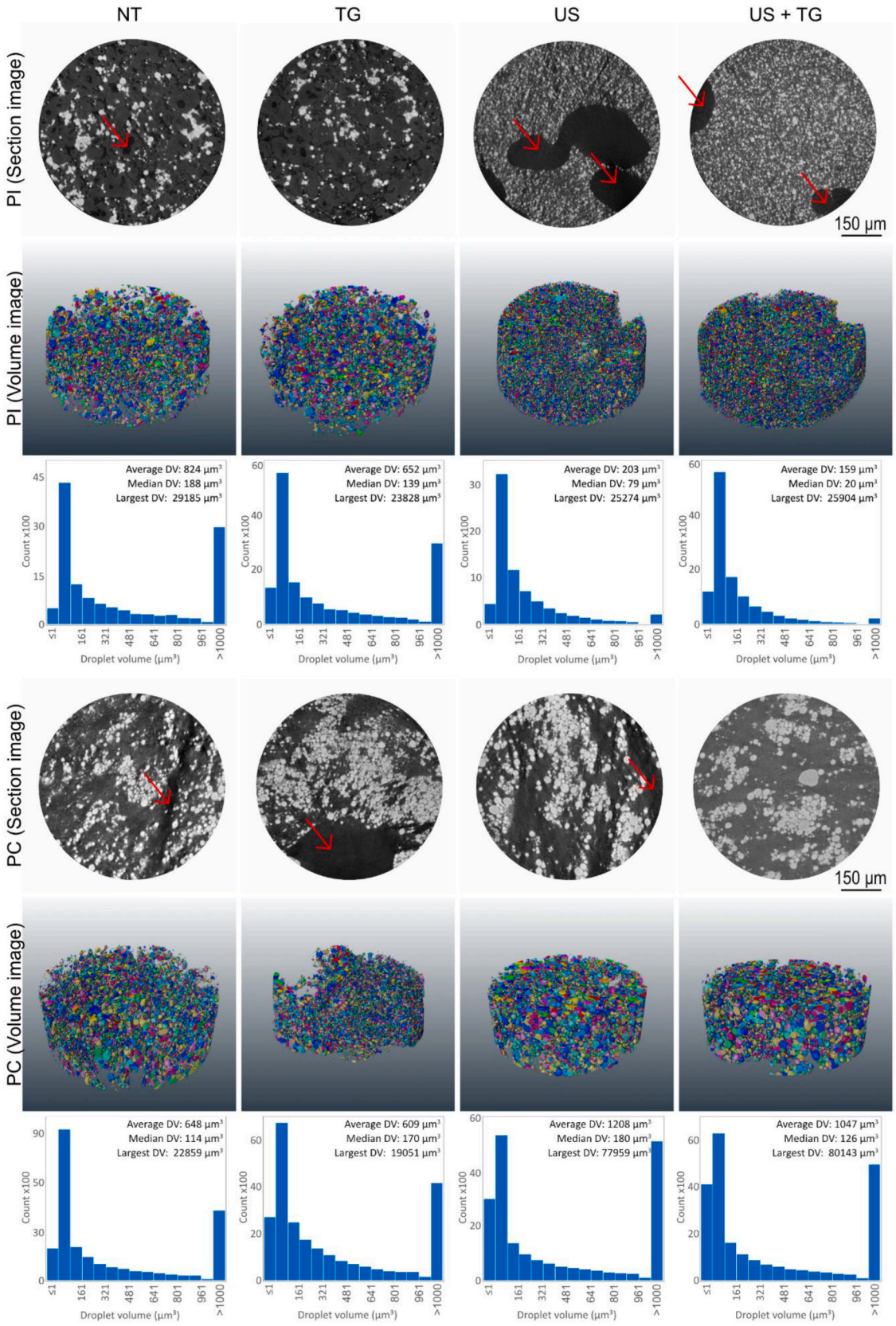


**Fig. 4.** Sectional images of gels made from pea protein isolate (PI) and pea protein concentrate (PC) after different pre-treatments: ultrasound (US), transglutaminase (TG), a combination of both (US/TG), and a control with no pre-treatment (NT). Computed tomography (CT) reconstructions show oil droplets (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 $\mu m^3$. 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 $\mu m^3$ 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 $\mu m^3$) followed by the sample treated with TG (652 $\mu m^3$), US (203 $\mu m^3$), and US combined with TG pre-treatment (159 $\mu m^3$). 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 $\mu m^3$) followed by the sample treated with US and TG (1047 $\mu m^3$). No significant difference ($p > 0.05$) was found between the sample without any pre-treatment (648 $\mu m^3$) and the TG pre-treatment only (609 $\mu m^3$). 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.,

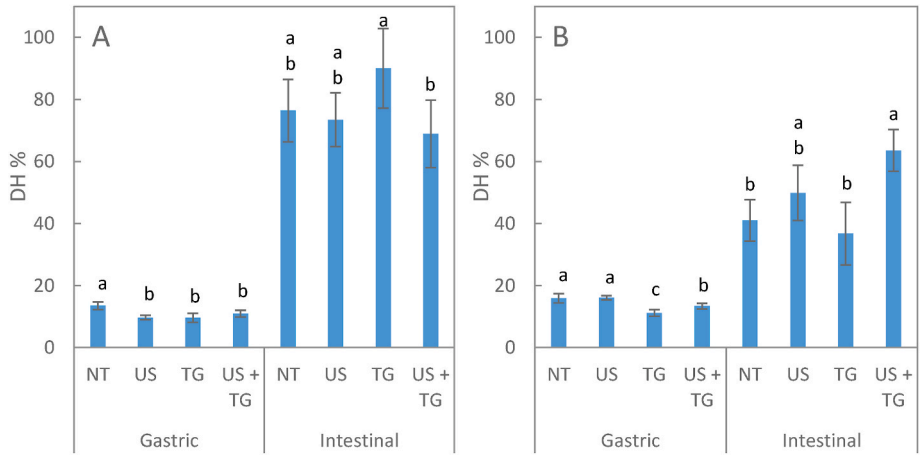


**Fig. 5.** Degree of protein hydrolysis (DH) of the different gels made from pea isolate (A) or pea concentrate (B) at the end of the gastric and intestinal phase, including gels after different pre-treatments: ultrasound (US), transglutaminase (TG), a combination of both (US/TG), and a control with 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

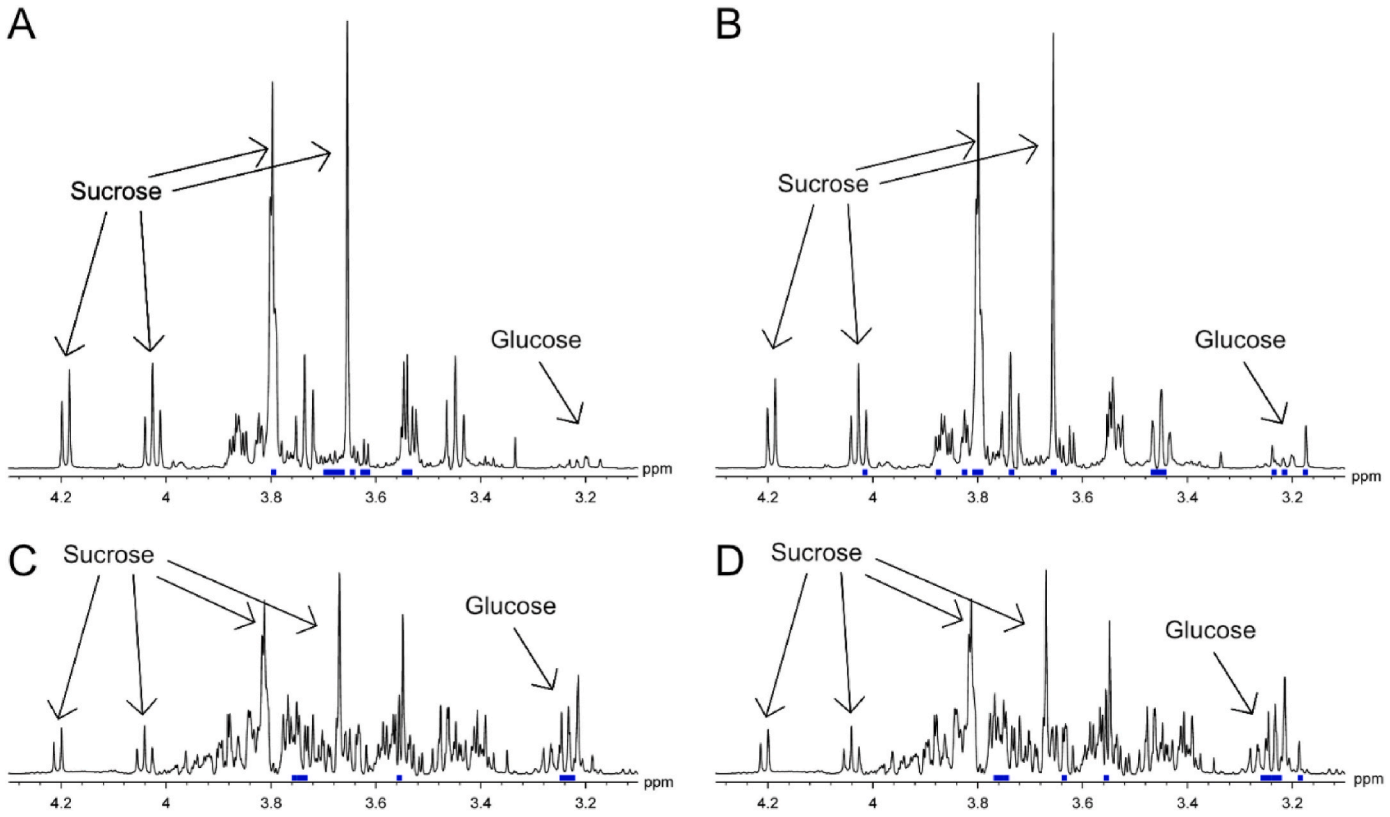


**Fig. 6.** Sugar region (3.1–4.3 ppm) of the average $^1$H NMR spectra of each PLS-DA model. Post-acquisition processing included e.g. airPLS-based baseline correction and icoshift-based alignment. The top-ranking features in the sugar region according to each corresponding PLS-DA model are indicated 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

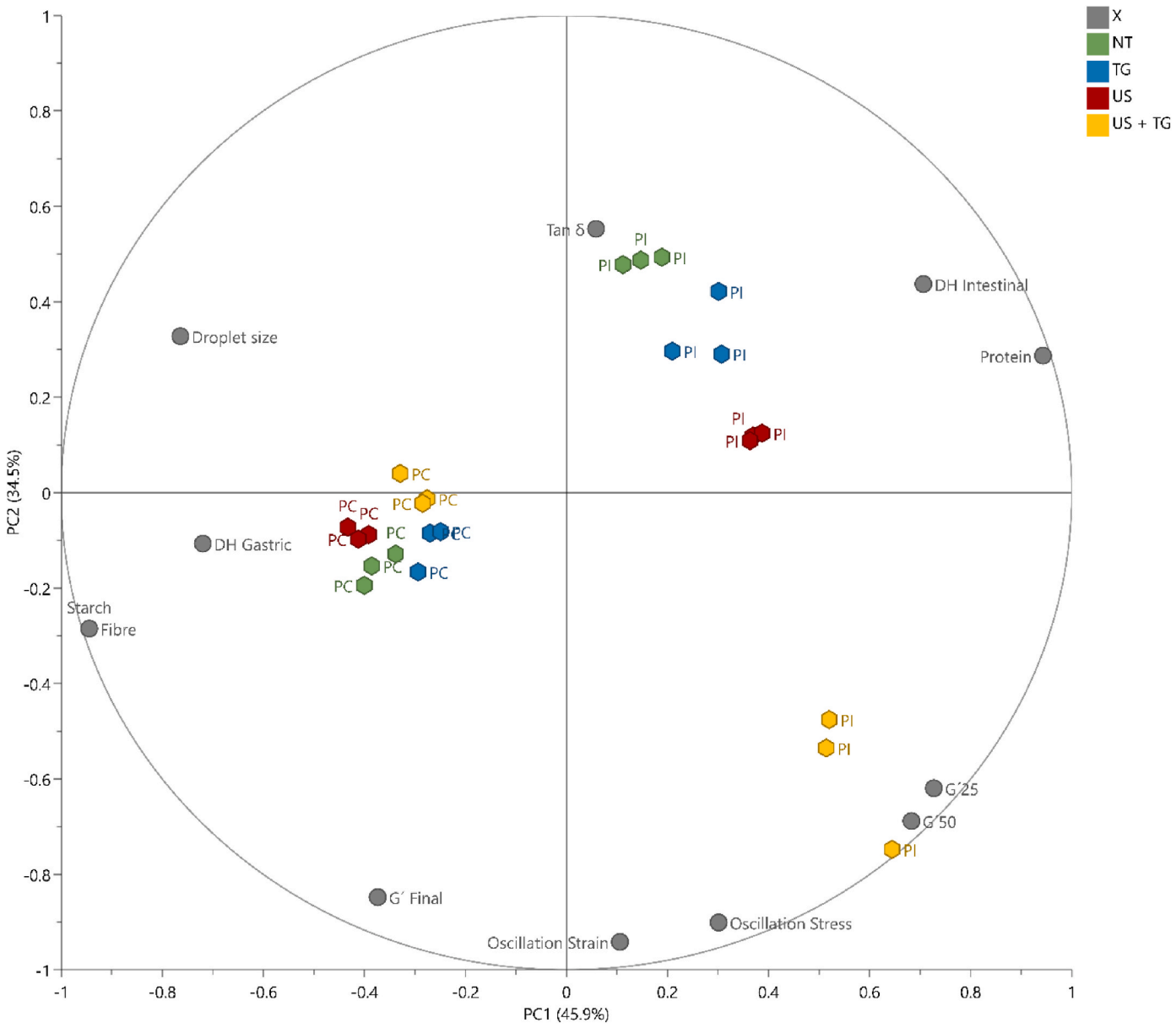


**Fig. 7.** Principal Component Analysis (PCA) biplot visualising the effect of pre-treatments on the structural features and digestibility of pea protein emulsion gels. The analysis includes gels made from pea protein isolate (PI) and pea protein concentrate (PC) treated with transglutaminase (TG), 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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