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Structural modulation of pea protein and multiscale organization of casein-pea protein hybrid gels

Raiane Rodrigues da Silva
Doctor Scientiae

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Thesis submitted to the Food Science and Technology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

Adviser: Antonio F. de Carvalho

Co-adviser: Federico Casanova

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ABSTRACT

SILVA, Raiane Rodrigues da, D.Sc., Universidade Federal de Viçosa, March, 2026. **Structural modulation of pea protein and multiscale organization of casein-pea protein hybrid gels**. Adviser: Antonio Fernandes de Carvalho. Co-adviser: Federico Casanova.

The growing interest in plant-based proteins, driven by environmental concerns and food security, has stimulated the development of plant-based products. However, sensory and technological limitations still pose significant challenges. In this context, hybrid systems combining plant and animal proteins emerge as an alternative to reduce the consumption of animal proteins while improving the sensory and technological characteristics associated with plant-based products, making it essential to understand the interactions and organization of these mixed matrices. Therefore, this study aimed to investigate, using a multiscale approach, the structure of hybrid gels formed by casein and pea protein, as well as to assess the effect of pH shifting on pea protein structure and its implications for the formation and organization of these gels. Through the multiscale approach, it becomes evident that the large-scale structural organization is the main factor governing key properties such as rheology and water mobility in hybrid gels. Moreover, these proteins, when forming a gel, tend to create individual networks without interacting with each other. Thus, structural modulation using pH shifting was employed. pH shifting increased pea protein solubility, induced structural changes in the protein, and consequently improved water-holding capacity and gel hardness, demonstrating its potential to enhance pea protein functionality in hybrid systems. The results highlight that the formation and properties of hybrid gels are strongly related to protein structure, emphasizing the need for additional strategies to develop hybrid products with sensory and functional attributes aligned with consumer expectations.

Keywords: hybrid gels; plant protein; sustainability

RESUMO

SILVA, Raiane Rodrigues da, D.Sc., Universidade Federal de Viçosa, março de 2026. **Modulação estrutural da proteína de ervilha e organização multiescalar de géis híbridos de caseína-proteína de ervilha**. Orientador: Antonio Fernandes de Carvalho. Coorientador: Federico Casanova.

O crescente interesse por proteínas de origem vegetal, impulsionado por preocupações ambientais e segurança alimentar, tem estimulado o desenvolvimento de produtos plant-based. No entanto, limitações sensoriais e tecnológicas ainda representam desafios significativos. Nesse contexto, sistemas híbridos que combinam proteínas vegetais e animais surgem como uma alternativa para reduzir o consumo de proteínas de origem animal, ao mesmo tempo em que melhoram as características sensoriais e tecnológicas associadas a produtos plant-based, tornando essencial compreender as interações e a organização dessas matrizes mistas. Assim, este trabalho teve como objetivo investigar, sob uma abordagem multiescala, a estrutura de géis híbridos formados por caseína e proteína de ervilha, bem como avaliar o efeito do deslocamento de pH na estrutura da proteína de ervilha e suas implicações na formação e organização desses géis. Através da abordagem em multiescala é possível perceber que a organização estrutural em larga escala é o principal fator que governa propriedades-chave, como a reologia e a mobilidade da água nos géis híbridos. Além disso, estas proteínas, quando em um gel, tendem a formar sistemas individuais, sem interação umas com as outras. Sendo assim, uma modulação estrutural utilizando deslocamento de pH foi empregada. O deslocamento de pH aumentou a solubilidade da proteína de ervilha, provocou alterações estruturais na proteína de ervilha e consequentemente melhorou a capacidade de retenção de água e a dureza dos géis, demonstrando seu potencial para aprimorar a funcionalidade da proteína de ervilha em sistemas híbridos. Os resultados evidenciam que a formação e propriedades de géis híbridos estão fortemente relacionadas à estrutura proteica, ressaltando a necessidade de estratégias adicionais para desenvolver produtos híbridos com atributos sensoriais e funcionais alinhados às expectativas dos consumidores.

Palavras-chave: géis híbridos; proteína vegetal; sustentabilidade

LIST OF ILLUSTRATIONS

CHAPTER II. RESEARCH ARTICLE ONE

CHAPTER III. RESEARCH ARTICLE TWO

CHAPTER IV. REVIEW ARTICLE

LIST OF TABLES

CHAPTER II. RESEARCH ARTICLE ONE

CHAPTER III. RESEARCH ARTICLE TWO

CHAPTER IV. REVIEW ARTICLE

SUMMARY

CHAPTER I.
GENERAL BACKGROUND

General introduction

In recent years, concerns regarding sustainability have increased. This concern is reflected in the UN 2030 Sustainable Development Agenda, where one of the goals is to take action to combat climate change (United Nations, 2015). Thinking about the food sector, one way to mitigate the impact of climate change is to reduce animal product consumption. Livestock farming is responsible for 65% of total greenhouse gas emissions, thus, reducing the consumption of animal products can have a positive effect on the food industry on climate change (Gil et al., 2024). Besides that, the Sustainable Development Agenda aims to achieve food security for the population. However, with the actual habits based on animal product consumption, especially the protein levels, and with the increase of the population, which can reach 10 million people by 2050, current production systems may not be able to meet the growing demand for food sustainably (Gil et al., 2024; United Nations, 2015).

In this context, plant-based proteins, such as pea protein, offer a promising alternative, as they produce lower greenhouse gas emissions, require less water and land, and can yield more protein per unit of land compared to animal production. This makes them an important strategy for promoting a more sustainable food system, both from social and environmental perspectives (Nascimento et al., 2023). Nevertheless, society's acceptability and the complete migration to plant-based products can be challenging due to the poor taste. Also, from the industry perspective, the vegetable proteins have low solubility, limiting their applicability. To overcome these problems, the formulation of a hybrid mixed with animal proteins can be an alternative, since reducing the drawbacks while decreasing animal-protein consumption (R. R. da Silva et al., 2025).

Regarding hybrid gel systems formed by pea protein and casein micelles, several studies have been reported in the literature using different gelation methods and protein ratios. In general, when these two proteins are combined in a gel system, they tend to form independent networks with limited interactions between them, which may significantly influence the resulting gel texture (Beghdadi et al., 2022; J. L. Mession et al., 2017; Nascimento et al., 2024; J. Silva et al., 2019).

To promote stronger interactions between the protein fractions, conventional modification strategies, including thermal treatment, pH shifting, and enzymatic modification, may be employed. These approaches can induce structural alterations such as partial unfolding, exposure of hydrophobic groups, and rearrangement of intermolecular bonds, thereby increasing protein–protein interactions (de Sousa et al., 2026; R. R. da Silva et al., 2025). Besides traditional methods, non-thermal technologies can also be applied to change the protein structure and induce interaction. From a sustainable perspective, non-thermal technologies, such as ultrasound, pulsed electric field, cold plasma, and high-pressure processing, are more energy-efficient, cost-effective, reduced use of chemical reagents, and have a lower negative impact on nutritional components (Jadhav et al., 2021; Nascimento, Queiroz, et al., 2023; Safwa et al., 2023).

Therefore, this study aimed to investigate, using a multiscale approach, the structure of hybrid gels formed by casein and pea protein, as well as to assess the effect of pH shifting on pea protein structure and its implications for the formation and organization of these gels.

Research hypothesis and aim

The hypothesis for this study was that the structural organization of hybrid gels can be effectively modulated by altering the structure of pea protein and adjusting formulation parameters, such as protein ratio, thereby influencing the interactions between pea protein and casein micelles. To test this hypothesis, the following objectives were established:

i. To verify, through a literature review, the potential of non-thermal technologies to modify the structure of pea protein and enhance its techno-functional properties.

ii. To understand the structuration of casein and pea protein hybrid gels formed by acid gelation with glucono-delta-lactone, at specific ratios, through multi-scale analysis and the impact of temperature on their interactions.

iii. To evaluate the effectiveness of the pH shifting treatment in pea protein and its impact on hybrid gel systems formed by pea protein and casein in different protein ratios, induced by acid gelation

Thesis outline

Chapter I brings an overview of pea protein and the importance of hybrid systems for the food industry. Also, it highlights the importance of structural modification and compares the traditional methods with non-thermal technologies, elucidating the research problem, as well as the hypotheses and objectives of the thesis.

Chapter II is a literature review about pea protein structure and the use of non-thermal technologies in the structural modification of it, presenting a strategy to increase the use of pea proteins in food industries as a substitute for animal proteins.

Chapter III investigates multiple scales, the effects of protein ratio and temperature on the structural organization and rheological properties of mixed hydrogels formed by casein micelles and pea protein through acid gelation.

Chapter IV elucidates the effect of pH-shifting on pea protein structure and its impact on the structure of hybrid gels formed with casein micelles in different ratios.

Chapter IV summarizes the main findings of this research and provides recommendations for future studies.

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CHAPTER II.

RESEARCH ARTICLE ONE

Multi-scale organization and rheology of casein and pea protein mixed hydrogels formed by acidification: effects of ratio and temperature

Silva et al.

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Multi-scale organization and rheology of casein and pea protein mixed hydrogels formed by acidification: effects of ratio and temperature

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Abstract

The growing demand for sustainable food alternatives is driving increased research into mixed protein systems. In view of this scenario, this study aims to investigate the structural organization and rheological properties of mixed hydrogels formed by casein micelles (CMs) and pea protein through acid gelation, as well as to understand the effects of protein ratio and temperature on gel structure. The objective is to elucidate how these variables influence network formation at multiple scales, providing insights into the design of novel food structures. Small-angle X-ray scattering (SAXS), nuclear magnetic resonance (NMR), and rheological analyses were used to assess gel properties. The results indicate that mixed gels exhibit non-monotonic rheological behavior, with strong structural changes depending on the CM:pea ratio. At smaller scales (submicron sizes), there is no significant difference between pea or casein aggregates formed in mixed gels compared to those in pure gels. However, at larger scales (micron to tens of microns), the presence of pea in casein gels (or vice versa) has a significant impact on the protein network structure and gel properties, as seen in pore sizes and rheological behavior. Furthermore, temperature plays a crucial role, with effects observed at temperatures above 40 °C, mainly in casein-rich systems. This study provides a new perspective on the structuring of mixed protein gels and contributes to the development of hybrid food products.

Keywords: mixed hydrogels; casein protein; pea protein; rheology; structure; SAXS.

1. Introduction

Plant proteins, such as soy, peas, beans, and chickpeas, have been widely used in the food industry in recent years. Among these, pea protein has gained significant recognition, primarily due to its low allergenicity, high nutritional value, availability, low cost, and sustainable production (Lam et al., 2018; Shanthakumar et al., 2022). Pea protein consists of globulin and albumin, which make up approximately 70–80% and 10–20% of the protein content in pea seeds, respectively. Globulins are salt-soluble storage proteins and can be divided into three groups: legumin (300–400 kDa), vicilin (150–170 kDa), and, in smaller quantities, convicilin (70 kDa) (Kornet, Penris, et al., 2021; Lam et al., 2018). Albumin, on the other hand, is a small water-soluble protein (4–26 kDa) responsible for seedling growth (Kornet, Penris, et al., 2021; Shanthakumar et al., 2022).

The biggest challenge associated with plant proteins is their off-flavors, which reduce consumer acceptability. Additionally, their poor techno-functionality, such as low solubility, complicates their use as ingredients in food formulations (Nascimento et al., 2023). To overcome these disadvantages, combining plant proteins with dairy proteins, which offer higher sensory acceptability and superior techno-functional properties, has become a viable option (Oliveira et al., 2022; Silva et al., 2019).

Bovine milk protein is composed by casein (80% wt) and milk serum proteins (20% wt), primarily β-lactoglobulin, α-lactalbumin, and bovine serum albumin (BSA) (Walstra et al., 2006). Casein is a phosphoprotein composed of four different types: αs1-casein, αs2-casein, β-casein, and κ-casein (~23.6, 25.2, 23.98, and 19.55 kDa, respectively). In milk, caseins are present in the form of micelles, which consist of casein molecules, water, and salts, primarily calcium phosphate, which acts as a binding agent between submicelles. These micelles are held together through hydrophobic and electrostatic interactions (Krishna et al., 2021; Silva et al., 2019; Walstra et al., 2006). Due to their structure, casein micelles (CMs) can undergo various modifications depending on operating conditions, e.g., pH, temperature, ionic strength, and enzyme activity, which is of paramount importance for their application in the dairy industry (Silva et al., 2019).

One important application of protein-based food systems is gelation, which plays a crucial role in products such as yogurt, cheese, and gelatin. Hence, the

application of different proteins and gelation techniques continues to be explored for the development of new food products (Felix et al., 2017). Acid gelation, also known as cold gelation, is one of the techniques used to produce protein gels. This process typically involves glucono-delta-lactone (GDL), a weak acid that dissociates, releasing protons into the solution (Xia et al., 2024). These protons reduce the protein's electrostatic repulsion by protonating a charged carboxyl group. Consequently, the proteins can approach each other and form a three-dimensional macromolecular network (Chihi et al., 2018; Oliveira et al., 2022; Nascimento et al., 2024).

The mixed gel formed by plant and milk proteins has been studied by many researchers, yet focusing on its physical properties, especially rheology (Ben-Harb et al., 2018; Chihi et al., 2018; Felix et al., 2017; Mession et al., 2017a, 2017b; Silva et al., 2018; Silva et al., 2019; Oliveira et al., 2022; Xia et al., 2024; Nascimento et al., 2024). In a previous study, Nascimento et al. (2024) identified a particular property of gels formed by casein and pea protein, particularly at a 20:80 ratio. This finding highlighted the need for a more detailed investigation of the structural formation of these gels.

In our study, we applied a multi-scale approach to examine the structure of casein and pea protein mixed gels, considering the impact of specific ratios and temperature. Small-Angle X-ray Scattering (SAXS) was used due to its ability to provide insights into the nanoscale structural arrangement of proteins within the gel network, complementing traditional rheological and macroscopic techniques. Thus, this study aims to understand the structuration of casein and pea protein mixed gels formed by acid gelation with glucono-delta-lactone, at specific ratios, through multi-scale analysis and the impact of temperature on their interactions.

2. Material and methods

2.1. Materials

Micellar casein (CM) powder (Promilk 852B, 85.4% protein w/w) was kindly donated by Ingredia SA (Arras, France), and pea protein (PP) powder (Nutralys F85F, 84% protein w/w) was kindly provided by Roquette SA (Lestrem, France). The protein content of the powders was as reported by the respective companies.

2.2. Suspension preparation

Casein and pea protein suspensions were prepared by hydrating the powders in ultra-pure water at a protein concentration of 16% (w/w), based on a previous study conducted by our group (Nascimento et al., 2024). Rehydration was carried out overnight at 25 °C with continuous stirring at 600 rpm. To prevent microbiological growth, 0.003% sodium azide was added to each suspension.

After rehydration, the final suspensions were prepared by mixing casein and pea protein suspensions at ratios of 80:20 (CM:pea protein) and 20:80 (CM:pea protein) for 2 h at 25 °C and 600 rpm. The pure suspensions were also analyzed.

2.3. Gelation process

Gelation was induced by adding glucono-delta-lactone (GDL) (Sigma-Aldrich, USA) at concentrations of 1.2% (w/w) for suspensions with a high PP concentration (20:80 and 0:100) and 2% (w/w) for suspensions with a high CM concentration (80:20 and 100:0). The different concentrations were necessary due to the varying buffering capacities of the suspensions. At the beginning of gelation, the pH was 7.3 for the 0:100 and 20:80 ratios and 6.8 for the 100:0 and 80:20 ratios. The suspensions were then mixed for 1 min at 400 rpm. Afterward, they were placed in a water bath at 30 °C for 4.5 h, the time required to reach pH 5.2. As demonstrated by Nascimento et al. (2024), the gel structure at this pH is representative of the structures formed between pH 5.2 and 4.6, as rheological properties did not exhibit significant variation within this range.

2.4. Suspension characterization

To ensure that the proteins were properly distributed in the liquid phase, the suspensions were characterized.

2.4.1. Particle size analyses

The particle size distribution of individual and mixed systems was measured using Dynamic Light Scattering (DLS) after diluting the suspensions 100-fold in ultrapure deionized water. The DLS measurements were performed using

a DynaPro Nanostar (Wyatt, CA, USA) at a 90° scattering angle with a wavelength of 658 nm at 25 °C.

The apparent hydrodynamic diameter (D_h) was determined using the Stokes-Einstein equation (Equation 1). The analyte's translational diffusion coefficient (D_t) was obtained through automated nonlinear least squares fitting of the autocorrelation function, which quantitatively characterizes the time-dependent fluctuations in light scattering intensity.

$$D_h = \frac{K_b T}{3\pi\eta D_t} \qquad \text{Equation 1}$$

where K_b is the Boltzmann's constant; T is the temperature; and η is the suspension viscosity.

2.4.2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was used to determine the molecular weight distribution following the methodology described by Beghdadi et al. (2022). Samples were first diluted to 8 mg/mL in ultrapure water, followed by a second dilution in different buffers: native (125 mM Tris-HCl, 20% glycerol, 0.4% bromophenol blue), reduced (0.055 M Tris-HCl, 2% SDS, 5% β-mercaptoethanol, 7% glycerol, 0.0025% bromophenol blue), and non-reduced (125 mM Tris-HCl, 4% SDS, 20% glycerol, 0.4% bromophenol blue). A molecular weight marker of 260 kDa was also used. The mini gels consisted of a 4% stacking gel (water, 0.5 M Tris-HCl pH 6.8, 30% acrylamide, 25% SDS, and 10% ammonium persulfate) and a 12% separating gel (water, 1.5 M Tris-HCl pH 8.8, 30% acrylamide, 25% SDS, tetramethylethylenediamine, and 10% ammonium persulfate). A volume of 20 µL of each sample was loaded per well, and electrophoresis was performed using a running buffer (0.025 M Tris, 0.192 M glycine, 0.1% SDS) at pH 8.3, applying 150 V for 1 h. After protein migration, the gels were fixed by immersion in a solution of 10% acetic acid and 30% ethanol, followed by staining with 0.15% Coomassie® Brilliant Blue R-250 dissolved in 10% acetic acid, ethanol, and water for 30 min under agitation. The gels were then destained in a 10% acetic acid solution for 1 h. After this process, the gels were scanned and analyzed using ImageJ. The molecular weight distributions were subsequently determined based on the gel under reduced conditions.

2.5. Rheology measures

Rheology was used to assess the effect of temperature on gel structure. The analysis was conducted using a strain-controlled rheometer (ARES, TA Instruments, USA) equipped with a cone-plate geometry (0.06 rad angle, 112 µm gap). The linear viscoelastic region was determined through a strain sweep test (0.01 to 100%) at 1 Hz and 30 °C, allowing the identification of the appropriate strain to be applied during the rheological tests. The flow behavior was evaluated through three cycles of varying shear rates from 0.1 to 300 s^{-1} to assess the thixotropic behavior.

Following the addition of GDL to the suspensions, samples were loaded into the rheometer, and gelation was carried out for 4.5 h at 30 °C. To further investigate the interactions governing the mixed gel systems, a temperature sweep test was performed according to the methodology described by Andlinger and Kulozik (2023). The temperature was initially set to 20 °C and maintained for 10 min to allow sample equilibration, ensuring a standardized starting temperature after gelation in the rheometer. This was followed by a temperature sweep from 20 °C to 60 °C, with a heating rate of 5 °C·min^{-1}.

2.6. Nuclear magnetic resonance (NMR)

Gels were prepared as described in section 2.3, with water replaced by deuterium oxide. The samples were then analyzed using solid-state nuclear magnetic resonance (NMR) spectroscopy. Proton dynamics (^{1}H NMR) were measured on a 9.4T AVIII Bruker spectrometer equipped with a 4 mm probe, operating at a spinning frequency of 700 Hz. To investigate the effect of temperature, NMR relaxation times (T_1) of water protons were measured for different gel ratios at 20 °C, 30 °C, and 40 °C. Data analysis was performed by spectral observation and comparison of the water peak and protein peak (2.79 ppm) using TopSpin 4.0 software (Bruker, USA).

2.7. Small-angle X-ray scattering (SAXS)

The gels were prepared as described in section 2.3 and placed in fixed capillaries for SAXS analysis. Measurements were conducted following the methodology of Nogueira et al. (2023) at the SOLEIL synchrotron facility (Gif-sur-

Yvette, France) on the SWING beamline, operating at ~12 keV photon energy. The scattered intensity was recorded using a detector positioned ~6.2 m from the sample. For each sample, initial data acquisition was performed at a short exposure time (~0.2 s) to prevent any radiation damage (aggregation), which could introduce artifacts at low scattering wavevector (q) values. Subsequently, data were collected at longer exposure times (~15 s) using a larger beam stop to improve the signal-to-noise ratio at high q values without damaging the detector. The intensities recorded at both exposure times were radially averaged and combined to generate a scattering curve covering a q-range of 1.03 × 10^{-3} to 0.18 $Å^{-1}$. Prior to merging, inconsistent intensity values were discarded. For each sample, the intensity scattered by the solvent (ultra-pure water) in the same capillary was measured and subtracted from the gel sample pattern. Measurements were conducted at 20 °C, 30 °C, and 40 °C. A unified model was applied to fit the scattering data, and the radius of gyration (Rg) of aggregates of various sizes was determined using the fitting method according to Equation 2.

$$I\,(q) = background + \sum_{i=1}^{N}\left[G_i \exp\left(-\frac{q^2 R_{gi}^2}{3}\right) + B_i exp\left(-\frac{q^2 R_{g(i+1)}^2}{3}\right)\left(\frac{1}{q_i^*}\right)^{P_i}\right]$$

Equation 2

where $$q_i^* = q\left[\mathrm{erf}\left(\frac{qR_{gi}}{\sqrt{6}}\right)\right]^{-3}$$

and i represents the structural level; R_g is the intensity-weighted average radius of gyration of the scatters; P_i is the power law exponent; G_i is the Guinier scale, and B_i is the perfector for power-law scattering at structural level i (Chen et al., 2022).

The Bouchoux model was also applied to fit the scattering data for gels with a higher casein content. The intensity $I(q)$ is given by Eq. 3:

$$I(q) = c[\phi_0 v_0 (\Delta p_0)^2 P_0(q) + \phi_1 v_1 (\Delta p_1)^2 P_1(q) + \phi_2 v_2 (\Delta p_2)^2 P_2(q)] \quad \text{(Equation 3)}$$

where c is a constant that accounts for the total concentration of the caseins; ϕ_n is the volume fraction occupied by the structural element n in the

dispersion; v_n and Δp_n are the volume and average scattering contrast of each structural element, respectively; $P_n(q)$ are the form factors of the different objects, modeled using the expression of Aragón & Pecora (1976).

2.8. Statistical analysis

The data were analyzed using analysis of variance (ANOVA) with Statistica (StatSoft Inc., Maisons-Alfort, France) to assess the influence of ratios and temperature. When a significant difference ($p < 0.05$) was detected, Tukey's HSD test was applied at a 5% confidence level to differentiate means. All experiments were performed at least twice independently.

3. Results and discussion

3.1 Diluted suspension characterization

The aggregation state of pea protein, both in individual and mixed systems, was determined by measuring the hydrodynamic radius using dynamic light scattering (DLS). The results are presented in Figure 1.

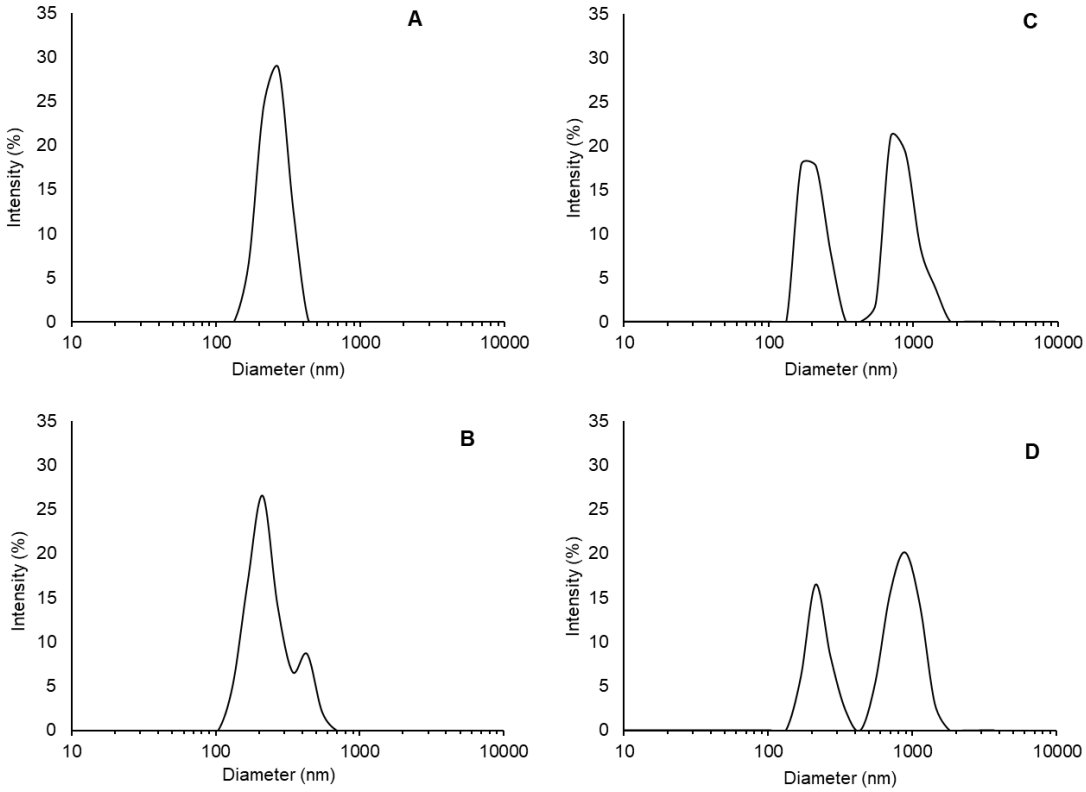
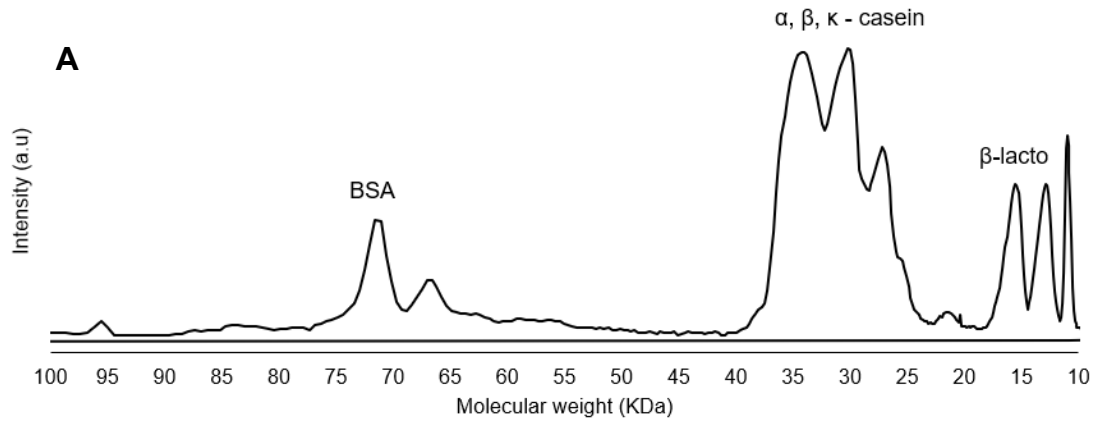


Figure 1. Particle size distribution for different ratios: 100:0 (CMs/pea protein) (A), 80:20 (B), 20:80 (C), 0:100 (D).

For the 100:0 ratio (pure casein), the monomodal distribution showed a hydrodynamic diameter of 269 nm, which aligns with the findings of Beghdadi et al. (2022), who reported a diameter of 280 nm. In the 0:100 ratio (pure pea protein), the suspension exhibited two peaks: one around 212 nm and another at approximately 880 nm, consistent with the results of Kornet et al. (2021). Since the expected hydrodynamic diameter for pea proteins is approximately 15 nm (Beghdadi et al., 2022), these peaks correspond to larger aggregates. Previous studies have suggested that these aggregates form during the production of pea protein isolate, which involves precipitation, purification, and drying (Barac et al., 2010; Beghdadi et al., 2022; Chihi et al., 2016). In the mixed gels (ratios 80:20 and 20:80), the observed particle sizes matched those previously reported for pure protein samples, indicating that no new stable aggregates were formed. Since even a small fraction of aggregates can significantly influence DLS measurements, electrophoresis was also employed to further characterize the protein suspensions.

The electrophoresis technique was applied to determine the protein composition of the powders. Figure 2 presents the protein band profiles in the gel, showing the composition of casein (Figure 2A) and pea (Figure 2B) proteins. Protein molecular weights were estimated based on migration distances (R_F values). For casein, the molecular weights of the α-, β-, and κ-casein fractions ranged from 35 to 25 kDa. The presence of serum proteins in the casein powder was also detected, indicated by the first peak corresponding to bovine serum albumin (BSA, 71 kDa) and the last peak associated with β-lactoglobulin (15 kDa). The presence of these proteins in the casein powder may be due to residual contamination from the production line.

Regarding the pea protein suspension (Figure 2B), the electrophoresis profile revealed the globulin fractions convicilin, legumin, and vicilin. The first peak in the molecular distribution corresponds to convicilin (Con), a storage protein with 73 kDa. Legumin (Leg), a protein with a sedimentation coefficient of 11S, consists of three subunits: an acidic subunit (Leg-α, 37 kDa), a basic subunit (Leg-β, 17 kDa), and a Leg-αβ subunit covalently linked by a disulfide bond (63 kDa). The vicilin (Vic) protein also contains three subunits: vic-α (45 kDa), vic-β (29 kDa), and vic-γ (13 kDa). Similar results were found by Beghdadi et al. (2022) and Shand et al. (2007), who investigated the impact of heat treatment on pea protein gel formation.



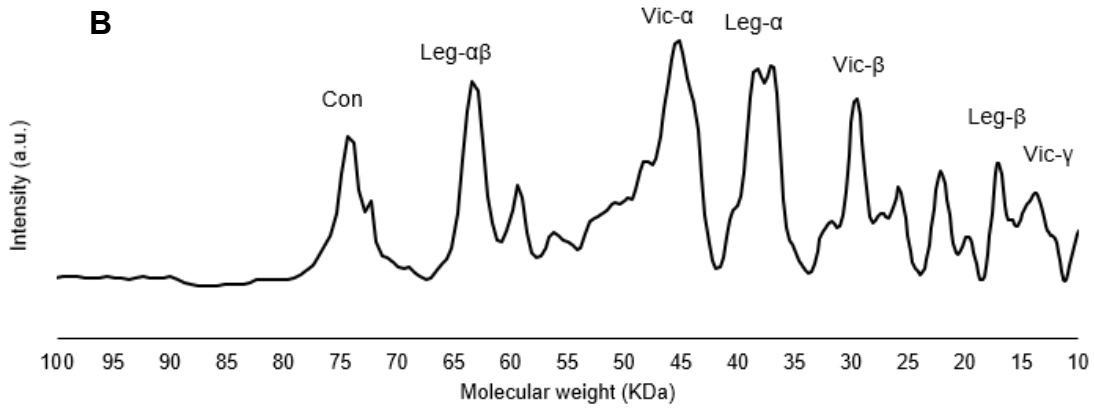


Figure 2. Molecular weight distribution of pure casein suspension (A) and pure pea protein suspension (B) at 16% (w/w).

At the top of the electrophoresis gel (Figure 3), some samples did not penetrate the gel, even after treatment with SDS and β-mercaptoethanol. This suggests the presence of large aggregates in the pea suspension that are covalently linked. These results indicate that, in addition to disulfide and non-covalent interactions, covalent bonds are responsible for the formation and cohesion of these protein aggregates (Sun & Arntfield, 2012).

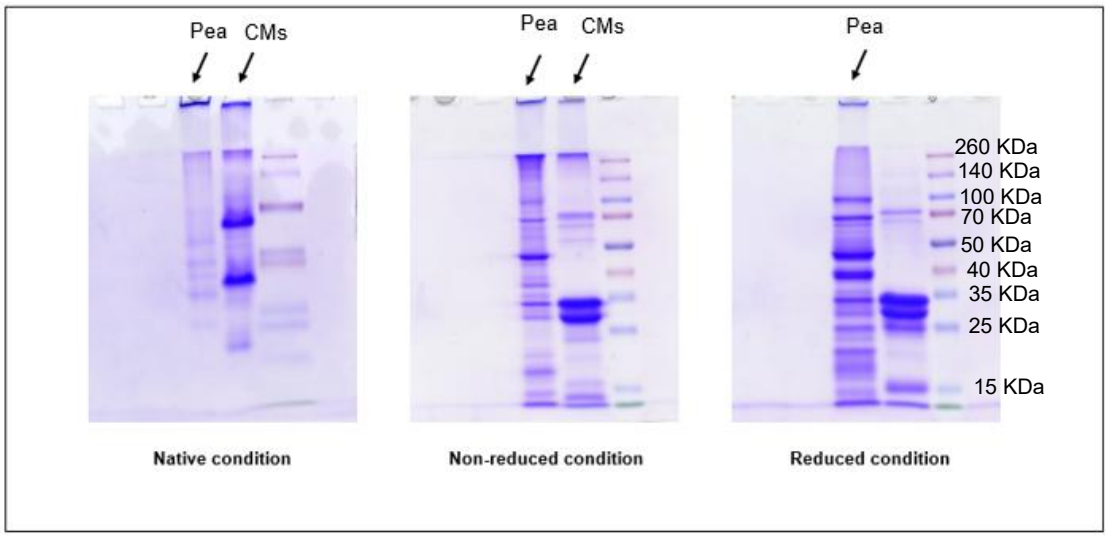


Figure 3. Electrophoresis gel of pure casein and pure pea protein suspensions under native, non-reduced, and reduced conditions.

3.2 Gel characterization

3.2.1 Nuclear magnetic resonance (NMR)

Solid-state ^{1}H NMR was used to analyze the effects of the CM:pea protein ratio and temperature on water dynamics and protein mobility. Water in gels can

be classified into two groups: bound water, which is located near the protein surface, and free water, which exists a few nanometers away from the protein surface. In gels or dense protein systems, free water dynamics are influenced by the size of the gel's cavities, which can range from hundreds of nanometers to the micron scale in diameter (Baumgartner et al., 2002; Mariette et al., 2002). The present study primarily focuses on free water, as it is significantly more abundant than bound water in gels (Baumgartner et al., 2002; Mariette et al., 2002).

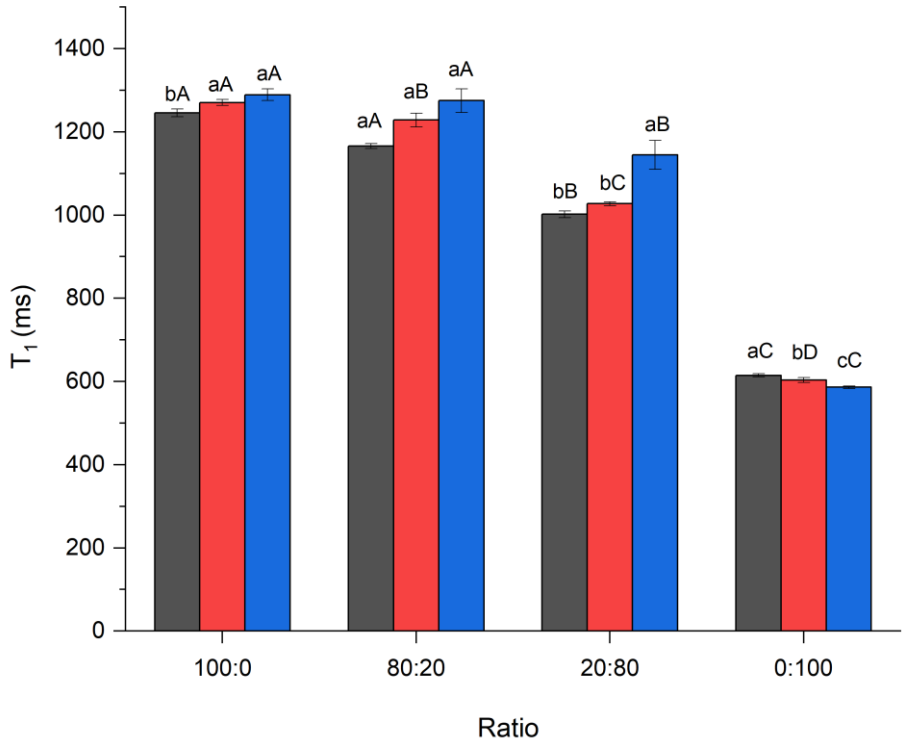


Figure 4. Spin-lattice relaxation times (T1) of water (4.79 ppm) as a function of gel ratio. Black columns: 20 °C; red columns: 30 °C; blue columns: 40 °C. Different lowercase letters indicate significant differences between protein ratios at different temperatures. Different uppercase letters indicate significant differences within the same temperature across different protein ratios. The significance level of the Tukey test was 5%.

As shown in Figure 4, water dynamics in the gels depend on the CM:pea protein ratio and temperature. Higher water relaxation times (T1) indicate greater water mobility within the gel structure. At 20 °C, T1 values increase with the casein concentration, with the 100:0 ratio exhibiting the highest T1 (Figure 4). On

the other hand, the pea protein gel (0:100 ratio; 0% casein) shows the lowest T1, indicating reduced water dynamics. The increased water mobility in casein-rich gels is attributed to the presence of larger water-filled cavities (also referred to as "pore size") (Ruan et al., 1997). Although the total amount of matter in both systems is similar, the organization of proteins substantially influences water dynamics. The data suggest that CM gels form a more heterogeneous structure (on the submicron to micron scale), creating larger cavities between proteins and allowing for greater water mobility. In contrast, pea protein gels form a more homogeneous network, restricting water movement.

Regarding the different CM/pea protein ratios, Figure 4 shows that T1 does not exhibit a linear evolution as a function of the ratio. The difference in T1 between the pure casein gel (100:0) and the gels with 20% pea protein (80:20) or 80% pea protein (20:80) is relatively small compared to the difference between the 80% pea protein gel (20:80) and the pure pea protein gel (0:100). This means that even a small amount of casein significantly disrupts the homogeneity of the pea protein gel, creating pores almost as large as those found in the pure casein gel. Additionally, replacing a large proportion of casein with pea protein (shifting from 20% to 80% pea) results in only a moderate reduction in T1. This indicates that the gels with 20% and 80% pea protein exhibit similar porosity, in contrast to the more compact network of the pure pea gel.

The temperature dependence of water in the casein gel shows a small, roughly linear shift in T1 with increasing temperature. This linearity suggests that heating does not induce significant structural transformations in the network, implying that casein does not drastically alter water behavior. Moreover, no substantial shift is observed in the gels composed solely of pea protein. This shows that in pea protein gels, the smaller pores can strongly counteract the effect of temperature on water dynamics.

Figure 5 shows the ^{1}H T1 values of pea proteins (measured using the pea protein peaks at 2.79 ppm), which reflect the dynamics of both the proteins and the free water in close proximity to them. In contrast to the T1 values of water, Figure 5 displays an almost linear increase in proton T1 as a function of the CM/pea protein ratio. This suggests that adding 20% casein to a pure pea protein system does not induce significant changes in the protein network at a local scale. The same trend is observed at all temperature values, indicating that the

reorganization of the protein network in terms of porosity is less pronounced at smaller scales.

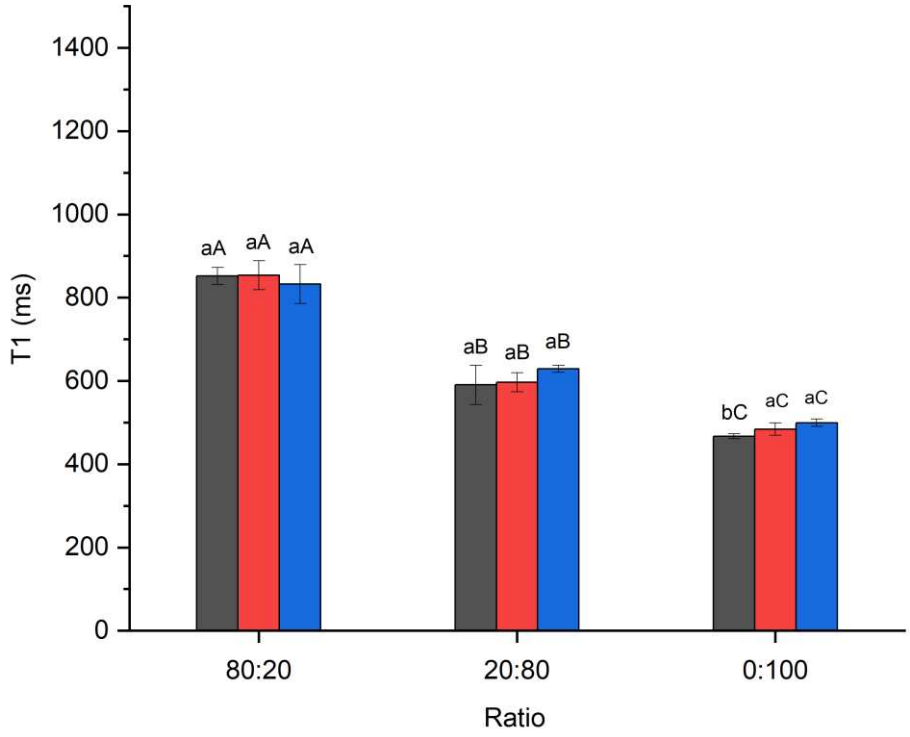


Figure 5. Spin-lattice relaxation times (T1) of pea protein (2.79 ppm) as a function of gel ratio. Black columns: 20 °C; red columns: 30 °C; blue columns: 40 °C. Different lowercase letters indicate significant differences between protein ratios at different temperatures. Different uppercase letters indicate significant differences within the same temperature across different protein ratios. The significance level of the Tukey test was 5%.

3.2.2 SAXS

3.2.2.1 SAXS curves visualization

Small-angle scattering is a technique that can provide multiscale information about the protein network in gels (Chen et al., 2022; Pedersen et al., 2022). Several differences between the ratios can be observed in Figure 6.

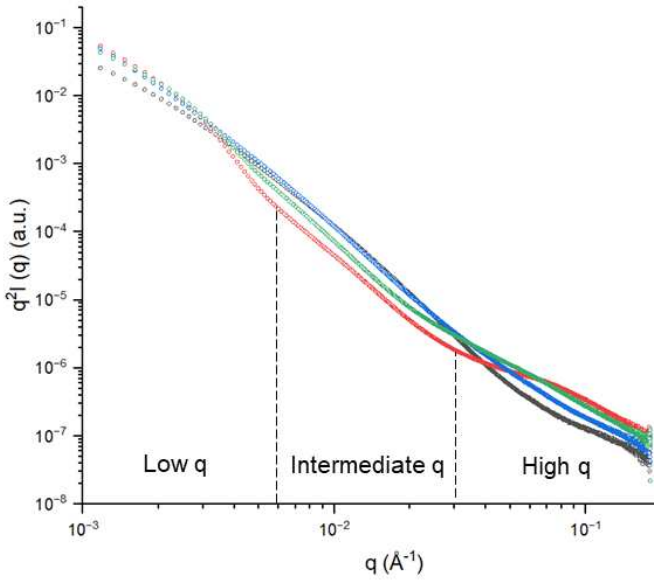


Figure 6. SAXS profiles of mixed gels formed by CMs:pea protein gels at 20 °C. Red circles: 100:0; green circles: 80:20; blue circles: 20:80; black circles: 0:100

An overview of the superposed SAXS data (Figure 6) shows distinct profiles across different ratios. In the low to intermediate q region, the SAXS profiles exhibit noticeable variations, indicating that the presence of just 20% pea protein in casein gels, or 20% casein in pea protein gels, induces structural changes. In the high q region, a "shoulder" feature appears to decrease roughly in proportion to the casein content in the sample. This may be attributed to the fact that the "shoulder" in this region is strongly influenced by the inter-distance between calcium phosphate clusters associated with casein (CCP) (Bouchoux et al., 2010). This explains why casein-rich gels exhibit some similarity in this region despite clear differences in the intermediate region. A more detailed interpretation of this data is provided in the next section.

Regarding the temperature effect, Figure 7A presents the X-ray scattering profiles at 20 °C and 30 °C. In this temperature range, only the low q region shows minor modifications for the pure casein sample and the two mixed gels, suggesting that the temperature effect observed by NMR primarily affects large-scale structures.

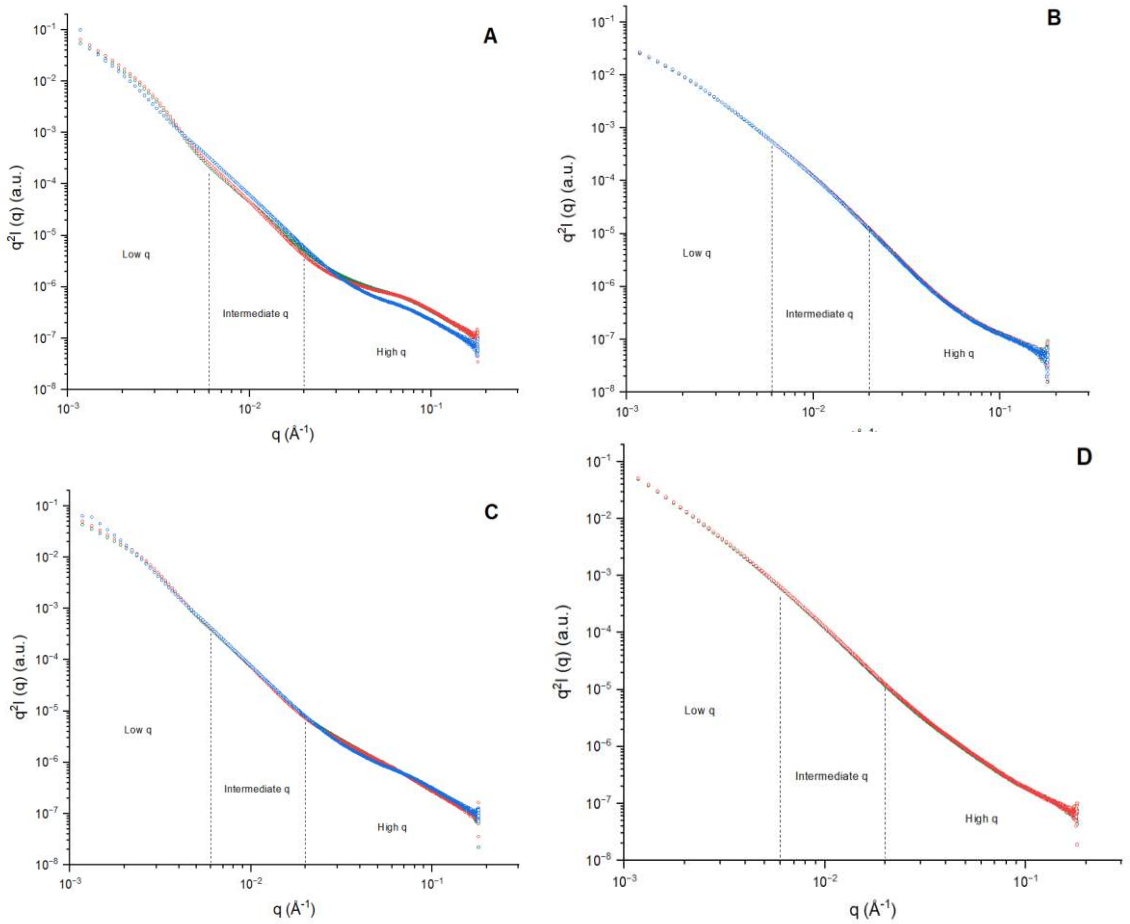


Figure 7. SAXS profiles of mixed gels formed by CMs:pea protein gels as a function of concentrations: A) 100:0 B) 0:100 C) 80:20 D) 20:80. Green circles: 20 °C; red circles: 30 °C; blue circles: 40 °C.

Temperature (°C)	Sample	Region 1 (Low q)				Region 2 (Intermediate q)				Region 3 (High q)			
		ϕ_0 (Å)	C	σ (Å^2)	N	ϕ_1 (Å)	C	σ (Å^2)	N	ϕ_2(Å)	C	σ (Å^2)	N
20 °C	100:0	800	5.0E-11	0.33	1.0	120	1.07E-05	0.33	20	20	1.514E-05	0.2	550
20 °C	80:20	800	2.50E-05	0.33	1.0	120	1.07E-05	0.33	50	20	1.51E-05	0.2	350

Table 1. Fits of the Bouchoux model to the SAXS data. ϕ, C, σ, N represents the volume fraction occupied by the structural element, constant, polydispersity, and relative number density, respectively.

Temperature (°C)	Sample	Region 1 (Low q)				Region 2 (Intermediate q)				Region 3 (High q)			
		G (cm^{-1})	R_g (Å)	B (cm^{-1})	P	G (cm^{-1})	R_g (Å)	B (cm^{-1})	P	G (cm^{-1})	R_g (Å)	B (cm^{-1})	P
20 °C	80:20	0.001	3500	250	1.2	150	720	8.00E-04	3.1	30	30	2.00E-05	4.3
20 °C	20:80	0.01	3500	150	1.2	150	700	8.00E-04	3.1	10	30	2.00E-05	4.3
20 °C	0:100	538.13	1428.40	2.37E-02	2	500	329.30	3.98E-05	2	188.25	150.91	4.50E-06	3.8

Table 2. Fits of the Unified model to the SAXS data. G, R_g, B, and P represent the Guinier scale, the intensity-weighted average radius of gyration of scatters, the prefactor of power-law scattering at structural level i, and the power law exponent, respectively.

3.2.2.2 SAXS curves fitting and interpretation

To gain a more comprehensive understanding of the structural variations in the gels, two different models were used to fit the data. Since the CM gel (100:0) appears qualitatively similar to previously published data for native casein micelles (Bouchoux et al., 2010), this sample was fitted using the “casein sponge model.” This model describes micelles as sponge-like porous structures (Table 1) with three main characteristics: i) The low q range (below 6 x 10^{-3} $Å^{-1}$), which corresponds to the distance between CMs and their micellar envelope, with a diameter of approximately 100 nm; ii) The intermediate q range (6 x 10^{-3} $Å^{-1}$ to 2 x 10^{-2} $Å^{-1}$), which correlates with smaller micelle structures or inhomogeneities (20 – 40 nm in diameter) that are referred to as "hard" since they remain incompressible under osmotic pressure; and iii) The high q range (above 7-8 x 10^{-2} $Å^{-1}$), where the inter-distance between calcium phosphate nanoclusters (CCP) covered by proteins is responsible for a characteristic “shoulder” in the q range of 0.042-0.27 nm^{-1}.

The model fit suggests slightly larger micelle diameters (ϕ_1 (Å)) for the different structural regions (120 nm for the “hard regions” and 20 nm for the CCP regions), indicating that at pH 5.2, the casein structure may exhibit structures approximately 30% larger than native micelles at neutral pH. Moreover, the relative quantity of CCPs (N) is considerably low (Table 1), which is expected, as micelles begin to lose their CCP centers at pH 5.2 (Day et al., 2017). This reduction may contribute to an increase in internal cavities within the micelle structure.

For the 80:20 ratio, the presence of just 20% pea protein in the casein gel induces a significant structural modification. The change is so pronounced that it was not possible to fit the data using the sponge model (see SI for details). Instead, for this sample, as well as the other samples containing pea protein, a more general unified fit model was applied. This model assumes that the gel structures exhibit fractal behavior, described by Porod exponents (Chen et al., 2022), which may better represent pea protein gels. Since the structure of pea protein gels is not well understood, the interpretation of the data focuses on relative differences between samples rather than absolute values. The values extracted using this model (Table 2) for regions 1 and 2 are quite similar between the 80:20 and 20:80 samples, with the main difference being an increase in prefactors. The small variations in R_g for regions 1 and 2 suggest that the SAXS

signal in these regions is primarily dominated by pea proteins. Additionally, increasing the amount of pea protein in the mixed gels does not significantly affect the structure of aggregates at smaller scales. A greater difference is observed in region 3, likely due to the more marked reduction of CCP casein clusters when transitioning from a casein-rich sample (80:20) to a casein-poor sample (20:80). Furthermore, transitioning from a pea-protein-rich gel (20:80) to a pure pea protein gel (0:100) induces significant structural modifications across multiple scales. At larger scales (regions 1 and 2), there is a notable decrease in the inter-distance radius, R_g (Å), by approximately 110%. These SAXS data suggest that the inhomogeneities formed by pea proteins in the pure pea gel are smaller, which explains the reduction in water cavities within the gels and, consequently, the decreased dynamics of free water.

3.2.3 Rheology measurement

Rheological properties describe a material's behavior when subjected to normal and tangential stresses (Rao, 2013). These properties are typically characterized by the elastic modulus (G') and the viscous modulus (G''), which are parameters for evaluating the structural networks of materials (Rao, 2013; Stojkov et al., 2021). Previous studies have shown that protein interactions and gel structure depend on the ratio of each protein during acid gelation. For instance, Roesch et al. (2004) observed this effect in acid gelation of soy and skim milk suspensions. Similarly, Silva et al. (2018) and Silva et al. (2019), in their studies on casein and plant protein heat gelation, found that casein and pea protein do not co-aggregate during gelation, demonstrating that structural modifications are ratio-dependent. Our previous study (Nascimento et al., 2024) also confirmed that the CM:pea ratio is an important factor in gel properties. Additionally, no drastic changes were observed in the rheological profile due to pH variations within the range of pH 5.4 to pH 4.6, the latter being the isoelectric point of casein.

Figure 8A presents the G' values of the samples as a function of temperature after gel formation at pH 5.2. At 20 °C, G' for the pure casein gel was 2523.5 Pa. However, when 20% pea protein was incorporated into the system (80:20 ratio), the gel strength was 505.45 Pa. This suggests that the presence of pea protein domains in casein-rich gels considerably weakens the cohesive forces of

the gel. For the other pea protein concentrations, G’ increases, indicating that, in the present case, G’ in casein gels is more dependent on long-range interactions than in pea gels. The addition of 20% pea protein to a casein-rich gel (80:20) reduces stiffness, whereas incorporating 20% casein into a pea-rich gel has minimal impact. This observation is in line with the NMR data, which shows that casein gels exhibit larger inhomogeneities than pea gels. A gel with smaller structural distances (smaller "pores") has a greater number of contact points between pea proteins at a smaller scale. Thus, adding casein domains to such gels is less likely to disrupt the long-range pea protein network.

The impact of temperature is slightly more pronounced in casein-rich gels than in pea-rich gels before 40 °C. However, after 40 °C, the casein-rich gel undergoes a sharp decrease in G’, indicating significant structural changes. The error bars also increase with rising temperature, suggesting that the casein network evolves rapidly, progressively reducing the reproducibility of the experiments. This behavior is further supported by the tan δ values, which represent the ratio between the viscous and elastic moduli (tan δ = G’’/G’), which is related to the bonds present, independent of their quantity (Gastaldi et al., 2003). As can be seen in Figure 8C, the 0:100 and 20:80 gels remain more stable across all temperature ranges, while the 100:0 and 80:20 gels show an increasing tan δ with rising temperature.

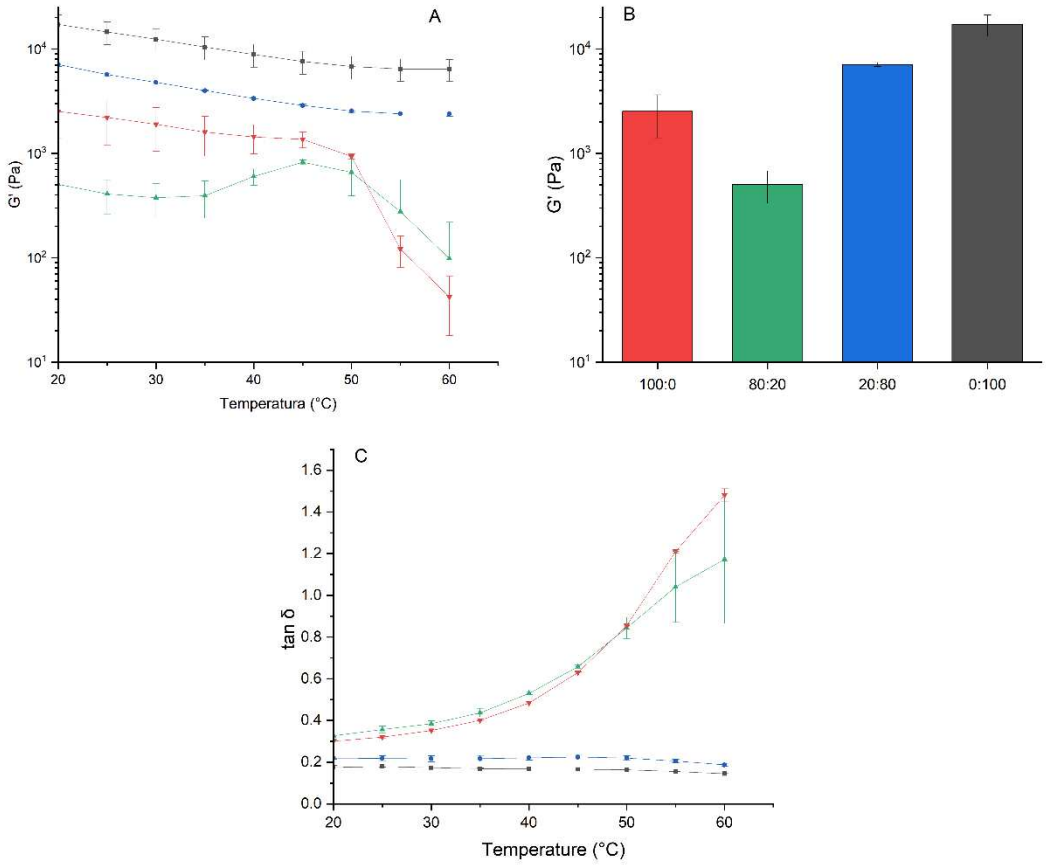


Figure 8. Temperature dependence of G' (A), G' at 20 °C (B), and tan δ (C) of mixed gels with CMs:pea protein ratios of 100:0 (red), 80:20 (green), 20:80 (blue), and 0:100 (black).

4. Conclusion

This study investigated the effects of protein ratio (CMs/pea protein) and temperature on the rheology and multi-scale structure of mixed gels. The addition of 20% pea protein to casein gels had a significant impact on gel organization, reducing stiffness compared to pure casein gels. The data indicate that casein and pea protein form independent network domains, with no evidence of molecular interactions between them. The results suggest that large-scale structural organization is the dominant factor governing key properties such as rheology and water mobility within the gels. Since no interactions between the proteins were observed, future studies could explore strategies to enhance pea-casein interactions, particularly using green technologies such as pulsed electric fields, cold plasma, and ohmic heating. Understanding the structure of mixed gels and improving their interactions could pave the way for the development of new foods

enriched with plant proteins, meeting growing consumer demand and driving transformations in the food industry.

CRediT authorship contribution statement

Raiane Rodrigues da Silva: Writing – original draft, Methodology, Investigation. Davide Odelli: Writing – original draft, Investigation. Amandine Descamps: Methodology, Investigation. Luisa Azevedo Scudeller: Writing – original draft, Formal analysis. Bertrand Doumert: Methodology, Investigation. Javier Perez: Methodology, Investigation. Guillaume Delaplace: Writing – original draft, Validation, Resources, Funding acquisition, Conceptualization. Antonio ˆ Fernandes de Carvalho: Writing – original draft, Validation, Resources, Funding acquisition, Conceptualization. Paulo Peres de Sa ´ Peixoto Junior: Writing – original draft, Validation, Supervision, Formal analysis, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

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CHAPTER III.

RESEARCH ARTICLE TWO

Effect of pH shifting on the physicochemical properties of pea proteins and its effect in hybrid gel with casein micelles

Silva et al.

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Effect of pH shifting on the physicochemical properties of pea proteins and its effect in hybrid gel with casein micelles.

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Abstract

Hybrid systems combining animal and vegetable proteins are gaining attention in the food industry to promote plant protein consumption. However, these proteins often show incompatibility, making it challenging to form stable colloidal systems like gels. pH shifting has emerged as a strategy to structurally modify vegetable proteins and can be a strategy to enhance their interaction with animal proteins. Thus, this study evaluated the effect of pH shifting on pea protein and its influence on hybrid gels formed with casein in different ratios (80:20, 50:50, and 20:80, casein:pea protein), using acid gelation. The treatment improved pea protein solubility by inducing structural changes, which led to reduced particle size, increased zeta potential, and altered intrinsic fluorescence, especially in pure pea protein suspensions. Additionally, these structural modifications enhanced the water retention capacity of the gels and altered their texture, increasing hardness in gels with higher pea protein content. Overall, the results demonstrate the potential of pH shifting to improve the functionality of pea protein and its compatibility in hybrid protein systems.

Keywords: Pea protein; Casein; pH shifting; Structure; Sustentability;

1. Introduction

Climate change has become a growing global concern, with food production being a significant contributor to this issue. It significantly impacts greenhouse gas (GHG) emissions, freshwater scarcity, eutrophication, land degradation, and biodiversity loss (C. Chen et al., 2022). In the United Nations 2030 Sustainable Development Goals, the achievement of certain targets, such as those related to Climate Action and Life on Land, is highly dependent on consumption patterns (C. Chen et al., 2022; United Nations, 2015). Furthermore, population growth and concerns about food security related to food production, especially protein, increase the need for changes in eating habits (Nascimento et al., 2023).

It is not easy to change a society's eating habits. Generally, a gradual shift is needed. As a result, industries and research teams are looking more and more at hybrid systems as a means for introducing new eating practices (C. Wu et al., 2021). As an example, several studies have been investigating the replacement of animal protein with vegetable protein in the food system (Lee et al., 2022; Omrani Khiabanian et al., 2020; Yan et al., 2022; Yuliarti et al., 2021), since animal production demands more land and water use, emits more greenhouse gases, and has a lower conversion rate into dietary protein (Nascimento et al., 2023).

Among vegetable proteins, pea proteins have been gaining attention due to their high productivity, low cost, and excellent amino acid profile, especially due to the presence of lysine and tyrosine (Shanthakumar et al., 2022b), thus being a great option for replacing animal proteins. However, the combination of proteins from different origins, such as milk and pea proteins, might drastically alter some colloidal systems characteristics (Wu et al., 2021). When pea and milk proteins, such as casein, coexist in the same system, their thermodynamic incompatibility induces a competitive dynamic between them, impacting the colloidal systems structure, for example, in gel formation (Ben-Harb et al., 2018; Nascimento et al., 2024). To enhance the interaction between these two proteins and minimize their incompatibility, several techniques have been employed, such as thermal treatment (J. L. Mession et al., 2017; Nascimento et al., 2024), ultra-sound (Nascimento, Queiroz, et al., 2023), and high hydrostatic pressure (Serrano León et al., 2024). A potential new approach to further improve their interaction could involve structural modifications induced by pH shifts.

The pH shifting technique consists of the adjustment of pH to extreme conditions, whether acidic or basic, and afterward a return to the neutral pH of the medium

as a way of inducing the “molten globules”, which during protein unfolding, are recognized as an intermediate conformational state and have the same secondary structural as in the native state (Zhu et al., 2021). In the last years, the pH shifting effect in vegetable proteins has been evaluated. Comparing the effect of the pH shifting in the pea protein hydrogel formed by heating, Zhu et al. (2021) noticed that the pH shifting to pH 12 causes changes in the gel microstructure, resulting in a uniform polymer-like gel network microstructure, with a higher WHC, depending on the treatment holding time. Li el al. (2020) studying the modifications cause by pH-shifting at different pH (2, 4, 10, and 12) in peanut protein isolate heat induced gel, noticed that the modification was capable to decreased particle size, increased solubility, free sulfhydryl group content and surface hydrophobicity in pH 10, thus being an effective treatment for the formation of gels with different structural properties. In systems formed by soy/potato (Sun et al., 2025), mung bean protein (Jeong & Cho, 2024) and soy protein (Tan et al., 2021), the pH shifting alone or combined with other methods has also proven to be effective in modifying proteins and consequently in gels structuring.

This study aims to evaluate the effectiveness of the pH shifting treatment in pea protein and its impact on hybrid gel systems formed by pea protein and casein in different protein ratios, induced by acid gelation. The approach of this study involves first the pea protein modification using pH-shifting treatment (pH 12) and then the mixture with casein at different ratios (80:20, 50:50, and 20:80). The effects of this modification are evaluated in the protein suspensions and gel structure formed by acid gelation, and from the best of our knowledge, being the first time investigated.

2. Materials and methods

2.1 Materials

Pea proteins (Nutralys, F85F, 83%) was kindly donated by Roquette (Lestrem, France), and Micellar Casein Isolate (Lacprodan Micelpure 86.5%) was donated by Arla Food Ingredients (Århus, Denmark). The protein content was determined by the Kjeldahl method (Kjeldahl, 1883), with nitrogen conversion factors (N) of 6.25 and 6.38 to pea protein and casein, respectively.

2.2 Methods

2.2.1. Suspension preparation

The protein suspension was prepared by diluting the protein isolates in deionized water to a concentration of 12% (w/w). This concentration was chosen to simulate a high-protein yogurt. The suspensions were stirred for at least 12 hours in a mechanical agitator to guarantee complete protein hydration. Sodium azide (0.003%) was added to the suspensions to avoid microbiological growth.

2.2.2. pH modification

Pea protein suspension modification was done by adjusting the pH to 12 with 3M NaOH and maintaining it under agitation for 24 hours. After this period, the pH was adjusted to 7 using 3M HCl [19]. The time was determined by a preliminary test, taking into consideration the protein solubility after the pH modification.

After the modification, the pea protein suspension (PPS) was mixed with casein micelles suspension (CMs) in different proportions of proteins, following Table 1. The mixture was stirred for 30 minutes before the analysis. The systems without modification were also evaluated.

Ratio	Protein concentration in the suspension
100:0	100% casein
80:20	80% casein and 20% pea protein
50:50	50% casein and 50% pea protein
20:80	20% casein and 80% pea protein
0:100	100% pea protein

Table 1. Protein proportions in the different ratios

2.2.3. Solubility

The solubility test was performed according to Li et al. (2020), with modifications. The suspensions were centrifuged at 3600 x g for 15 min, and then the protein content was determined using the biuret method. The protein content was also determined before centrifugation. The solubility was then calculated by Equation 1. Bovine serum albumin (BSA) was used as the standard protein.

$$Solubility\ (\%) = \frac{Protein\ content\ in\ supernatant}{Total\ protein\ content} \qquad \text{Equation 1}$$

2.2.4. Particle size and zeta potential

The particle size distribution and zeta potential were measured with the Zetasizer Nano ZS (Malvern Instrument Ltd., UK) according to Li et al., (2020). First, the suspensions were diluted 100 times, and then, 1 mL of the sample was injected into the capillary cells. After, the zeta potential and particle size distribution were tested. All measurements were conducted at 25 °C in three independent tests.

2.2.5. Polyacrylamide Gel Electrophoresis

The protein profile was determined by the electrophoresis technique, following the methodology described by Beghdadi et al., (2022). The samples were prepared by a first dilution to 10 mg/mL in deionized water and after added to the buffer for native conditions (Tris-HCl 0,5M pH 6,8, pH 6.8, glycerol, Bromophenol Blue) and reduced conditions (Tris-HCl 0,5M pH 6,8, SDS, glycerol, β-mercaptoethanol, Bromophenol Blue). The gels Midi (7x10) were composed of 4% stacking and 15% separating gels. After gel solidification, 10 µL of the samples was placed in the well and the running of the gel was carried out using a running buffer (0.025 M Tris, 0.192 M glycine, 0.1% SDS) at pH 8.3, applying 150 V for 1 hour. After the protein migration, gel staining was done by immersion in a solution of 0.15% Coomassie® Brilliant Blue R-250 dissolved in acetic acid, methanol, and water for 30 minutes under agitation, followed by discoloration in acetic acid solution (10%).

2.2.6. Intrinsic fluorescence

The intrinsic fluorescence of tryptophan was analyzed according to the method described by Nascimento et al. (2023) with minor modifications. Samples were diluted in deionized water to a final concentration of 10 mg/mL and transferred to a 96-well microplate. The excitation wavelength was set at 280 nm, and the emission spectra were recorded from 280 to 500 nm.

2.2.7. Gel preparation

The gel preparation was performed according to the method described by Nascimento et al. (2024). To promote the gelation, glucono-delta-lactone (GDL) was added to the suspension and stirred for one minute to ensure complete solubilization. Ultimately, the suspension was incubated in a water bath at 30 °C for 4.5 h, allowing the pH to gradually decrease to 4.5.

2.2.8. Water holding capacity (WHC)

The water holding capacity was measured according to Nascimento et al. (2024), with minor modifications. 10 g of the gels were prepared in a centrifuge tube at 30 °C. After the formation, the tube was centrifuged at 3600 x g for 15 min, and the supernatant was carefully removed and weighed. The WHC was calculated by Equation 2.

$$WHC\ (\%) = \frac{mb - ms}{mb} \qquad \text{Equation 2}$$

Where mb is the gel mass before centrifugation, and ms is the mass of the supernatant.

2.2.9. Texture analysis

The gel textural characteristics were analyzed according to Batista et al. (2022) using the universal machine test (Instron Corporation, USA), after 1 day of storage at 4 °C. A cylindrical probe with 12 mm diameter was displaced perpendicularly under the gel, with a 250 N load cell, compression distance of 60% of the initial height, test speed of 1 $mm.s^{-1}$, with two penetration cycles, and three repetitions. The hardness, gumminess, and springiness have been evaluated.

2.2.10. Statistical analysis

To confirm the impact of pH shifting on the ratios, the data were analyzed using analysis of variance (ANOVA) with Statistica software (StatSoft Inc., Maisons-Alfort, France). The data were further examined using Tukey's HSD test at the 5% confidence level to distinguish between means when a significant difference ($p < 0.05$) was seen. Every experiment was carried out at least three times on its own.

3. Results and discussion

3.1. Solubility

Protein solubility (Figure 1) is an important parameter to characterize proteins. Through this parameter, it is an indication of protein techno-functional properties, such as gelation [23]. In the untreated ratios, as the pea proportion in the system increases, solubility decreases relative to casein. The same tendency can be seen in a study developed by Nascimento et al. (2023), where CMs had a solubility of 64.0% and pea proteins of 42.8%. The solubility of vegetable proteins, especially pea protein, tends to

be low, making their application a challenge, thus highlighting the need for additional treatments, such as pH shifting to improve their techno-functional characteristics and subsequent application in the food industry.

Therefore, applying pH shifting treatment, all the ratios showed an increase in protein solubility. When the proteins are submitted to extreme conditions, such as alkaline pH, the structure that is normally folded is then unfolded, leaving a high repulsion in the system. After the return to natural conditions (pH 7), the proteins tend to fold again, however, the strong interactions that previously existed are reduced, thus forming a protein in the state named “molten globule”, which is an intermediate state during the unfolding, with same secondary structure as the native one but with a disrupted tertiary structure. This modification is capable of increasing techno-functional properties like solubility, probably as a result of charged proteins and water having more ionic interactions [15,20,23]. Besides that, some protein subunits can be dissociated from the protein aggregates, contributing to increasing the solubility [24].

After the pH shifting, the ratio 0:100 had a higher increase, increasing from 26.89% to 55.54%. In a study developed by Jiang et al. (2017), the solubility of pea protein suspension after pH shifting modification to 12 was 54.94%, was similar to the result of our study, which demonstrates that pH shifting modification has a great impact on pea protein solubility and thus can be applied to improve protein techno-functional properties. The less impacted ratio was 80:20, being the solubility after the pH shifting treatment of 45.84%. Compared to the other ratios, the smaller increase can be explained by the small amount of modified pea protein in the system. Therefore, the contribution of CMs to solubility is much greater than that of pea protein.

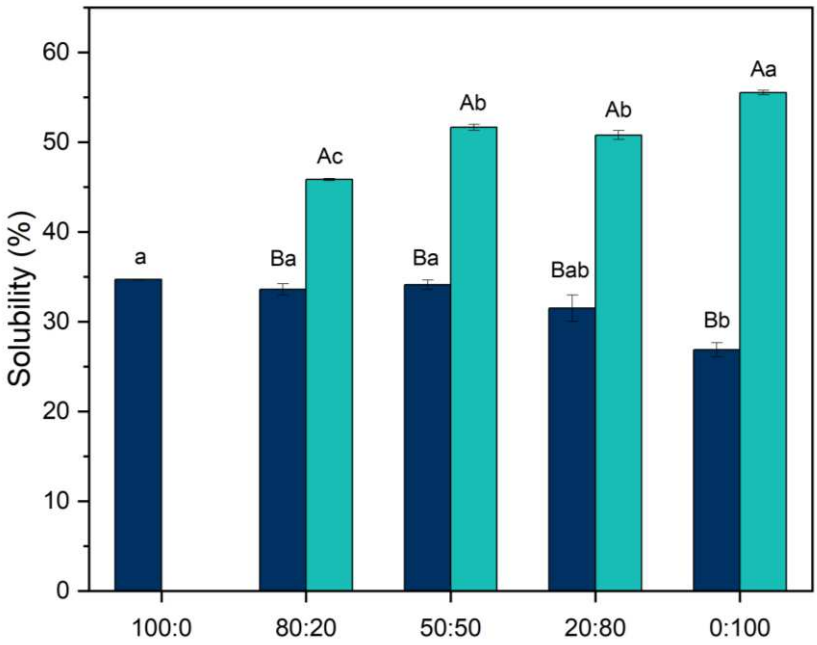

Figure 1. Protein solubility before and after pH shifting. Dark blue represents the suspensions before the modification, and light blue after the modification. Uppercase letters indicate differences between the treatments in the same ratios, while lowercase letters indicate differences between the ratios under the same treatments.

3.2. Particle size and zeta potential

The particle size is an important parameter to infer the effect of a treatment on the techno-functional properties of a protein. One characteristic that can be influenced by particle size modification is solubility, since the small protein aggregates have a higher contact area, the aggregates can interact more with water, increasing their solubility and subsequently having a significant effect on the techno-functional characteristics (Jiang et al., 2017). Thus, the particle size of the protein suspensions was evaluated, and the results are presented in Figure 2.

Before the modification, the ratios 0:100, 50:50, and 20:80 presented a bimodal distribution. In the ratio 0:100, the first peak is around 122.4 nm, and the second one is around 615.1 nm. Already in the ratio of 50:50, the size in the first peak was the same, with an increase in the intensity, and in the second peak, the particle size was 955.4 nm. In the ratio 20:80, the first peak was around 61.2 nm, and the second one was 553.2 nm. This two-peak distribution is a characteristic of pea protein powder, due to the presence of aggregates. Normally, during the powder obtention, the pea flour

passes through a harsh processing condition, forming aggregates (Beghdadi et al., 2022).

After the modification, there was a reduction in the particle size of the hybrid systems (80:20; 50:50; 20:80). Also, the ratios 50:50 and 20:80, which before presented two peaks distribution, after the treatment presented only one peak, due to particle size reduction. This reduction can be due to the dissociation of protein aggregates, thus reducing their size and modifying the distribution profile (Jiang et al., 2017). In the ratio 80:20, the particle size was also reduced. In the ratio 0:100, no modification can be noticed, which may indicate that the improvement in solubility did not occur just because of the dissociation of small protein units from the aggregates, but rather due to a conformational change, which can be confirmed by the zeta potential.

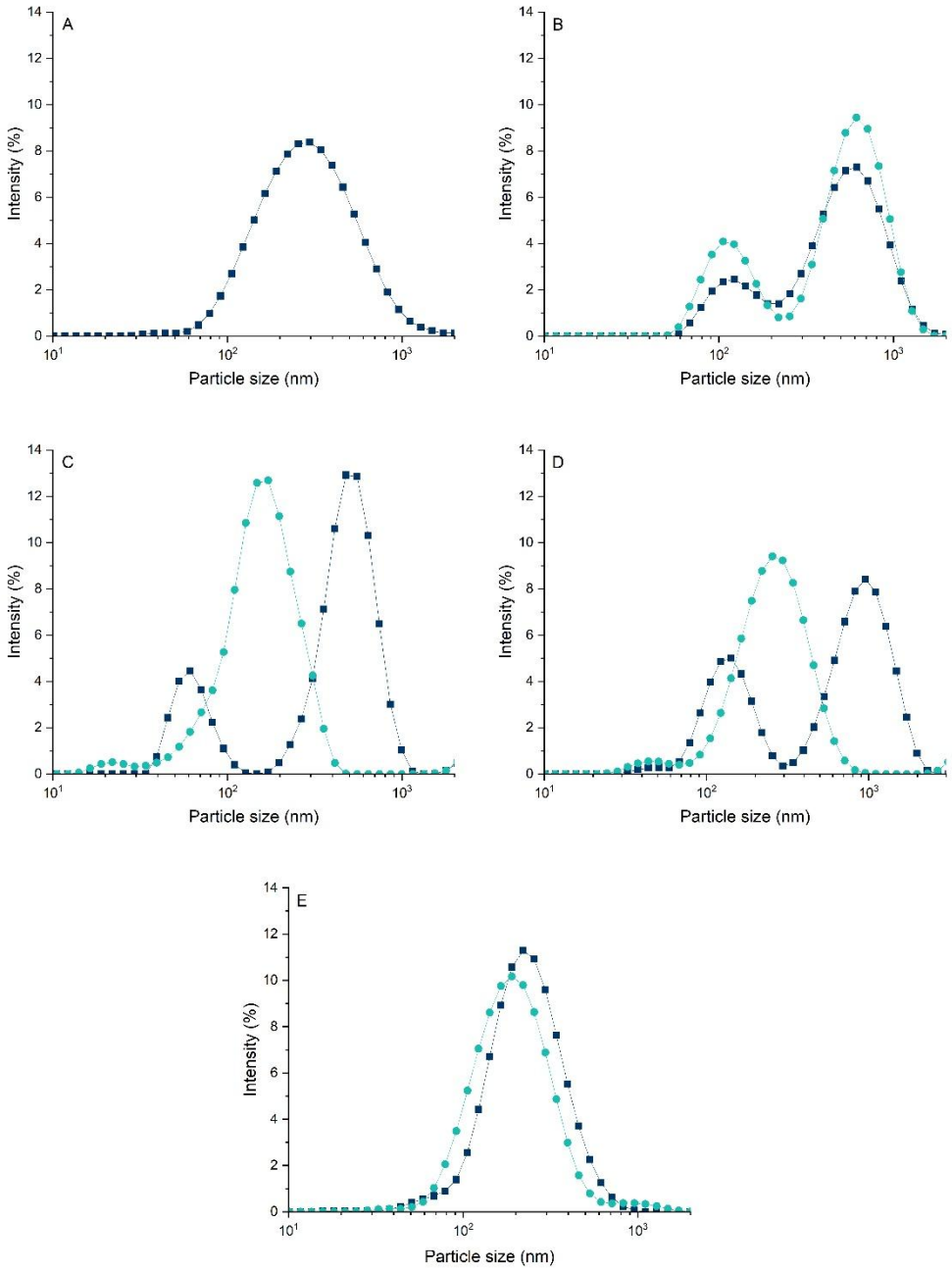

Figure 2. Particle size distribution of protein suspensions. A) 100:0 B) 0:100 C) 20:80 D) 50:50 E) 80:20. Dark blue represents the suspensions before the modification, and light blue after the modification.

The zeta potential (Figure 3) is an indicator of suspension electrostatic stability, which quantifies the particles' resulting surface charge (Nascimento et al., 2023). All the suspensions presented a negative charge, which means that these particles

contained more negatively charged amino acids on the surface than positively charged (Nascimento et al., 2023; Sun et al., 2025). Before pH shifting, the hybrid systems, 80:20, 50:50, and 20:80, presented the highest values for zeta potential, being -21.71 mV, -22.25 mV, and -26.62 mV, respectively. Then followed by the ratio 100:0 (-27.19 mV) and the lowest was the pure pea protein suspension (0:100: -31.58 mV), being consistent with previously published results (Nascimento et al., 2023; Post et al., 2012).

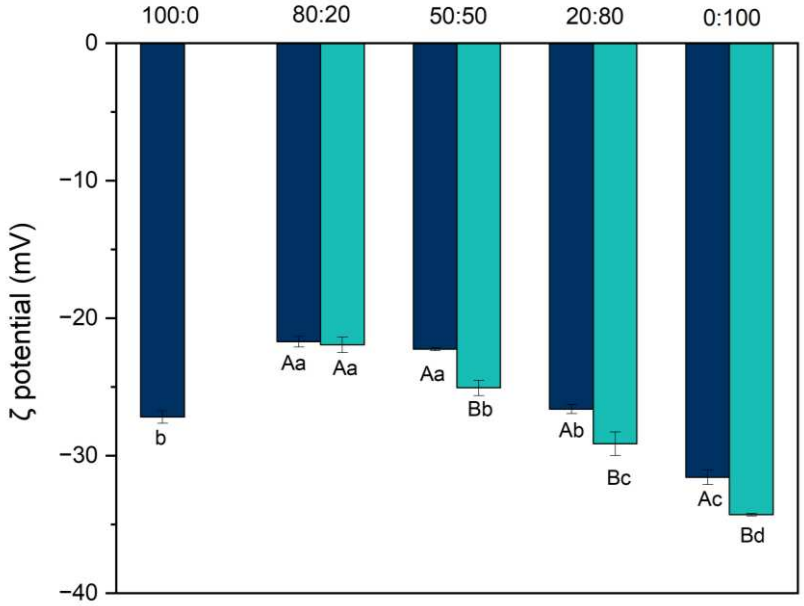

Figure 3. Zeta potential (mV) of protein suspensions. Dark blue represents the suspensions before the modification, and light blue after the modification. Uppercase letters indicate differences between the treatments in the same ratios, while lowercase letters indicate differences between the ratios under the same treatments.

After the pH shifting, the zeta potential increased for most of the suspensions, except 80:20, where, probably, the small amount of modified pea protein in the system was insufficient to significantly alter the magnitude of the electric charge on the particle surface. As the amount of pea proteins increased, the zeta potential also increased, reaching a value of -34.09 ± 0.09 mV for the ratio 0:100, showing that the increase in solubility is due to a structural change. After the return to pH 7, due to the molten structure, groups that were previously buried or participating in protein stabilization may be more exposed and negatively charged, increasing the zeta potential of the particles.

(Sun et al., 2025). This result can also be confirmed by the intrinsic fluorescence that is presented later in this study.

The particle charge can also be correlated with the solubility. Indeed, according to Li et al. (2020), as the absolute value of zeta potential rises, the repulsive interactions between the molecules also increase, causing the system to become more stable by a reduction in their aggregation and ultimately improving their solubility. When the zeta potential is greater than or equal to ± 30 mV, more stable the system is, and the less particles tend to aggregate.

3.3. Electrophoresis

The pH-shifting effect in the pea protein subunits can be visualized by the electrophoresis profile (Figure 4). Pea proteins are composed mainly of two protein fractions: albumin (15-25%) and globulin (49-70%), with globulin being the most abundant fraction. Regarding this fraction, globulin can be divided into several subunits depending on the sedimentation coefficient, 11s (legumin) and 7s (vicilin) (Grossmann, 2024).

Pea legumin (11S) is a hexameric globular protein with a molecular mass ranging from approximately 310 to 400 kDa. Each subunit of legumin has a molecular mass of ~65 kDa and is composed of two polypeptide chains, an acidic α-chain (38–40 kDa) and a basic β-chain (19–22 kDa), linked by disulfide bonds (Grossmann, 2024; Jiang et al., 2017; Nascimento, et al., 2023). Instead, vicilin is a trimeric protein, and the interaction between them is independent of disulfide bridges. However, when the cleavage occurs, some subunits can appear (Vαβγ ~50; Vαβ, ~30–36 kDa; Vα, ~20 kDa; Vβ, ~13kDa; Vγ, ~12kDa) (Emkani et al., 2023). Also, another important vicilin protein is covicilin. Covicilin is a trimeric structure and appears in SDS-page gel around 70 kDa (Grossmann, 2024).

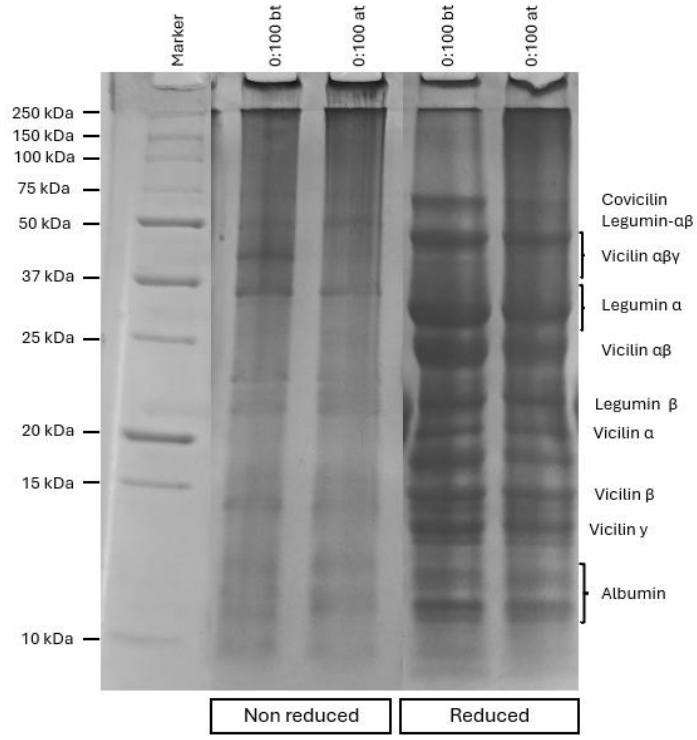

Figure 4. SDS-page gel analysis under non-reduced and reduced conditions. Bt: before pH shifting and at: after pH shifting.

Regarding the gel in native condition, comparing the ratio 0:100 after the modification, clear changes in the band patterns can be observed. The bands related to legumin-αβ and vicilin-αβγ had a decrease in intensity. The molten state can promote the disruption of S-S bonds, leading to the cleavage of vicilin-αβγ and legumin-αβ, increasing the intensity of the subunits (Zhang et al., 2022). Jiang et al. (2017), analyzing the effect of different pH levels in the pH shifting, also observed the reduction in the legumin-αβ intensity after the pH shifting at pH 12.

Comparing the native condition and reduced condition, a clear increase in the number of bonds in the gel can be observed. This increase is due to the use of the β-mercaptoethanol, which acts as a reducing agent and promotes the cleavage of the disulfide bonds of the proteins (Jiang et al., 2017). One of the bands that is influenced by the use of β-mercaptoethanol is legumin-αβ, which disappeared, being converted into the bands referring to legumin-α and legumin-β. Other bands, such as vicilin-α, vicilin-β, and vicilin-γ, appear under reduced conditions due to the cleavage of S-S bonds by β-mercaptoethanol. Probably, its effect was more intense than the pH shifting effect, causing a more significant effect in the S-S bonds.

3.4. Intrinsic fluorescence

The intrinsic fluorescence of tryptophan (Trp) can indicate changes in the tertiary structure due to its sensitivity to the polarity of the microenvironment (S. Zhang et al., 2022). Comparing the samples before the pH-shifting treatment (Figure 5), the intrinsic fluorescence decreases as the pea amount increases. The difference between the samples can be attributed to the distinct amounts of tryptophan residues in the protein structures. Casein contains approximately 1.55% more tryptophan than pea protein, which contributes to the higher fluorescence intensity observed in the 100:0 sample (Nascimento et al., 2023).

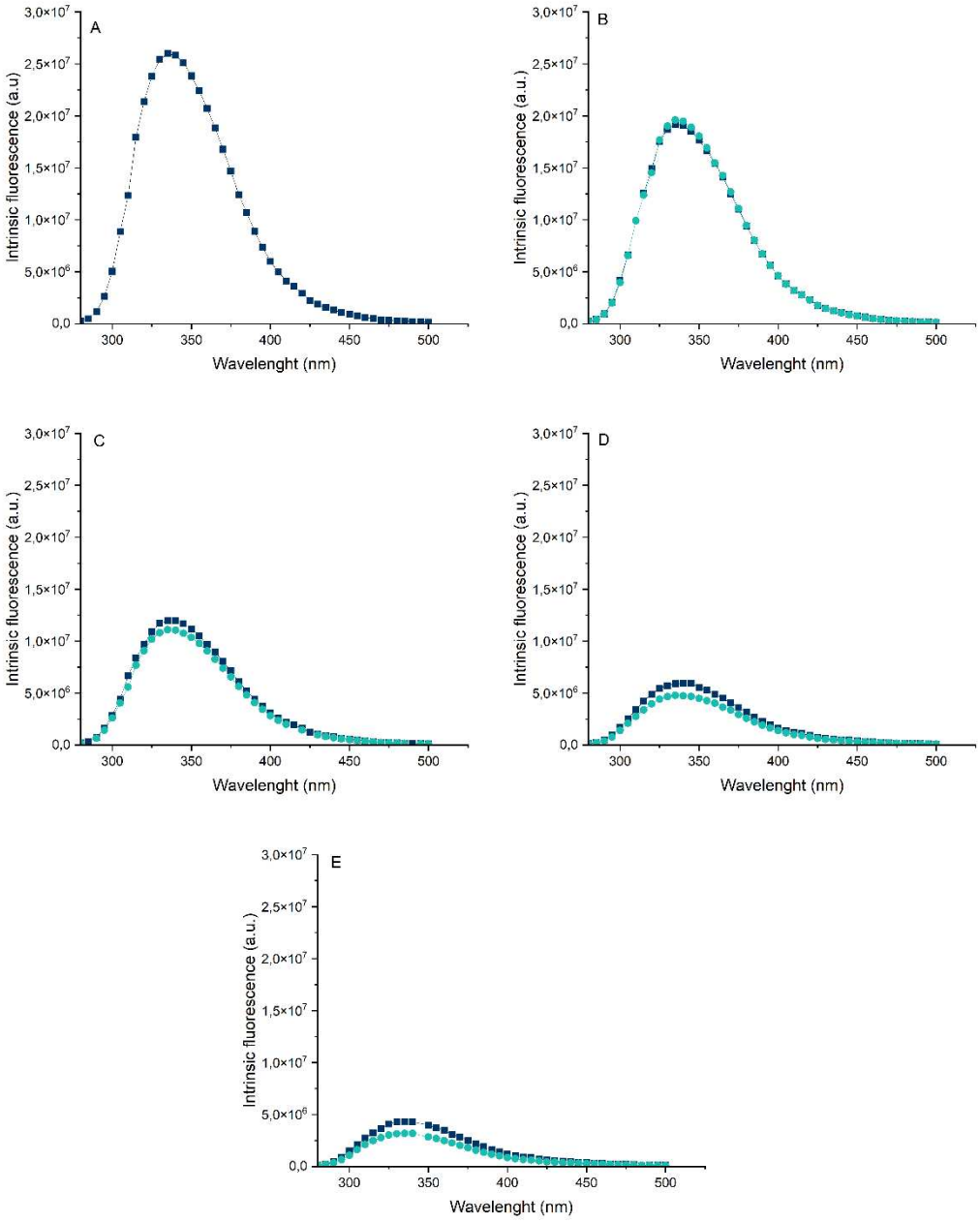

Figure 5. Intrinsic fluorescence of protein suspensions. A) 100:0 B) 80:20 C) 50:50 D) 20:80 E) 0:100. Dark blue represents the suspensions before the modification, and light blue after the modification.

Regarding the pH shifting treatment, no red or blue shift was observed in the emission spectra, with the highest value at 335 nm. However, a decrease in fluorescence intensity was detected after the modification. In the 80:20 ratio, no significant

structural changes were observed, probably because the amount of modified pea protein in the system was insufficient to alter the Trp microenvironment. In contrast, the 50:50 and 20:80 ratios showed a slight reduction in fluorescence intensity, with the most pronounced decrease found in the 0:100 sample. This suggests that the pH-shifting treatment induced structural modifications in the protein, modifying the microenvironment of Trp. Controversially, J. Zhang et al. (2022) observed an increase in intrinsic fluorescence when pea protein isolate was subjected to pH-shifting modification. However, in the referenced study, the pH-shifting time was relatively short (1 hour), which may have limited the extent of Trp residue exposure. In contrast, the decrease in fluorescence intensity observed in our study may be attributed to a quenching effect, potentially caused by increased solvent accessibility or interactions with quenching agents (Nascimento et al., 2023). Despite these quenching effects, the reduction in fluorescence intensity still suggests that the pH-shifting treatment promoted the exposure of Trp residues. This interpretation is further supported by complementary results obtained in this study, such as solubility and zeta potential measurements.

3.5. Water Holding Capacity (WHC)

Water holding capacity (WHC) is the ability of a protein to retain and hold water when centrifugal force is applied. This characteristic is extremely important in food texture, such as yogurt and cheese (Jeong & Cho, 2024). For the pure systems, before the pH shifting treatment, the WHC was 58.10 ± 0.86% and 77.70 ± 2.13% (Figure 6) from ratios 100:0 and 0:100, respectively. These results are lower than the results presented by Nascimento et al., (2024), which were 82.6% and 98.3%, however, they follow the same pattern, being the casein gel capable of holding less water than pea protein gel. According to the authors, this difference can occur because of the interconnection inside the gels. Pea protein gel has a stronger interconnected network, enabling it to retain more water in its structure. On the other hand, casein gels have large pores in their structure and are capable of holding less water. Related to the mixed systems, no significant difference was noticed between the ratios 80:20 and 20:80, however, at the ratio 50:50, the WHC was the smallest, 62.54% ± 0.58%. In hybrid systems formed by pea protein and casein protein, both proteins tend to form independent systems, generating a less cohesive gel network, and reducing water retention (Oliveira et al., 2022; Xia et al., 2024).

After pH shifting treatment, the ratio 0:100 had the highest WHC, 92.39% ± 0.55%. Zhu et al. (2021) studying the pH-shift effect in pea protein gel noticed that after the pH-shifting the gels tend to form a more uniform pore size of 3–5 μm in the gel, and because the more uniformly distributed network, the structure tends to hold more water because its molecules are more tightly retained within this dense structure.

Between the hybrid systems, the ratio 80:20 had the highest WHC, 89.08% ±1.43%. In the ratios 50:50 and 20:80, a reduction in WHC was noticed from 62.54% to 59.89% and from 69.58% to 54.62%, respectively. When a significant proportion of pea protein is incorporated into a casein gel, it disrupts the cohesion of the casein network due to lower structural and functional compatibility, probably reducing the WHC (Xia et al., 2024). However, in the ratio 80:20, the modification of pea protein structure probably increased the interaction with water, having a dominant effect on WHC.

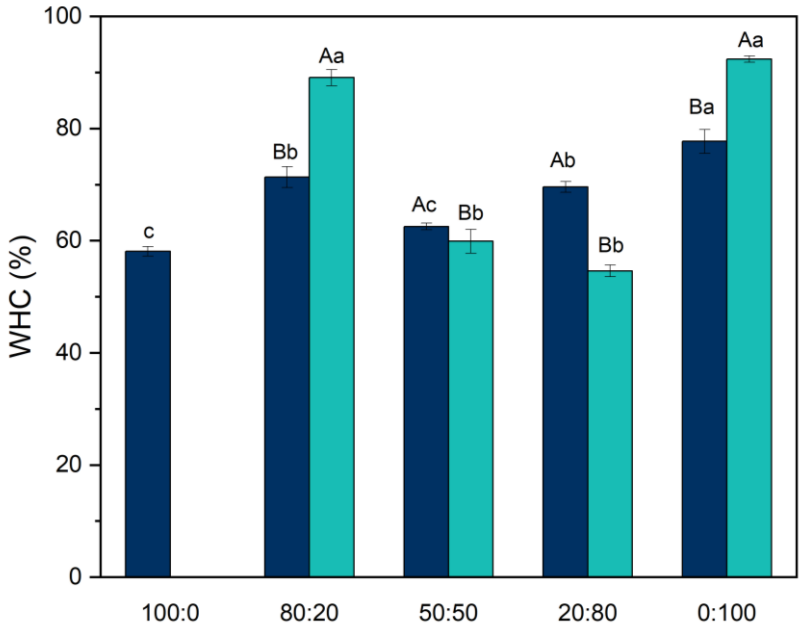

Figure 6. Water holding capacity of hybrid gels. Dark blue represents the gel without modification, and light blue is the gel produced after pH shifting. Uppercase letters mean differences between the treatments in the same ratios, and lowercase letters mean differences between the different ratios under the same treatments.

3.6. Texture profile analysis (TPA)

During a TPA test, samples are subjected to two successive compressions, mimicking the biting action of the human mouth. This process allows for the evaluation

of the sample's textural properties and their behavior during mastication (Xia et al., 2024). Hardness describes the mechanical strength of a material. Its value is calculated by the maximum peak formed when a hard body penetrates the gel (Masiá et al., 2023; Xia et al., 2024). In Table 2, among the ratios without modification, the gel hardness is reduced when more pea protein is added to the system, changing from 2.673 ± 0.0222 N to 0.145 ± 0.01 N for the ratio 100:0 and 0:100, respectively. This difference can be related to the WHC of the gels. The casein gel has a smaller WHC, thus allowing a more rigid structure. Instead, the pea protein has a higher WHC, causing the hardness reduction. Xia et al. (2024), studying acid gels formed by pea proteins and casein micelles, also noticed that after the addition of pea proteins to a casein micelle gel, the hardness is reduced.

Sample		Hardness (N)	Chewiness (N)	Resilience	Gumminess (N)	Springiness (mm)	Cohesiveness
Before pH shifting	100:0	2.6735 ± 0.0224 ^{a}	17.0898 ± 0.4235 ^{a}	0.8947 ± 0.0103 ^{a}	1.1628 ± 0.0503 ^{a}	11.9540 ± 0.2416 ^{a}	0.4342 ± 0.0288 ^{abA}
	80:20	2.1129 ± 0.0455 ^{bA}	13.0187 ± 2.0603 ^{bB}	0.7998 ± 0.0165 ^{abA}	0.8699 ± 0.1400 ^{bA}	10.9078 ± 0.2395 ^{aA}	0.4125 ± 0.0670 ^{abA}
	50:50	0.8241 ± 0.0080 ^{cA}	5.7489 ± 0.2305 ^{cA}	0.7987 ± 0.0074 ^{abA}	0.2983 ± 0.0733 ^{cA}	7.8289 ± 0.5428 ^{aA}	0.3778 ± 0.0964 ^{aB}
	20:80	0.1116 ± 0.0104 ^{dB}	0.4765 ± 0.1002 ^{dB}	0.7516 ± 0.0387 ^{bA}	0.0324 ± 0.0070 ^{dB}	-	0.2857 ± 0.0800 ^{bcA}
	0:100	0.1452 ± 0.0100 ^{dB}	0.5379 ± 0.0531 ^{dA}	0.6747 ± 0.0193 ^{cA}	0.0369 ± 0.0040 ^{dA}	-	0.2273 ± 0.0400 ^{cA}
After pH shifting	80:20	1.3828 ± 0.0571 ^{aB}	8.6147 ± 0.7808 ^{bA}	0.7573 ± 0.0217 ^{aA}	0.5747 ± 0.0515 ^{bA}	11.2789 ± 0.0283 ^{aA}	0.4138 ± 0.0371 ^{bA}
	50:50	0.4215 ± 0.0479 ^{bB}	2.4497 ± 0.3426 ^{bA}	0.8108 ± 0.0093 ^{aA}	0.1651 ± 0.0217 ^{bB}	7.9378 ± 0.6187 ^{aA}	0.3933 ± 0.0601 ^{bA}
	20:80	0.5267 ± 0.0135 ^{bA}	39.8140 ± 3.0960 ^{aA}	0.8431 ± 0.0330 ^{aA}	2.6644 ± 0.1999 ^{aA}	9.5650 ± 0.2996 ^{a}	5.2000 ± 0.4228 ^{aA}
	0:100	0.6179 ± 0.0694 ^{bA}	3.0360 ± 0.6526 ^{bA}	0.7849 ± 0.0318 ^{aA}	0.2028 ± 0.0434 ^{bA}	7.6442 ± 1.1931 ^{a}	0.3333 ± 0.0882 ^{bA}

Table 2. Texture profile analysis of hybrid gels. Uppercase letters mean differences between the treatments in the same ratios, and lowercase letters mean differences between the different ratios under the same treatments.

Comparing the ratios 80:20 and 50:50, after the modification, the hardness was reduced from 2.113 N to 1.383 N and from 0.824 N to 0.422 N, respectively. In the 80:20 ratio, after the modification, the gel was capable of holding more water in the structure, probably due to the opening of the pea protein structure, causing the hardness reduction. Related to the ratio 50:50, despite structural alterations in the pea protein, as indicated by the decrease in particle size and the rise in zeta potential, the protein's thermodynamic incompatibility was unavoidable. Probably, after the pH shifting, pea proteins tended to interact even more with themselves, decreasing the WHC and the gel hardness.

For the 20:80 after the modification, the hardness increased. This suggests that in mixed gels with a higher proportion of pea protein, the modification in pea protein structure may enhance protein-protein interactions during gelation, overcoming the negative effect that caseins can have on the system, increasing gel hardness. The same behavior was verified in the ratio 0:100. Studying the effect of pH-shifting on soy and potato protein and also their mix, Sun et al. (2025), also observed an increase in the hardness of the gel, due to the increase of SH groups, after the pH shifting, which is favored by the formation of disulfide and covalent bonds during gel structuration.

Gumminess is the energy needed to break up a semi-solid food in the mouth before swallowing (Hwang et al., 2012). Before the modification, the gumminess is reduced as the amount of pea protein increases in the system. This reduction can be attributed to the structural characteristics of pea protein, which, compared to casein, tends to form looser, more porous networks that require less effort to break down. After the modification, for the ratios 20:80 and 0:100, the gumminess of the system increases, from 0.032 N to 2.664 N and from 0.037 N to 0.203 N, respectively, showing that the modification in these ratios can favor more pea-pea interactions, increasing the hard structure of the gel. Conversely, for the 80:20 and 50:50 ratios, the presence of pea protein in a system with greater or equal amounts of casein can cause a disruptive effect on the gel, resulting in the reduction of gumminess.

Related to the springiness, which indicates how well the gels return to their original height after the first downward compression (Xia et al., 2024). Comparing the different ratios, the springiness decreases as the amount of pea in the system decreases, indicating that the gels with higher amounts of casein have a more elastic and integrated gel structure (Zhu et al., 2021). Comparing the ratios before and after the

modification, the springiness increases. Probably, the modification is capable of increasing the gel elasticity.

4. Conclusion

This study has demonstrated that pH shifting can be an effective tool for improving the techno-functional properties of hybrid systems formed by casein and pea proteins. Related to the hybrid systems, depending on the majority protein (more casein or more pea protein) in the suspension or gel, the properties are different. For the ratio 80:20, the pH shifting effect was less significant. However, the presence of the modified pea protein in the casein gel was able to improve the interactions between the proteins, increasing the WHC and reducing hardness. For the 50:50 and 20:80 ratios, even with the increase in solubility, after gelation, the gel was able to hold less water in its structure, and the gel hardness decreased. In the ratio 0:100, structural modification promoted by the pH shifting resulted in a significant increase in solubility and WHC, resulting in a gel with different structural characteristics. The different effects of pH shifting in the pea protein indicate that the structural and functional compatibility between the proteins directly influences the behavior of the hybrid systems. The results highlight the potential of pH shifting in the modification of pea protein and, consequently in hybrid systems. The use of this technique is a promising process in food formulation, especially in high-protein hybrid products aimed at balancing the functionality of casein and the sustainability of pea proteins.

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CHAPTER IV.
REVIEW ARTICLE

Structural and techno-functional modifications of pea protein fractions by non-thermal technologies

Silva et al.

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Structural and techno-functional modifications of pea protein fractions by non-thermal technologies

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Abstract

Animal-based food production is highly associated with greenhouse gas emissions, land degradation, and animal welfare issues. In response to growing environmental and ethical concerns about this production system, consumers are increasingly adopting more sustainable dietary habits. This shift includes reducing the consumption of animal-derived products in favor of plant-based alternatives, with legumes standing out as a promising source of protein. Pea proteins have gained attention due to their high productivity, low allergenicity, and non-genetically modified (non-GMO) status. Pea is primarily composed of globulins (legumin 11S and vicilin 7S) and albumins 2S, each exhibiting distinct structural and techno-functional properties that influence their behavior in complex food formulations. To overcome formulation challenges, non-thermal technologies (NTTs) such as ultrasound (US), cold plasma (CP), high hydrostatic pressure processing (HPP), and pulsed electric fields (PEF), have emerged as innovative tools for modulating protein structure and improving their techno-functional properties. Despite the promising capacity of NTTs to modify the protein structure and techno-functionalities, the current research remains limited, particularly regarding the impact of these NTTs on isolated protein fractions and their effects in improving plant-based formulations. Consequently, this literature review critically explores the potential of the NTTs to modulate the structure of pea protein fractions, thereby enhancing their techno-functionalities and broadening their utilization in complex food systems.

Keywords: Pea protein; Techno-functional properties; Pulsed electric Field; Ultrasound; Cold plasma; High pressure processing.

Highlights

- Each pea protein fraction has a different structure, giving them different technological properties
- Most non-thermal treatment studies target pea protein isolates, not the fractions.
- The response of pea protein fractions subjected to NTTs remains unexplored.
- Tailored applications can be developed by linking NTT effects to specific fractions

1. Introduction

Concerns regarding the environmental impact of human activities are increasing, particularly in the agrifood sector, which is a substantial contributor to these issues (Valdelomar-Muñoz & Murgado-Armenteros, 2024). Among the various challenges posed by this sector, the consumption of animal-derived foods stands out, as its production is responsible for significant greenhouse gas emissions, land and water use, and deforestation (Nascimento et al., 2023). In response, policymakers are encouraging consumers to change their eating habits, emphasizing the adoption of sustainable food sources, particularly plant-based products. Furthermore, increasing consumer awareness of the health and environmental benefits of plant-based diets is driving this shift (Gil et al., 2024).

Consequently, plant-based materials are of great interest due to their lower carbon footprint and interesting nutritional composition, particularly due to their high protein content (Akharume et al., 2021; Hertzler et al., 2020). Among plant sources, peas have attracted much attention due to their high productivity, low allergenicity, and non-genetically modified (non-GMO) status. Pea (*Pisum sativum* L.) is one of the oldest crops cultivated for human consumption. It is currently grown in 84 countries and is the second most cultivated crop (Lu et al., 2020). According to FAO, the global production of dry peas was approximately 14.17 million tons in 2022, with the main producers being Canada, Russia, China, India, and Ukraine (FAO, 2023).

The growing interest in peas is largely due to their nutritional profile and various applications in the food industry. Depending on cultivar, agricultural conditions, and maturity at harvest, pea seeds contain 60-65% carbohydrates, 23.1-30.9% protein, and 1.5-2.0% lipids. Pea seed is also composed of micronutrients such as vitamins, minerals, phytic acid, polyphenols, saponins, and oxalates (Lam et al., 2018). From a protein composition perspective, the growing global interest in peas as a sustainable protein source is reflected in scientific literature. Indeed, as shown in Figure 1, over the last decade, the number of studies on pea proteins has grown considerably, reflecting increased interest in protein extraction strategies, structural properties, and potential applications in food systems.

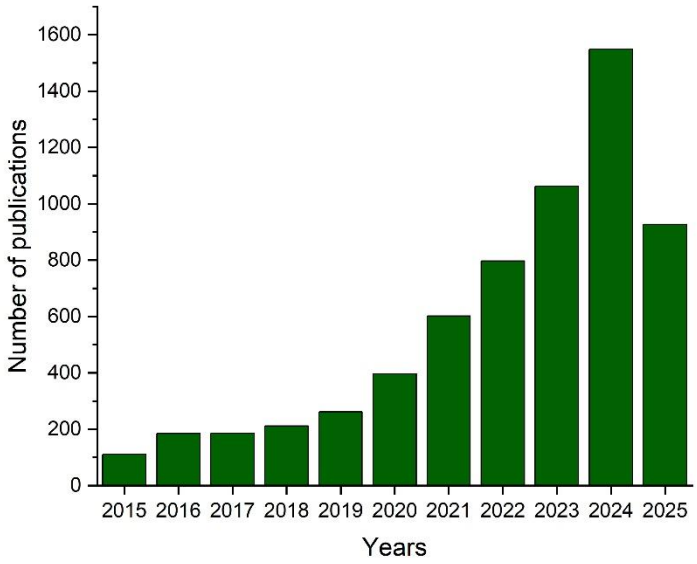

Figure 1. Number of publications related to pea protein from 2015 to 2025. Data was collected using Scopus with the keyword "pea protein" on April 10th, 2025.

Pea seed is primarily composed of globulins (49-70%), including legumin 11S and vicilin 7S, and albumins 2S (15-25%), with minor amounts of prolamins and glutelins (Grossmann, 2024). Regarding their three-dimensional structures, these protein fractions differ in solubility and techno-functional properties. Indeed, globulins are mainly responsible for gelation and emulsification (Q. Shen et al., 2023; Tahir et al., 2024), while albumins contribute to solubility and foaming capacity (R. Kornet et al., 2022). The minor fractions, prolamins, and glutelins, play a lesser role in the overall techno-functionalities of pea protein, but there is a lack of studies related to these fractions (Lam et al., 2018). However, using these proteins in food systems is often hindered by their native structural characteristics and sensitivity to extraction methods, which can adversely affect their solubility, functionality, and overall performance (Lam et al., 2018; Tanger et al., 2020). Moreover, limited research has explored the utilization of isolated pea protein fractions, limiting their full application in food formulations.

Numerous studies have investigated technological strategies to modify the native structure of proteins in order to improve their techno-functional properties and broaden their applications in the food industry, such as conventional heating, pH shifting, and enzymatic treatment. Among these, heating treatments are the most widely used in the food industry. However, these techniques can reduce the

nutritional value of proteins and are less sustainable, for example, in heat processing, fossil fuels are used for heat generation, which increases their environmental impact (Toepfl et al., 2006; M. Yang & Wang, 2025). To overcome these issues, non-thermal technologies (NTTs), such as pulsed electric field (PEF), cold plasma (CP), ultrasound (US), and high-pressure processing (HPP), have emerged as an alternative, as they are more energy-efficient, cost-effective, have a lower impact on nutritional components, and present a reduced environmental footprint (Jadhav et al., 2021; Safwa et al., 2023).

From an energy-efficient perspective, the comparison of non-thermal technologies with conventional heating processes is highly dependent on the process goal, the type of system, the type of material to be treated, and other specific processing parameters, and there is still a gap in the literature regarding comparing the costs and energy efficiency (Landi et al., 2025; Raso et al., 2022). Toepfl et al. (2006) state that non-thermal technologies such as PEF and HPP can increase energy efficiency by reducing overall energy consumption. In the PEF case, the reduction of energy consumption was demonstrated by Landi et al. (2025) comparing the PEF efficiency against high-temperature short-time pasteurization in orange juice. The authors demonstrated that PEF reduces electricity and fuel consumption by 20% and 60%, thus increasing energy efficiency and reducing greenhouse gas emissions by ~30%. In the HPP, a decrease of 20% in energy consumption was observed for sterilization (Toepfl et al., 2006). In CP, energy efficiency is also higher, since the energy is directly used to generate reactive species with lower energy dissipation (M. Yang & Wang, 2025). The energy efficiency, together with shorter processing times, less water consumption, and improved product quality and shelf life, can offset the higher initial and operational costs of NTTs, making them economically viable (Raso et al., 2022; Toepfl et al., 2006; M. Yang & Wang, 2025).

Concerning the NTTs nutritional impact, especially the heat-sensitive nutrients, the NTTs have minimal or no impact on their stability. Since these technologies have low processing time and normally operate in ambient temperature or low temperature, compared to conventional heating, preserving the sensorial aspects. However, with long processing time or high intensity, some modifications in sensorial characteristics can happen (Jadhav et al., 2021).

Regarding NTTs use in the pea protein modification, PEF enhanced protein solubility, increasing gelling and emulsifying capacity by inducing structural rearrangements and reducing protein aggregation (Guo et al., 2024; Melchior et al., 2020). Similarly, CP has been reported to enhance solubility and modify surface hydrophobicity of pea protein, altering the three-dimensional structure by breaking intermolecular interactions. This effect is attributed to the hydroxyl (-OH) radicals generated during CP treatment, which cleave peptide and disulfide bonds in pea protein (Chuang et al., 2025; Zhang et al., 2021). The US has been widely investigated for its ability to modify the conformation of pea proteins through acoustic cavitation effects, improving their techno-functional properties (Nascimento et al., 2023; Xiong et al., 2018). Finally, HPP, depending on the level of pressurization applied, can promote the dissociation of pea protein aggregates or induce reversible unfolding and exposure of functional groups, increasing the technological application of different proteins, including pea proteins. The published works on NTTs and their effects on protein structures reveal promising opportunities to enhance key techno-functionalities of pea proteins, such as solubility, emulsification, gelation, and foaming properties, and, consequently, their applications in a wide range of food formulations.

However, most of these studies have been conducted on pea protein isolates as a whole, overlooking the fact that individual fractions (globulin, vicilin, legumin, and albumins) differ significantly in their molecular structure, amino acid composition, and functional potential. This review therefore provides a critical analysis of pea protein fractions, with a particular focus on globulins (legumin and vicilin) and albumins, and examines how different NTTs may differentially modulate their structural and techno-functional properties. By emphasizing fraction-specific responses, this review offers new insights for tailoring protein functionality and advancing the development of next-generation plant-based and hybrid food systems.

2. The protein fraction of pea seed

The protein content in pea ranges from 23.1-30.9% (Barac et al., 2010; Shen et al., 2022; Wu et al., 2023) and is the second most abundant macromolecule after carbohydrates. Pea proteins contain all essential amino acids, accounting for approximately 23.6% of the total amino acid content, higher than in

soy and wheat (Asen et al., 2023). Among the essential amino acids, lysine, leucine, and phenylalanine are the most prevalent, with lysine levels notably higher in peas compared to other pulses (Asen et al., 2023; Zeidanloo et al., 2019). However, sulfur-containing amino acids such as methionine and cysteine are present in lower amounts, which limits the overall protein quality (Lu et al., 2020). Regarding non-essential amino acids, aspartic acid, glutamic acid, and arginine are the most abundant in pea proteins (Zeidanloo et al., 2019). Compared to other conventional pulse and legumes, pea proteins are not associated with major allergenic issues in the population (Lu et al., 2020; Nascimento et al., 2023), even if allergenic proteins in pea were already detected and characterized (Dreyer et al., 2014; serGeant & Moneret-Vautrin, 2015; Taylor et al., 2021). In addition, peas have not undergone genetic modification, which is advantageous for consumers seeking non-GMO products. However, their techno-functional properties are generally lower than those of soy, and the presence of beany flavor, mainly generated from the oxidation of unsaturated lipids, can negatively affect consumer acceptance (Guo et al., 2024; Shen et al., 2022).

Pea protein fractions (**Figure 2)** are divided into four groups, *i.e.,* globulin, albumin, prolamin, and glutelin, with globulin and albumin being the most abundant (Lu et al., 2020). Globulins (55 to 65% of total pea proteins) are salt-soluble proteins and classified into three fractions based on their sedimentation coefficient, mainly legumin (11S) and vicilin (7S), with small amounts of convicilin (8S) (Shen et al., 2022). The legumin/vicilin ratio ranges from 0.4 to 2.0, and variability could occur due to agricultural practices, cultivars, environmental conditions, and extraction methods (Lam et al., 2018; Shen et al., 2022).

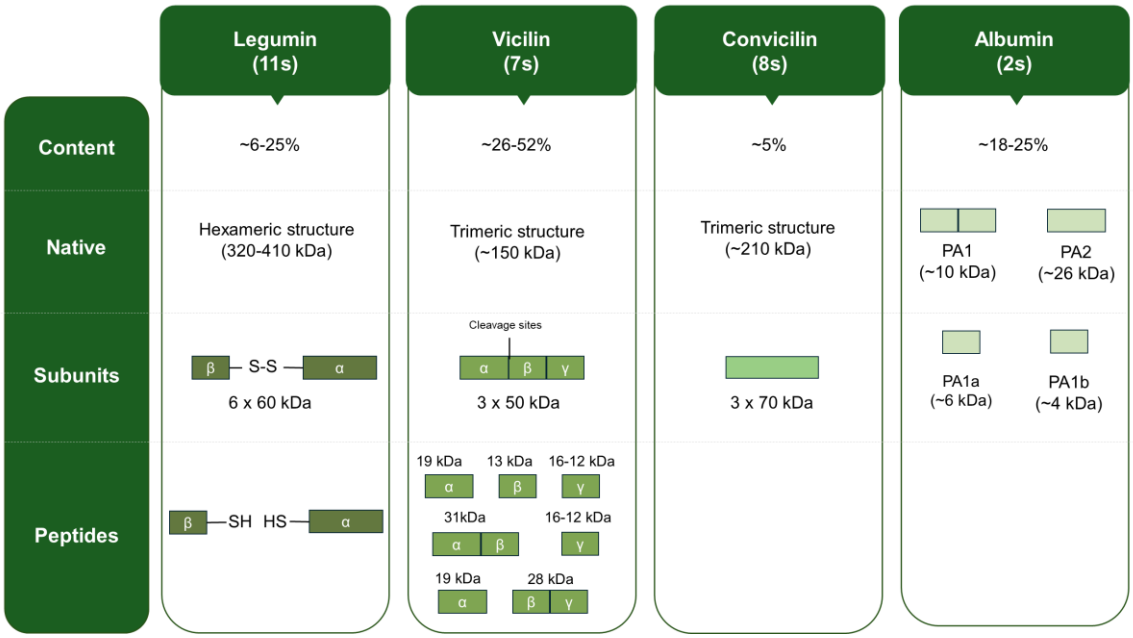

Figure 2. Structural organization of pea protein fractions. The figure summarizes the main pea protein fractions: legumin (11S), vicilin (7S), convicilin (8S), and albumin (2S). For each fraction, the content corresponding to the total pea protein is presented (content), followed by its native quaternary structure and molecular weight (native). The subunit composition is shown under subunits, and the main peptides are illustrated under peptides. PA1 and PA2 represent the pea albumin fractions. PA1a, PA1B, -α, -β, and -γ represent the pea protein subunits. Disulfide bonds (S–S) and free sulfhydryl groups (–SH) are also represented.

Legumin (11S) is a hexameric storage protein with molecular weights ranging from 320 to 410 kDa. Each monomer unit consisted of an acidic (~40 kDa, pI ~4.5-5.8) and a basic (~20 kDa, pI:6.22-8.0) polypeptide covalently linked by a disulfide bond, forming a subunit of approximately 60–65 kDa. These subunits assemble into a quaternary structure stabilized by non-covalent interactions (Gravel & Doyen, 2023; Grossmann, 2024; Kornet et al., 2021). The hydrophilic α-chains are located on the molecular surface, while hydrophobic regions are buried within the core, limiting their interaction with water and enhancing the structural stability of the protein (Lu et al., 2020). Legumins remain in their native conformation between pH 7 and 9 and exhibit moderate heat stability, with thermal denaturation ranging from 80-94 °C, depending on the extraction conditions (Gravel et al., 2023; Hansen et al., 2022; J.-L. Mession et al., 2013). Legumin is particularly rich in sulfur-containing amino acids, with approximately five cysteine

residues per 60 kDa subunit (Husband et al., 2024; O'Kane et al., 2004). Also, it contains higher levels of acid and basic amino acids compared to vicilin (Husband et al., 2024).

Vicilin (7S) is a glycosylated trimeric protein with a molecular weight of ~150 kDa. Its subunits consist of αβγ (50 kDa), αβ (31 kDa), βγ (28 kDa), α (19 kDa), β (13 kDa), and γ (16-12 kDa), linked by hydrophobic interactions (Gravel et al., 2024; Lam et al., 2018). Vicilin is less hydrophobic than legumin due to its lower content of sulfur-containing amino acids such as cysteine and methionine (Grossmann, 2024). Convicilin 8S, present in relatively small amounts in pea, has a trimeric structure with a molecular weight of ~ 210 kDa, composed of subunits of 70 kDa (Gravel et al., 2024). In terms of amino acid composition, convicilin contains cysteine and features a strongly charged N-terminal extension, distinguishing it from vicilin. Nevertheless, it shares approximately 80% sequence similarity with native vicilin (Lam et al., 2018; Lu et al., 2020).

Albumin (2S) (18–25% of total pea proteins) is less extensively studied than globulins (Lam et al., 2018). Albumins include two main components: pea albumin 1 (PA1), comprising PA1a (~6 kDa) and PA1b (~4 kDa), and pea albumin 2 (PA2), a dimer of ~26 kDa subunits (Gravel et al., 2023; Shen et al., 2022). These proteins are highly soluble and exhibit a wide range of isoelectric points (~4.2–8.1). Rich in cysteine, albumins contribute to the functional properties of pea protein due to their capacity to form disulfide bonds (Grossmann, 2024). This fraction also includes proteins with biological functions, such as lipoxygenases, lectins, and trypsin inhibitors (Grossmann, 2024; Lam et al., 2018).

Prolamins (4–5%) are storage proteins that are more commonly found in cereals than in legumes, such as peas (Shen et al., 2022). They are primarily composed of glutamine and proline, which strongly influence their solubility and structural characteristics. Based on these properties, prolamins are classified into four groups: α- (the most abundant), β-, γ-, and δ-/ω-prolamins (Song et al., 2021). Unlike many other proteins, prolamins do not coagulate under heat, but they can be hydrolyzed into proline and ammonia (Lu et al., 2020). Glutelins (3-4%) were also present in small concentrations in pea flour (Shen et al., 2022). Regarding their amino acid composition, glutelins are rich in phenylalanine, valine, tyrosine, and proline, which contribute to their insolubility (Lu et al., 2020).

The different pea protein fractions, like other legume and pulse proteins, can be fractionated according to Osborne's classification. Indeed, globulins are soluble in saline solutions, albumins in water, prolamins in alcohol, and glutelins in diluted alkaline or acid solution (Lu et al., 2020; Shen et al., 2022). Using these properties, different strategies, mainly alkaline extraction-isoelectric precipitation, salt extraction, and mild fractionation, were developed to generate pea protein-rich ingredients (Shanthakumar et al., 2022), as detailed in **Figure 3**.

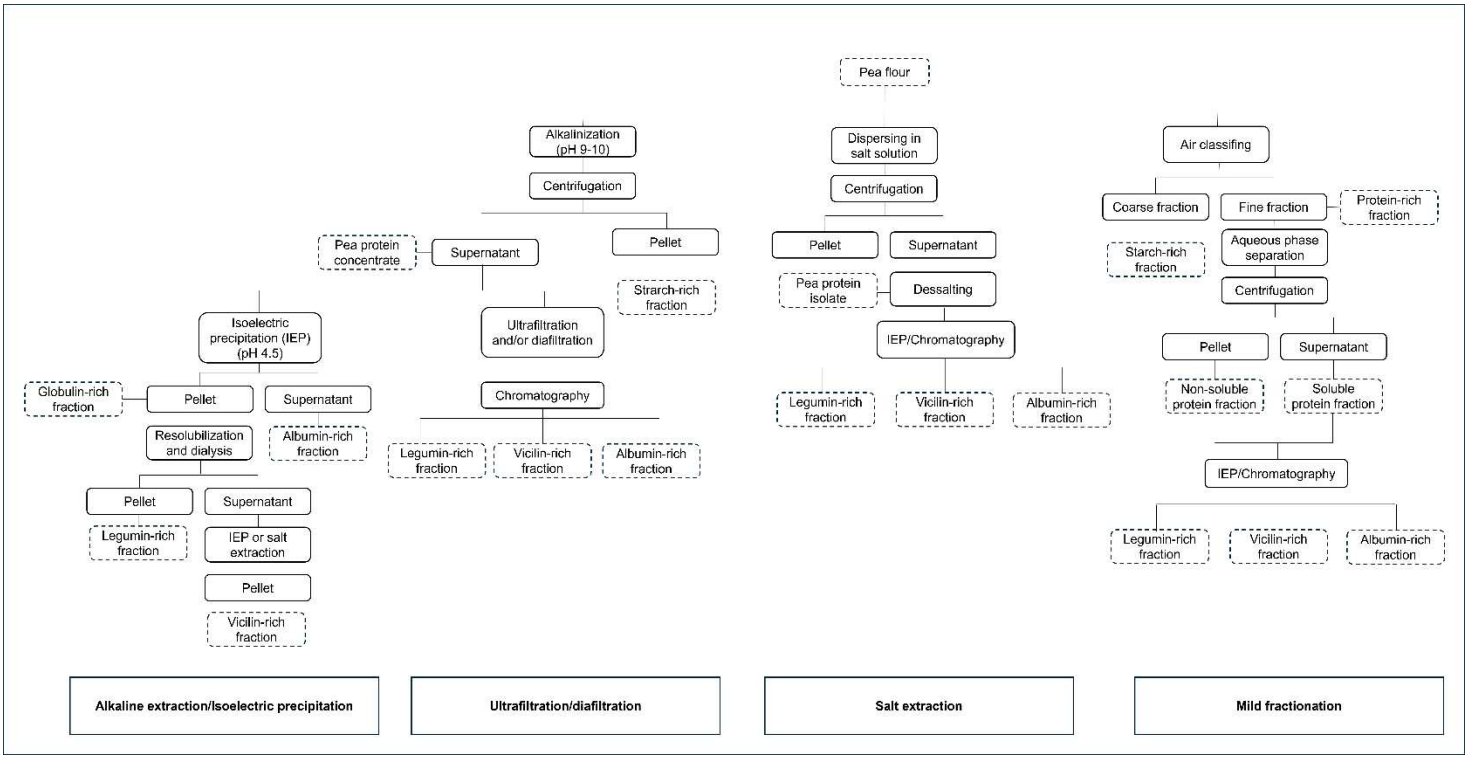

Figure 3. Protein extraction and concentration processes to generate pea protein-rich ingredients.

Alkaline extraction followed by isoelectric precipitation is widely used to generate pea protein isolate (PPI) (J. Yang et al., 2021). In this method, pea flour is dispersed in water and alkalinized by using NaOH or KOH at pH 9-10. The suspension is maintained at 50-60°C for 30 to 180 minutes and then centrifuged. The supernatant, rich in protein, is collected for isoelectric precipitation while the pellet, rich in starch and other non-soluble compounds, is discarded. The protein-rich supernatant is then subjected to isoelectric precipitation by adjusting the pH to the isoelectric point, generally around pH 4.5, followed by centrifugation. The supernatant is discarded while the pellet, corresponding to the protein isolate, is

washed, generally neutralized (pH 7), and dried, typically by freeze-drying at lab-scale or spray-drying at commercial-scale (Möller et al., 2022; Shanthakumar et al., 2022; Tanger et al., 2020). The resulting freeze-dried protein isolate is mainly composed of globulins, as albumins, which remain soluble across a wide pH range, staying in the supernatant (Kornet et al., 2020). This supernatant can also goes under another centrifugation and dialysis to promote the fractionation of the pellet into legumin and vicilin (Gravel & Doyen, 2023). Instead of the isoelectric precipitation, the ultrafiltration and/or diafiltration membranes can also be used to isolate specific fractions from the supernatant (Lam et al., 2018). Another technique that can be applied to separate the fractions and purify them is chromatography. This technique involves separating fractions based on molecular weight, net charge, hydrophobicity, and affinity to the stationary phase, and can be useful for producing purified fractions (Crévieu et al., 1996; Gravel & Doyen, 2023; J. L. Mession et al., 2012).

The salt extraction method is based on the salting-in and salting-out characteristics of proteins (Gravel & Doyen, 2023). In low salt concentrations (salting-in), where the ionic strength is low, protein solubility increases. However, as the ionic strength exceeds a specific threshold, the solubility decreases due to enhanced protein-protein interactions (H. N. Xu et al., 2015). Exploiting this principle, pea flour is mixed with a salt solution (typically in a ratio 1/10 v/w), stirred (30 min), and centrifuged (4500 xg/20 min). After that, the supernatant is desalted by dialysis and dried, applying freeze-drying (Li et al., 2022; Shanthakumar et al., 2022; Tanger et al., 2020). This method results in a mixture of globulins and albumins. Micellization, a similar technique, also relies on this principle. In this process, protein solubility in the salt solution is decreased through dilution, leading to the formation of protein micelles and enabling protein extraction (Shanthakumar et al., 2022; Stone et al., 2015).

The mild fractionated method was developed to have a lower environmental impact and combine dry and wet fractionation (Kornet et al., 2022). First, the pea flour, obtained by milling, undergoes air classification. The resulting fine fraction, rich in proteins, is then subjected to aqueous phase separation. This process involves mixing the pea flour with water, stirring the mixture, and centrifuging it at 4500 rpm for 30 minutes. After centrifugation, three visually distinct layers are formed. The upper layers are collected and further processed by ultrafiltration to

concentrate the proteins (Pelgrom et al., 2015; Zhou et al., 2025). According to Pelgrom et al. (2015), this method can provide a native high-protein concentrate with less water and energy use compared to a wet route.

Depending on the extraction method used, differences are observed in the protein content of the isolates, as well as in the extraction yield and purification efficiency achieved during the process. Comparing different methods, J. Yang et al. (2021), observed that a higher protein content was observed in the sample obtained by alkaline extraction followed by ultrafiltration (89.89%), while the lowest was found in air classification (54.68%). Other methods, such as alkaline extraction–isoelectric precipitation, salt extraction–dialysis, salt extraction–ultrafiltration, and micellar precipitation, resulted in protein contents of 88.83%, 86.76%, 86.33%, and 83.97%, respectively. Lower values were observed by Tanger et al. (2020), which determined the protein content of 74.5, 75.1, and 73.2% for alkali extraction–isoelectric precipitation, micellar precipitation, and salt extraction, respectively. In this study, the yield for these methods was 46, 25, and 39.5%. Due to solubilization, alkaline precipitation typically yields high protein yields (Asen et al., 2023).

The method described in this section can separate the distinct pea protein fractions. A detailed characterization of these fractions in terms of techno-functionalities is crucial for a better understanding of their behavior in different model food systems and complex food formulations.

3. Techno-functional properties of pea protein fractions

The techno-functional properties of protein are closely linked to its suitability for food applications. Extensive research has aimed to understand and improve these properties, particularly solubility and the capacity to form colloidal systems such as gels, emulsions, and foams (Asen et al., 2023; Lam et al., 2018). This techno-functionality is largely influenced by various factors, including the pea cultivar, which affects protein content, composition, and conformation, as well as the extraction method and physico-chemical environment (ionic strength, pH, temperature, etc.), which exposes the protein to external conditions that may alter its structure and properties (Cui et al., 2020). Also, when comparing the protein fractions, their functionality is highly diverse, as shown in Table 1. Therefore,

understanding how each fraction can act in a colloidal system is essential for applying them in the food industry.

Table 1. Solubility and colloidal properties of PPI and its fractions.

PPI	Legumin	Vicilin	Albumin	Globulin	Authors
Solubility (%)					
32.21	57.42	72.56	34.52	-	Tahir 2024
95.00	84.60	92.00	89.00	-	Shen 2023
-	92.12	90.14	97.44		Gravel 2023
-	68.30	96.00	-	96.00	Chang 2022
WHC (g/g)					
0.68	0.37	0.86	0.96	-	Tahir 2024
OHC (g/g)					
0.5	0.71	0.34	0.24	-	Tahir 2024
Gelling					
4.40 N	8.58 N	2.34 N	2.17 N	-	Tahir 2024
-	-	-	545 Pa	300 Pa	Kornet 2022
Emulsifying ability (%)					
67.52	77.45	55.03	59.87	-	Tahir 2024
-	12.28	-	28.70	43.69	Chang 2023
Foam capacity (%)					
143.80	206.67	180.23	185.62	-	Tahir 2024
-	36.79	183.33	-	82.21	Chang 2022
-	-	-	258.00	61.00	Kornet 2022
400.38	397.39	390.56	392.12	-	Shen 2023

When numerical values were not explicitly reported, they were extracted from figures using WebPlotDigitizer software (automeris.io).

3.1. Solubility

Protein solubility is a key functional property, particularly in industrial applications, as it reflects the balance between protein–protein and protein–solvent interactions. It is influenced by extrinsic factors such as solvent type, temperature, and protein concentration, all of which affect the protein's surface characteristics (Asen et al., 2023; Lam et al., 2018).

Tahir et al. (2024) used isoelectric precipitation combined with dialysis to generate legumin, vicilin, and albumin fractions, and their solubility was compared to a commercial PPI. The highest solubility (72.56%) was obtained for vicilin, while albumin, legumin, and PPI exhibited solubilities of 57.42, 34.52, and 32.21%, respectively. The higher solubility of vicilin can be attributed to a lower level of protein aggregation, driven by its lower degree of polymerization, higher

structural flexibility, and relatively lower molecular weight compared to other fractions. Additionally, a higher proportion of intermolecular β-sheets and a lower content of random coil structures contribute to improving solubility by influencing protein-water interactions. The presence of glycosylated subunits further contributes to enhancing the vicilin solubility. Similarly, Shen et al. (2023) observed higher solubility for vicilin compared to albumin and legumin, reinforcing the role of structural flexibility and smaller particle size in promoting solubility. However, in contrast to Tahir et al. (2024) no significant difference in solubility between PPI and vicilin was observed, which can be an effect of the history and isolation process, since the study of Shen et al. (2023) presented a higher solubility for the PPI (~95%).

Even greater differences are observed when comparing these results with those reported by Gravel & Doyen (2023), who found considerably higher solubility values for legumin (92.12%), vicilin (90.14%), and albumin (97.44%). These variations are unlikely to be attributed only to intrinsic structural differences among fractions. In their study, solubility was evaluated at pH 8 in the presence of 0.25 M NaCl, conditions that favor protein solubilization due to increased electrostatic repulsion and improved protein–water interactions.

The pH also significantly impacts protein solubility, as it can induce structural modifications that affect protein-water interactions. Chang et al. (2022) compared the solubility of pea globulin with legumin and vicilin. Under acidic conditions, solubility was 68, 57, and 37% for globulin, legumin, and vicilin, respectively. The highest solubility was measured in alkaline conditions (pH 9-10), where solubility exceeded 90% for globulin and vicilin and reached 73% for legumin. The lowest solubility (2-3%) in the pH range of 4.0-6.0 is attributed to the proximity of these pH values to the isoelectric point, which is estimated to be 4.59 for globulin, 5.26 for legumin, and 4.70 for vicilin. This study also indicates that the solubility and characteristics of globulins are not merely the sum of legumin and vicilin.

Djoullah et al. (2015) compared the solubility of globulin and albumin fractions and observed a U-shaped profile for globulin, while albumin was soluble from pH 2-10, with a small reduction at pH 5 (85% of solubility). The main difference between these fractions is related to the structure of albumin. Albumins present a hydrophilic nature and low surface hydrophobicity, which minimizes aggregation near the isoelectric point. For globulin, the lowest solubility (25%) occurred

at pH 4.0-6.0, corresponding to the pI, while the highest solubility was observed in alkaline conditions, exceeding 90%. In this study, the effect of ionic strength was also evaluated. Globulins showed greater ionic strength dependence, with maximum solubility observed at 50 mM NaCl (90%). Beyond this concentration, solubility decreased, likely due to the salting-in and salting-out effects.

Due to their distinct molecular structures, pea protein fractions display markedly different solubility profiles and respond differently to external conditions such as pH and ionic strength. For example, vicilin generally shows higher solubility than legumin, while albumins remain soluble across a wide range of conditions. These inherent differences suggest that each fraction may interact uniquely with NTTs, leading to fraction-specific structural modifications. Understanding these distinct responses is essential, as they can be strategically used for different applications as food ingredients.

3.2. Colloidal properties

3.2.1. Water and oil holding capacities

Water holding capacity (WHC) refers to the ability of a protein to retain water or absorb water. In the food industry, this parameter is extremely important as it influences sensory attributes such as texture, mouthfeel, and flavor (Shen et al., 2022). This capacity is governed by various molecular interactions, including ion-dipole, dipole-dipole, dipole-induced dipole, and hydrophobic interactions (Shanthakumar et al., 2022). Gaining a deeper understanding of how different pea protein fractions contribute to WHC is essential for expanding their application in food formulations.

A study by Tahin et al. (2024) demonstrated that WHC varies significantly among pea protein fractions. Albumin, vicilin, and legumin exhibited WHC values of 0.96, 0.86, and 0.37 g/g, respectively. These differences are closely linked to structural attributes such as surface hydrophobicity: legumin showed the highest hydrophobicity (427), compared to 144 for albumin, thus interacting less with water, decreasing the WHC. Additionally, legumin's elevated β-sheet content limits water accessibility, further reducing its WHC. Chihi et al. (2018) reported a WHC of 42% for a globulin-rich gel formed via acidification at 4% protein concentration, which was higher than that of legumin alone but lower than that of vicilin. This

indicates that the relative proportion of vicilin and legumin in the system can modulate the WHC, highlighting the importance of protein composition.

Oil holding capacity (OHC) describes a protein's ability to retain oil within its structure, another key functionality for applications in meat products, beverages, and dressings (Shen et al., 2022). OHC is primarily driven by lipid-protein interactions, where the aliphatic chains of lipids are associated with the nonpolar side chains of amino acids. Thus, proteins with higher hydrophobicity generally exhibit stronger oil-binding capacities (Shanthakumar et al., 2022).

According to Tahir et al. (2024), legumin, due to its higher hydrophobic group content and β-sheet structure, showed the highest OHC (0.71 g/g), followed by vicilin (0.34 g/g) and albumin (0.24 g/g). While these structural features reduce legumin's affinity for water, they enhance its oil-binding capability. However, even with the increased interest in the functional properties of pea protein fractions, few studies have investigated WHC and OHC in these fractions. The limited research available highlights the need for further studies to explore these properties in greater detail and their implications for food and industrial applications.

3.2.2. Gelling properties

Gelation refers to the ability of proteins to form a three-dimensional network capable of entrapping liquids such as water and oil within its structure (**Figure 4**). Various methods can induce gelation, with heat-induced and acid-induced gelation being the most commonly used in food applications (Shanthakumar et al., 2022; Shen et al., 2022). In heat-induced gelation, proteins such as those from peas are subjected to elevated temperatures, leading to the unfolding of their native structure and the exposure of hydrophobic amino acid residues. These exposed regions facilitate new intermolecular interactions, primarily hydrophobic, but also hydrogen bonding and disulfide bridges, depending on the specific protein fractions involved. If these interactions are sufficiently strong, a stable protein network is formed, resulting in gel formation (Nascimento et al., 2025). In acid-induced gelation, gelation is driven by a gradual decrease in pH through the addition of a weak acid, which slowly dissociates in the medium. As the pH drops, proteins begin to unfold, exposing reactive side chains. These destabilized chains

then interact via ionic, hydrophobic, and hydrogen bonds to form a cohesive gel network (Nascimento et al., 2024).

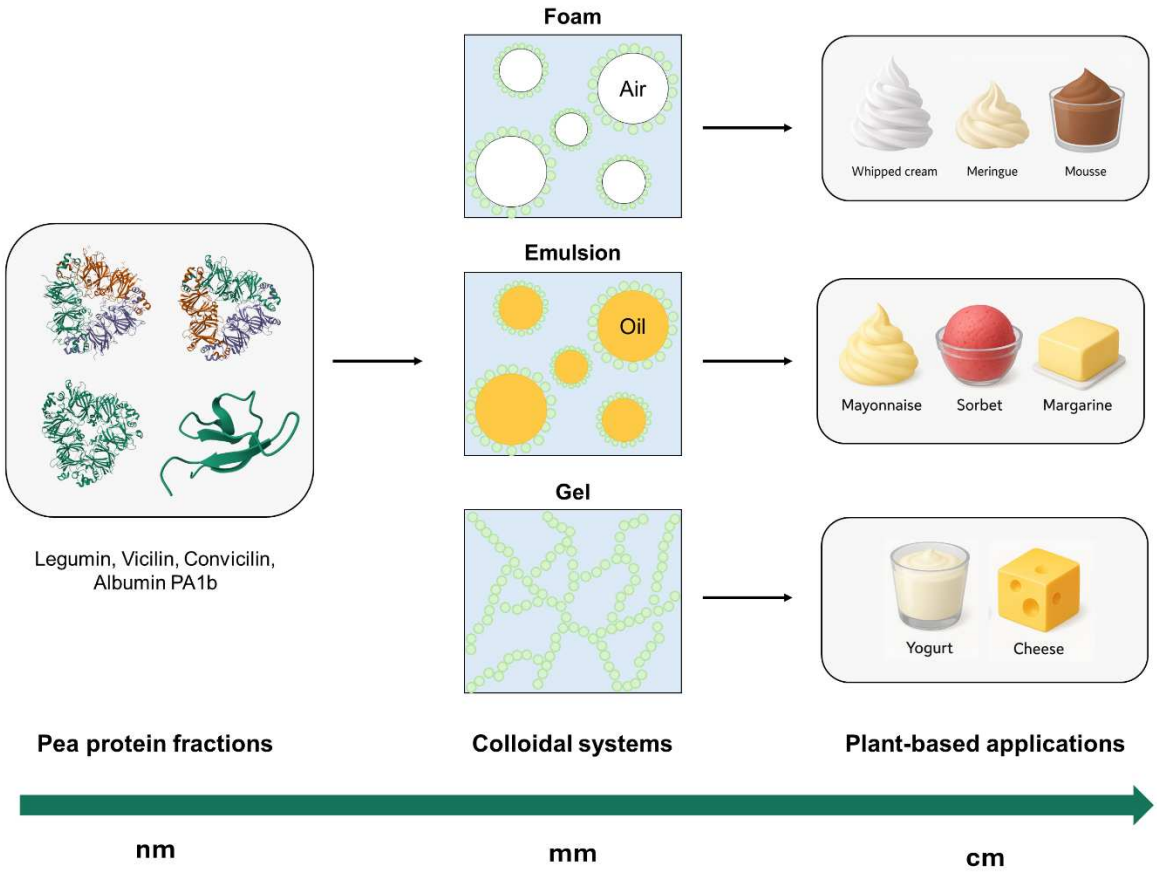

Figure 4. Pea proteins use in the development of colloidal systems and their applications in plant-based products. Protein structures obtained from RCSB PDB (https://www.rcsb.org/, accessed March 06, 2026): references: Albumin PA1b (1P8B), Convicilin (7U1J), Vicilin (7U1I), Legumin (3KSC). Green circles in the colloidal systems indicate pea proteins. Icons of plant-based applications were generated using ChatGPT (OpenAI). The figure exemplifies how pea protein fractions can be used in the development of colloidal systems, which are the structure of plant-based products.

In recent years, numerous studies have investigated the gelation properties of pea proteins. However, most have focused on whole protein isolates without differentiating between individual protein fractions. Given that each fraction exhibits distinct structural and functional characteristics, it is essential to study them separately to better understand their specific contributions to gel formation.

R. Kornet et al. (2021) examined the heat-induced gelation behavior of globulin and albumin fractions at a concentration of 15 wt%. Following gelation, the elastic modulus (G′) of the gels the globulin presented a solid soft behavior with an elastic modulus of 300 Pa, with a purity of 87.3%. On the other side, albumin behaves like a weak solid with a G' of 8 Pa, with a purity of 21.1%. However, for a better comparison, the authors purify the albumin, producing a rich fraction with a purity of 53.5%. In this condition, the albumin-rich fraction exhibited an elastic modulus of 545 Pa, demonstrating a greater gelling capacity per gram of protein than globulin. Moreover, albumin-based gels exhibited lower sensitivity to changes in pH and ionic strength, making them more versatile for diverse food applications.

Comparing the fractions, Tahir et al. (2024), reported higher gel strength in legumin-based gels compared to those formed from albumin or vicilin. In this study, heat-induced gels exhibited strengths of 8.58 N, 2.34 N, and 2.17 N for legumin, vicilin, and albumin, respectively. The authors attributed legumin's superior gel strength to the formation of disulfide bonds that occur upon heating the protein beyond its denaturation temperature. Notably, the study did not report the protein purity of the fractions used, which may affect the interpretation of the results.

Despite the limited number of studies specifically addressing the gelation properties of individual pea protein fractions, the available evidence highlights clear functional distinctions. Legumin, with its compact structure and has high cysteine content, tends to form stronger and more stable gels through disulfide crosslinking. In contrast, vicilin and albumin produce weak gel networks. These structural differences suggest that fraction-specific gelation behavior could be strategically harnessed in food applications. However, systematic studies comparing the gelation performance of these fractions under standardized conditions are still lacking, representing an important research gap for optimizing their use in plant-based food development.

3.2.3. Emulsifying properties

Emulsions are mixtures of two immiscible liquids, typically oil and water, where one is dispersed as small spherical droplets within the other (**Figure 4**). Proteins can act as emulsifiers due to their amphiphilic nature, enabling them to reduce interfacial tension and stabilize the emulsion system (McClements, 2015).

This property is referred to as emulsifying ability. Emulsion formation generally involves two steps. In the first step, protein is absorbed at the oil/water interface, while the second step involves a protein structural rearrangement, including partial denaturation, where hydrophilic regions orient toward the aqueous phase while hydrophobic regions embed in the oil phase (Shanthakumar et al., 2022). Two key parameters are used to assess protein emulsification performance, *i.e.,* the emulsifying ability (EA), which reflects the protein's capacity to form emulsions, and the emulsion stability (ES), which indicates the resistance of the system to phase separation over time (Ozkan et al., 2024).

Several studies have explored the use of pea protein fractions in emulsification. R. Kornet et al. (2022) compared the emulsifying performance of a globulin-rich fraction, an albumin-rich fraction, and a pea protein concentrate. Globulin-rich fraction and pea protein concentrate produced emulsions with droplet sizes below 1 μm, while albumin-rich fraction formed emulsions with two distinct droplet populations (0.3–3 μm and 3–30 μm). After treatment with sodium dodecyl sulfate, the larger droplet population in albumin-rich fraction disappeared, suggesting flocculation. In contrast, globulin-rich fraction and pea protein concentrate showed no signs of flocculation. Moreover, albumin-rich fraction exhibited additional destabilization phenomena such as coalescence and creaming, indicating inferior stabilizing performance at a protein concentration of 0.7% (w/w). The smallest albumin capacity to prevent the destabilization phenomenon is because albumins have a lower protein charge than globulins; thus, electrostatic repulsion is low, leading to destabilization. Besides that, due to the low protein charge, more albumins can fit at the interface, and consequently, more protein is required to stabilize it.

When considering the individual fractions, Tahir et al. (2024) reported higher EA values for legumin, followed by PPI, albumin, and vicilin. The authors linked higher EA with increased surface hydrophobicity, which, despite reducing solubility, improves adsorption at the oil–water interface. They also observed an inverse correlation between droplet size and surface hydrophobicity in legumin, suggesting that interfacial activity was more influenced by surface chemistry than by particle size. Although albumin had a smaller particle size and lower surface hydrophobicity than vicilin, it formed smaller droplets, resulting in better EA performance. Comparing the studies by R. Kornet et al. (2022) and Tahir et al.

(2024), when the globulin is fractioned into legumin and vicilin, different behaviors can be observed, demonstrating the necessity of analyzing the fractions individually to determine which one is more responsible for emulsifying properties. The same behavior can be observed in the study of Chang et al. (2022). In this study, the author compared the functional properties of legumin, vicilin, and total globulin fractions from peas and chickpeas under varying pH conditions (3.0, 7.0, and 9.0). Globulins exhibited higher EA than legumin and vicilin. Nevertheless, comparing the fractions, vicilin showed a higher EA than legumin, likely due to its lower molecular weight and more flexible structure. Despite this, legumin demonstrated superior ES, attributed to its strong interfacial anchoring capacity due to the more sulfur-containing amino acids, SH and SS groups, and larger molecular weight. Related to the pH conditions, EA and ES values were improved under alkaline conditions due to enhanced protein solubility and greater net surface charge, which facilitated faster diffusion to the interface.

Shen et al. (2023) further analyzed interfacial properties of PPI and its fractions, focusing on the dilatational rheological properties. reporting the highest EA for vicilin, likely due to its flexible structure that enables rapid interfacial rearrangement. However, legumin showed the highest ES, consistent with its higher surface hydrophobicity, which supports the formation of a more cohesive interfacial network. Albumin, in contrast, displayed low EA and ES, which the authors attributed to its simple, less interactive structure.

In summary, pea protein fractions exhibit significant differences in their emulsifying properties. Legumin exhibits strong emulsifying activity and stability due to its interfacial anchoring and hydrophobic characteristics. In contrast, albumin and vicilin, owing to their smaller molecular size, show rapid adsorption at the interface. However, because of their lower content of hydrophobic groups, the stability of the emulsions is reduced. These findings underscore the importance of selecting specific protein fractions to tailor emulsification performance in food formulations.

3.2.4. Foaming properties

Foam can be described as a system where gas is the dispersed phase, distributed within a liquid or solid continuous phase (**Figure 4**). Normally, in the food industry, the gas phase is air, nitrogen, or carbon dioxide, and the continuous

phase is an aqueous solution (Murray, 2020). This colloidal system is really common in the food industry and is an example: sponge cakes, chocolates, ice creams, bread, mousses, confectionery products, whipped creams, soufflés, and carbonated beverages (Murray, 2020; Zhan et al., 2022).

Foam formation depends on the reduction in interfacial tension between the two phases, and this phenomenon occurs due to the presence of surfactants, typically proteins or other small molecules. These molecules migrate from the bulk phase to the air/water interface, where they accumulate and cause a reduction in interfacial tension. As they unfold and reorganize, they form a viscoelastic layer that helps prevent drainage, coalescence, and disproportionation (Zhan et al., 2022).

In a study by Kornet et al. (2022), albumins and globulins were compared to investigate their foaming properties (overrun, air bubble size, and stability (half-life time)). Related to the foaming ability (FA) and air bubble size, albumin has higher FA and a smaller air bubble size, probably because of the protein size. Albumin has a molecular weight varying from 48-53 kDa, while globulin, from 170-380 kDa. The small size allows the protein to diffuse faster to the interface and have a lower surface adsorption energy. Regarding stability, albumins also showed a higher ability to stabilize foam, presenting a half-time life of 272 min, while globulins showed a 70 min. This difference results from the small air bubble size and stiffer interfacial layer.

Concerning globulin and its fractions, Chang et al. (2022) comparing acidic, neutral, and alkaline conditions, reported a significant influence of pH on FA, with acidic pH being more effective for all fractions due to the increased net charges in the protein surface. Observing the fractions, vicilin presented a higher FA in all the tested pHs, probably because the small Mw and less rigid structure allow the protein to fast adsorption in the interface. Regarding foaming stability (FS), globulins showed higher stability under alkaline conditions, whereas vicilin and legumin had greater stability under acidic conditions. Comparing the fractions, legumin showed better stability results (~60%) than vicilin (~30%) due to the structural differences, for example, the higher α-helix content, which can contribute to forming a thick and cohesive film, increasing stability.

In contrast, Tahir et al. (2024) reported a higher FA for legumin (206.67%) than vicilin (180.23%). In this study, the authors attribute this greater FA to two

main factors: one is the fact that legumin has greater surface-active properties, making it more capable of stabilizing air bubbles, and the second is better drainage resistance. Another difference from Chang et al. (2022), is the higher FS for vicilin and albumin compared to legumin. The higher FS of vicilin is probably because of the flexible structure, the absence of disulfide bonds, and cysteine residues, which favor the formation of a cohesive and resilient interfacial film. These discrepancies between the studies reflect differences in experimental conditions, protein isolation methods, and fraction purity, highlighting that the foaming performance of pea protein fractions is strongly system-dependent.

Even with the scarcity of studies correlating foaming properties with pea protein fractions, the available studies highlight these fractions' ability to be used in foam formation, presenting a promising alternative to animal proteins and synthetic molecules commonly used in the industry. However, compared to the traditional ingredients used in the food industry, the pea protein fractions functionalities are reduced, requiring structural modifications to improve their use. In this context, non-thermal technologies can appear as a solution.

4. Non-thermal technologies (NTTs)

The adoption of NTTs in the food industry has grown significantly in recent years, driven by the demand for safer, more sustainable, and higher-quality food products. Conventional thermal processing, while effective in microbial inactivation, is associated with several drawbacks, including the formation of harmful chemical compounds, such as acrylamide (Galani et al., 2017), and the degradation of heat-sensitive nutrients such as vitamins and polyphenols, compromising food quality (Fadimu et al., 2022; Safwa et al., 2023). Also, from a sustainable perspective, in the food industry, the generation of heat comes from the use of fossil fuels, thereby increasing greenhouse gas emissions (Toepfl et al., 2006; M. Yang & Wang, 2025).

NTTs are considered eco-friendly and cost-effective, which is particularly advantageous given the growing concern about environmental issues (**Table 2)** (Pan et al., 2022).

Currently, NTTs are widely used in the food industry for sterilization processes, including milk, juice, meat, fruits, and various other food products, depending on the technology (Chiozzi et al., 2022). Beyond sterilization and safety

assurance, ultrasound (US) has been used for the extraction of valuable compounds, as well as in homogenization and emulsification processes (Bhargava et al., 2021). PEF, in contrast, has been particularly applied in potato processing (*e.g.,* French fries and potato chips production) and tomato peeling (Raso et al., 2022; Taha et al., 2022). Even though these technologies are already applied in the food industry, many consumers remain unfamiliar with them, which can lead to reluctance in accepting products processed in this way. In addition, concerns related to product safety and the potentially higher price of treated foods may further reduce consumer acceptance. However, when the benefits of these technologies, such as enhanced food safety, improved sensory quality, and reduced environmental impact, are clearly communicated, acceptance tends to increase (Melios et al., 2025).

In addition to classic food applications, NTTs have been explored as a strategy to modify protein structures (Pan et al., 2022). The modifications caused by NTTs in pea protein structure **(Table 3)** are important for their use in the food industry, for example, in the formulation of vegan products, since its native structure makes its application difficult, mainly because of the low solubility and techno-functional properties are reduced compared with other proteins utilized in the industry. Each NTTs works through distinct mechanisms that induce structural modifications in proteins **(Table 2)**. The following sections will explore these technologies in detail, highlighting their principles, effects on pea protein fractions' structure, and potential applications in the food industry.

4.3. High hydrostatic pressure processing

High hydrostatic pressure processing (HPP), also known as cold pasteurization or pascalization, is a non-thermal technology first studied in 1899 for milk pasteurization (Hite & Giddings, 1914). HPP involves applying high levels of isostatic pressure, typically between 300 and 600 MPa, to food materials placed in a sealed pressure vessel (Gokul Nath et al., 2023; Houška et al., 2022). Treatment durations may range from a few seconds to 20 minutes. While temperatures can reach up to 100 °C due to adiabatic compression heating, HPP is classified as non-thermal, since it is conducted at or below 30 °C to preserve food quality (Barbhuiya et al., 2021).

The HPP equipment consists of a robust pressure vessel with secure closures, an intensifier pump to generate and maintain pressure, and instrumentation for precise control and monitoring of pressure and temperature (Safwa et al., 2023). HPP offers multiple advantages it is energy-efficient, preserves food sensory and nutritional properties, and improves food safety while supporting "clean label" product development (Barbhuiya et al., 2021; Gokul Nath et al., 2023). As a disadvantage, this method operates only in batch or semi-continuous mode, reducing productivity. Besides that, the food products need to be packed to go under the treatment and present some restrictions on products with low humidity (Gokul Nath et al., 2023).

Beyond microbial and enzyme inactivation, HPP has been widely studied for its ability to alter protein structures. Pressure-induced structural changes primarily affect non-covalent interactions such as hydrophobic, ionic, electrostatic, and hydrogen bonds. Moderate pressures (100–200 MPa) can dissociate multimeric protein complexes into subunits by disrupting hydrophobic interactions. At higher pressures (400–800 MPa), proteins may undergo reversible unfolding, leading to exposure of buried functional groups and a reorganization of tertiary structure (Barbhuiya et al., 2021; Zhang et al., 2023). These modifications enhance the techno-functional properties of plant proteins, such as pea protein, facilitating their application in food formulations (see Table 3).

Zhang et al. (2023) investigated the effects of pH (5, 7, 10), pressure (300 and 600 MPa), treatment time (5 and 15 min), and protein concentration (10-15%, w/v) on pea protein gelation. Comparing the protein concentrations (10 and 15%), gel formation at 10% occurs only with pressure increase (600 MPa) because at low protein concentration, the exposition of more active sites is required to form the gel, and the gel formed has a coarser and less compact structure. In different pH (5, 7, and 10) at the same concentration (15%), the pressure effect was also important to form the gel. At pH 5, even in low pressure (300 Mpa) and low time (5 min), the gel structure was formed, because the pH is close to the isoelectric point, thus, the electrostatic repulsion is low, allowing protein interaction. At pH 7, the gel network was formed only at 600 Mpa. In low pressure, the disruption of electrostatic and hydrophobic bonds can occur, and during the pressure release, the formation of new interactions can be insufficient to form the gel. However,

when the pressure increases, more active sites can be exposed, allowing gel formation.

Compared to heat-induced gel (90 °C for 30 min), HPP was able to form gels at pH 5, whereas heat-induced gels did not form at pH 5. Also, at pH 7, the protein concentration required to produce a gel was lower, demonstrating the effectiveness of HPP in inducing gel formation. Regarding the gel structure, the gel formed through HPP exhibits a higher storage modulus than the heat-induced gel, indicating a harder network with greater resistance to elastic deformation (Tang et al., 2024). This difference indicates that HPP can be better exploited for the development of food products, as it can be applied over a wider pH range and at lower protein concentrations.

The same behavior was observed by Hall & Moraru (2021) . The authors reported reduced solubility (from 57 to 30%), probably due to protein aggregation, and increased ES, FS, and WHC after applying 600 MPa for 4 min. They also observed that HPP induced gelation at high protein concentrations (15%, w/w), attributed to hydrophobic interactions between exposed residues following protein unfolding. In comparison with the heat treatment,

Chao et al. (2018) assessed the impact of pressures (200, 400, 600 MPa) on yellow field PPI. HPP had minimal effect on solubility but significantly influenced emulsifying and foaming properties, depending on pH and protein concentration. The effect of pH on ES is more relevant than protein concentration because it influences the net protein charge. Regarding HHP, high-pressure treatments generally increased ES, probably because moderate protein aggregation and higher net charges promoted stronger interfacial membranes and greater repulsion between oil droplets, thereby reducing coalescence and consequently increasing stability. Compared with different heat treatments reported by the same author, HHP was more effective in enhancing emulsion stability. In contrast, moderate heat treatment could improve ES under certain conditions by increasing protein flexibility and interfacial adsorption. In contrast, high-temperature treatments (>70°C) generally reduced ES due to excessive protein denaturation and weakened interfacial membranes (Chao & Aluko, 2018).

The authors also observed the HPP effect on FS (Chao et al., 2018). The FS increased at pH 3.0 and 50 mg/mL but declined at higher pressures due to protein aggregation. FS was more influenced by protein concentration than HPP,

with lower FS observed in treated samples, likely due to reduced protein flexibility and interfacial strength. However, when the samples were heat-treated, the stability increased at pH 5.0 and 7.0, especially at 50 mg/mL protein concentration, probably due to the presence of heat-induced polypeptides with increased net charge or formed of strong interfacial films through increased hydrogen bond, facilitating the protein–protein interactions.

To further enhance HPP-induced gelation, Zhang et al. (2025) investigated the incorporation of κ-carrageenan, a natural polysaccharide, into PPI gels. Increasing κ-carrageenan concentrations (0.1-1%) reduced the pressure required to form high-quality gels, improving energy efficiency. At 600 MPa and 1% κ-carrageenan, compressive strength increased 27-fold compared to HPP alone, and gel strength was five times higher than that of heat-induced gels, thus being able to produce robust pea protein gels using minimal additive concentrations.

In this section, it is evident that the HPP has the potential to replace thermal treatment to modify pea protein structure, being able to improve emulsion and gelling characteristics. However, as observed, no studies have investigated the relationship between pea protein fractions and the effects of HPP, highlighting a gap in the current literature. This gap is relevant since each fraction may have different responses to HPP, due to the differences in their structures. Therefore, future studies should not only test the global effect of HPP on pea protein isolates but also address these individual fractions to better understand their techno-functional potential and enable more targeted applications in plant-based food design.

Table 2. Mechanisms of action, advantages, and limitations of non-thermal technologies for protein processing.

Non-thermal technology	Mechanism of action	Advantages	Limitations
High-pressure processing	At high pressure, it disrupts non-covalent, hydrophobic, electronic, and hydrogen bonds, modulates solvation, and induces partial unfolding; at low pressure, it dissociates oligomeric proteins into subunits.	• Energy efficient • Homogeneity • Short processing time • Low temperature • No degradation of sensitive compounds	• High cost • Low productivity • Limited applicability
Ultrasound	Acoustic cavitation generates microbubble collapse, producing localized high temperature and pressure that induce chemical, mechanical, and thermal effects on proteins.	• Low cost • Low processing time • Improve efficiency	• Requires precise parameter control • Can produce hydroxyl radicals → oxidation of food components • Needs specialized equipment • Limited scalability
Cold plasma	Cold plasma generates reactive oxygen and nitrogen species (ROS/RNS) that chemically modify	• Low energy consumption • Design versatile • Cost-effective	• Surface-limited action • Potential alteration of sensory attributes

	amino acid residues, leading to peptide-bond cleavage, new functional groups, and altered protein surface properties.		• Complex equipment • Scalability issues
Pulsed electric field	The electric field interacts with protein dipoles, generating free radicals that alter hydrogen bonds, salt bridges, hydrophobic and Van der Waals interactions, modify protein charge, and induce partial unfolding without changing the primary structure.	• Low energy consumption • Minimal waste generation • Short processing times • Preserve heat sensitive nutrients	• High initial implementation cost • Scalability challenges • Electrode degradation and fouling • Product-specific

4.4. Ultrasound

Ultrasound (US) treatment involves the application of sound waves at frequencies above the human hearing threshold (>20 kHz). Its adoption in food processing has increased recently due to its sustainable nature, providing several advantages over conventional methods, including low cost, faster processing, and improved efficiency (Bhargava et al., 2021; Jadhav et al., 2021). On the other hand, the use of ultrasound requires high parameter control to avoid high pressure and temperature. When not controlled, these parameters can lead to protein denaturation and the production of hydroxyl radicals, which can oxidize food components, generate off-flavors, and decrease phenolic compounds. Besides that, it requires specialized equipment and has scalability limitations (Bernardi et al., 2021; Bhargava et al., 2021; Justino et al., 2024).

US is used in a wide range of industrial applications, including food sterilization, protein extraction, gelation, emulsion stabilization, cooking, foaming, and deaeration (Bhargava et al., 2021). This technology can be applied through direct or indirect methods. In the direct approach, a probe is immersed in the sample or placed in a flow cell, ensuring high cavitation intensity. However, this method may lead to food contact with equipment surfaces, raising concerns about contamination or metal leaching (Bernardi et al., 2021). In contrast, the indirect method uses an ultrasonic bath, where ultrasound is transmitted through a coupling liquid. While this avoids direct contact and minimizes contamination risks, it results in lower acoustic intensity(Bhargava et al., 2021; W. Chen et al., 2022).

The primary mechanism behind US-induced protein modification is acoustic cavitation. This phenomenon involves rapid formation, growth, and collapse of microbubbles under high-pressure acoustic waves. Bubble collapse generates localized high temperatures (up to 5000 K) and pressures (up to 30 MPa), which induce chemical, mechanical, and thermal effects that alter protein structure (Chen et al., 2022; Wei et al., 2025).

Xu et al. (2025) investigated the ultrasound effect at 20 kHz and varying power levels (30% [195 W], 60% [390 W], and 90% [585 W]) on PPI gelation. Except for the 30% power treatment for 10 min, all conditions yielded higher storage modulus (G′) values compared to the control, with the 60% power treatment being most effective. Gel hardness also increased with higher power levels and longer treatment times (10 and 20 min), likely due to reduced particle size,

increased surface area, and enhanced solubility, factors that improve water interaction and network formation. These structural changes led to more uniform and resilient gels, supported by increased β-sheet content.

In a related study, Gao et al. (2022) reported increased solubility of PPI due to partial disruption of hydrogen and hydrophobic bonds between protein subunits. This disruption caused macromolecules to swell and adopt distorted conformations, forming soluble aggregates. Nascimento et al. (2023) also observed improved gelation of PPI following US treatment. The enhanced complex modulus was attributed to reduced particle size and increased surface hydrophobicity, promoting the formation of a cohesive, interconnected protein network. Compared to casein-only gels, PPI gels demonstrated greater hardness, likely due to a higher number of junction zones. This suggests that pea protein could be a viable substitute for animal-derived proteins in food formulations.

The US also enhances gel strength in systems where gelation is enzyme-induced (e.g., via transglutaminase). Mozafarpour et al. (2022) observed increased gel strength in grass PPI treated with different amplitudes (25–75%) and times (5–20 min). However, excessive treatment (20 min at 75%) reduced strength due to SH group oxidation. US also improved EA and ES by reducing particle size, increasing solubility, and promoting surface hydrophobicity, enhancing protein diffusion and adsorption at oil-water interfaces and improving droplet stability through increased electrostatic repulsion.

These findings align with Ozkan et al. (2024), who treated PPI at 20 kHz and 25% amplitude for up to 30 min. EA and ES increased significantly compared to untreated PPI. FA and FS were also enhanced. Bahmanyar et al. (2025) confirmed similar trends at higher power levels (100-300 W), with increased EA, FC, FS, and surface area due to protein unfolding and exposure of hydrophobic regions. However, excessive power or duration can lead to protein denaturation and reduced solubility, negatively affecting colloidal properties (Gao et al., 2022).

Compared to heat treatment, US has been shown to be better at improving colloidal properties. Related to structural properties, US caused a higher reduction in particle size and an increase in hydrophobicity and solubility, with higher modification at higher ultrasound treatment. These modifications were able to increase WHC and OHC, as well as the emulsifying activity index, emulsifying capacity, foam stability, and gel strength. High-intensity ultrasound, due to acoustic

cavitation, caused a higher impact on protein structure by promoting the disruption of aggregates and exposure of hydrophobic groups. In contrast, heat treatment mainly affected the molecular weight distribution and promoted the formation of insoluble aggregates, which improved some properties compared to untreated samples, but was less effective than high-intensity ultrasound (Tahir et al., 2025). The authors also compared US and heat treatment with pH-shifting, another conventional technology. Acoustic cavitation was more effective at improving colloidal properties than pH-shifting. pH-shifting promotes protein unfolding and induces a molten globule state, which can expose hydrophobic groups but may also favor protein aggregation, thereby limiting improvements in colloidal properties.

The US effect on individual pea protein fractions has been demonstrated in a few studies. Sha and Xiong (2022) applied US (20 kHz, 50% amplitude, 5 s pulse cycles for 5 min) to legumin and vicilin fractions. For legumin, US increased solubility, reduced particle size, and elevated zeta potential. Vicilin showed reduced particle size and increased surface hydrophobicity, but solubility and zeta potential remained unchanged. These differences affected emulsion activity index and capacity: emulsion activity index increased more in legumin (30.8%) than in vicilin (29.6%). In contrast, emulsion capacity was higher in vicilin due to its more negative surface charge, enhancing electrostatic repulsion between droplets.

To summarize, the US is promising as an NTT for modifying pea proteins and has been applied to fractions such as legumin and vicilin in a few studies, offering benefits such as enhanced solubility, reduced particle size, and altered surface properties. These modifications improve gelation, emulsification, and foaming characteristics. Therefore, the US application on pea protein fractions, such as legumin and vicilin, remains limited and should be investigated in other fractions, for example, pea albumin, which may respond differently from globulins to acoustic cavitation and shear forces generated by US. Moreover, the impact of the US on minor fractions, such as prolamins and glutelins, remains completely unknown, despite their potential role in modifying texture and protein–protein interactions in complex food matrices. Therefore, future research should systematically assess how US affects each protein fraction to enable fraction-specific functional tailoring in plant-based formulations.

4.5. Cold plasma

Plasma, regarded as the fourth state of matter, was first described by Langmuir in 1925 (Jadhav et al., 2021; Safwa et al., 2023). Plasma is generated by supplying energy to a gas, causing ionization. This can occur under both atmospheric and low-pressure conditions (Safwa et al., 2023; Wei et al., 2025). Thermodynamically, plasma can be classified into two types: thermal plasma and cold plasma (CP).

In CP, energy is primarily supplied to electrons through an electrical discharge, avoiding excessive heating of the system. Due to the non-equilibrium nature of CP, electrons, being much lighter than ions and neutral atoms, are more readily energized and transfer their energy to other particles via collisions. Consequently, the overall temperature remains relatively low, typically ranging from 25 to 65 °C (Bayati et al., 2024; Harikrishna et al., 2023; Jadhav et al., 2021). CP systems vary based on discharge type, including radiofrequency, dielectric barrier discharge, plasma jet, and microwave plasma technologies (Bayati et al., 2024).

In protein structure, CP primarily modifies the structure by interacting with reactive oxygen species (ROS) and reactive nitrogen species (RNS), which chemically alter amino acid residues, thereby altering protein conformation. The structural changes result from peptide bond cleavage, the formation of new functional groups, and modifications of the protein's surface properties (Chuang et al., 2025). The extent of modification depends on multiple factors, such as the protein type, plasma conditions, reactive gases, and operational parameters (**Table 3**) (Kopuk et al., 2022; Marcinauskas et al., 2024). However, cold plasma also presents limitations, including its surface-limited action, complexity of equipment, and scalability issues, which must be considered when designing food applications.

Understanding these factors is crucial for optimizing the CP application to pea protein. Bu et al. (2022) investigated the impact of reactive species (NxOy/O3, O3, H2O2, and OH) on pea protein under different pH conditions (2 and 7). Of the four species, ozone (O3) and hydroxyl radicals (OH) significantly affected surface hydrophobicity and β-sheet content, improving gelation and emulsifying properties. These modifications result from the interaction between O3/OH and sulfur-containing amino acids, inducing oxidation that promotes

protein unfolding. Additionally, the formation of inter-chain disulfide bonds reduces the distance between proteins, enhancing steric interactions and unfolding, further boosting techno-functionalities.

Beyond species form, operational conditions, such as time and energy inputs, directly affect pea protein structure. Rout & Srivastav (2024) explored the effect of output voltages (25, 30, and 35 kV) and treatment times (2, 4, 6, and 8 min) on soy and PPI. Compared to untreated samples, all voltage levels increased the WHC, OHC, and solubility, with the optimal results observed at 30 kV. At this voltage, treatments lasting up to 4 min enhanced free sulfhydryl groups and surface hydrophobicity, while longer treatment times (over 4 min) reduced these parameters, likely due to protein unfolding and oxidation of hydrophobic amino acid residues. Moreover, CP treatment improved the gelation properties of PPI, increasing the storage modulus for treatments lasting 2, 4, and 6 min at 30 kV. S. Zhang et al. (2021) also reported improved gelling properties of pea protein concentrate following CP treatment. Before CP, pea protein concentrate could not form a gel at 90°C, but after treatment, it showed good gelation between 70 and 90°C. CP caused partial protein unfolding, exposing internal hydrophobic side chains and active sites that reacted with free radicals generated by CP. These reactions facilitated the formation of covalent bonds, leading to stronger gels with improved water-holding capacity and viscoelasticity.

Chuang et al. (2025) examined the impact of CP on the structure and formulation of pea protein vegan cheese. By varying power (90 W, 130 W, and 170 W) and treatment time (3, 6, 12, and 20 min), the authors identified the optimal conditions as 130 W for 12 min. Under these parameters, CP induced structural changes, including a decrease in β-sheet content, an increase in β-turn formation, and heightened surface hydrophobicity. These alterations contributed to improved protein techno-functionalities, including enhanced solubility, WHC, OHC, EA, and FA. The authors attributed these changes to the breaking of peptide bonds, the introduction of new functional groups, and the exposure of hydrophobic amino acids such as phenylalanine, tryptophan, and leucine. Additionally, CP improved the texture and melting properties of vegan cheese, demonstrating its potential as a valuable tool for the plant-based food industry. Both Chuang et al. (2025) and Mahdavian Mehr & Koocheki (2023) studied the effects of prolonged CP exposure. They found that while short CP treatments improved the techno-

functionality of pea protein, extended exposure (beyond optimal durations) resulted in a decrease in these properties, including reduced FA and FS. This decline was attributed to protein aggregation and the formation of strong hydrophobic intermolecular interactions between proteins.

Cold plasma has proven to be effective in modifying the structure and techno-functional properties of pea proteins, enhancing functionalities such as gelation, emulsification, and foaming. These modifications open up new opportunities for the application of pea protein in plant-based food products. However, current studies have been almost exclusively limited to protein isolates, while the effects of CP on specific pea protein fractions remain largely unexplored. This is a critical gap, as the mechanisms of CP involve interactions with ROS and RNS, which can oxidize sulfur-containing amino acids and induce conformational changes. Fractions richer in cysteine and methionine, such as legumin and albumin, are therefore expected to be particularly sensitive to CP treatment, potentially improving disulfide cross-linking. Conversely, vicilin, with its lower sulfur content and more flexible structure, may respond differently. Understanding these fraction-specific responses could allow the targeted use of CP to tailor functional attributes in plant-based formulations.

4.6. Pulsed electric field

Pulsed electric field (PEF) is an eco-friendly and sustainable technology with a growing range of applications, including inactivation of microorganisms, extraction of valuable compounds, improving the quality of potato chips, and modifying protein structure (Taha et al., 2022). It involves the application of short, high-voltage electric pulses (10-80 kV/cm) to materials placed between two electrodes (Pataro & Ferrari, 2020; Taha et al., 2022). Food materials that contain charged particles, such as ions, exhibit electrical conductivity. When an external electric field is applied, an electric current flows through the food, inducing a series of electrochemical and physicochemical changes that modify food properties (Malik et al., 2024). Due to its energy use, PEF is considered eco-friendly, with low energy consumption, minimal waste, and short processing times. However, the initial implementation involves high costs, scalability remains challenging, electrode degradation and fouling may occur, and the process is highly dependent on the

product, requiring specific parameters for each matrix (Raso et al., 2022; Taha et al., 2022).

PEF equipment typically comprises a treatment chamber, a pulsed power supply, and control and monitoring systems (Arshad et al., 2020). Treatment chambers can be static or continuous, depending on the type of material being processed. Static chambers are generally used for solid foods, while continuous chambers are suitable for liquids or semi-solids. In continuous mode, flow dynamics may be classified as coaxial, collinear, or cofield (C. Zhang et al., 2023). The power supply generates electrical pulses by charging a capacitor, with discharge regulated by a trigger or switch to control decay within the circuit (Taha et al., 2022). The control and monitoring systems are crucial for regulating parameters such as voltage, current, and temperature, which directly influence the effectiveness of the PEF treatment (Arshad et al., 2020). Further technical details on PEF equipment can be found in the reviews of Arshad et al. (2020) and Taha et al. (2022).

The PEF effect on proteins arises from the interaction of the electric field with molecular dipoles. During treatment, polar groups in proteins absorb energy and generate free radicals, which affect intramolecular interactions such as hydrogen bonds, salt bridges, hydrophobic interactions, and Van der Waals forces. Furthermore, PEF exposure influences the ionization of carboxyl (-COOH) and amine (-NH3+) groups, altering the protein's apparent charge and causing conformational changes. These changes can lead to protein unfolding and aggregation, which modify the protein’s secondary structure without affecting its primary structure (Malik et al., 2024; Shams et al., 2024; Taha et al., 2022, 2023; Yan et al., 2024).

Regarding the pea proteins, their structural modifications under PEF treatment depend on several factors, including electric field intensity, treatment duration, and sample pH (**Table 3**). Guo et al. (2024) and Chen et al. (2023) compared the effects of electric field intensity on pea protein structure. Guo et al. (2024) evaluated field intensities of 5, 7.5, 10, and 12.5 kV/cm at a frequency of 600 Hz, while Chen et al. (2023) studied intensities from 0 to 25 kV/cm at 1 kHz. In both studies, increasing the intensity up to 10 kV/cm resulted in modifications in the secondary and tertiary structures of pea protein. This was evidenced by a reduction in α-helix and β-turn structures, coupled with an increase in β-sheet and

random coil configurations. Furthermore, changes were observed in hydrophobicity, intrinsic fluorescence, and UV-vis absorption, indicating protein unfolding and enhanced flexibility. However, higher intensities led to aggregation, reducing functionalities. Guo et al. (2024) also reported increased WHC and ES, but a decrease in OHC and EA.

Chen et al. (2022) investigated the effects of frequency (50 Hz and 20 kHz) and field strength (5, 10, and 20 V/cm) on pea protein. Similar to previous studies, the authors observed modifications in the protein structure, including a reduction in α-helix content and an increase in β-sheet formation. Notably, at 50 Hz and 20 V/cm, these modifications were more pronounced in the gelation process, resulting in stable and ordered gel networks. These gels exhibited superior WHC (90.12g/100g) compared to conventionally heated gels (86.73 g/100 g), though they were more elastic and cohesive. Finally, Melchior et al. (2020) examined the effects of moderate electric field intensities through varying pulse numbers (20,000 and 60,000 pulses, pulse width: 5 µs) and pH (5 and 6). Their findings aligned with previous studies, showing protein unfolding and increased flexibility. However, at pH 6 and with 20,000 pulses, solubility decreased, and FA increased, with no significant effects on WHC and OHC. The authors suggested that the limited changes in techno-functional properties were likely due to the low content of sulfhydryl groups, as the electrical current was insufficient to induce significant modifications compared to other studies.

Consistent with previous studies, De Gol et al. (2025) also demonstrated the ability of PEF (24 kV/cm, 50 Hz, pulse width 20 µs) with different inlet temperatures in modifying pea protein extract. Besides the PEF effect, the authors compared it with thermal treatment at different temperatures, 51, 54, 57, and 60 °C. The thermal treatment induced protein aggregation, reduced solubility, and limited gelation capacity. On the other side, PEF treatment promoted protein unfolding, increasing solubility (+8%), exposing tryptophan residues, and consequently enhancing gelation ability. Thermomechanical tests confirmed a 35% increase in gelling properties for PEF-treated proteins, indicating stronger intermolecular interactions and superior modification of protein functionality compared to thermal treatment. This demonstrates that replacing thermal technologies with non-thermal approaches can effectively modify proteins, not only enhancing their

functionality in colloidal systems but also minimizing drawbacks associated with thermal processes.

Overall, the PEF has demonstrated effectiveness in modifying pea protein, with the effect being dependent on PEF parameters. As observed for the other non-thermal treatments, the PEF effect on pea protein fractions remains unexplored, which demonstrates a gap for future research.

Table 3. Effect of non-thermal technologies (NTTs) on pea protein structure and techno-functional properties.

Food processing	Treatment parameters	Free-SH	Surface hydrophobicity	Solubility	Holding capacity	Techno-functional properties	Reference
HPP	**Pressure:** 300 and 600 MPa; **Time:** 5 and 15 min	-	-	-	-	Increase as a function of pressure level and time, enhancement of gel formation	(Zhang et al., 2023)
	Pressure: 200, 400, and 600 MPa. **pH:** 3, 5, and 7	-	-	No significant difference	-	200 MPa and pH 3 induced better emulsion quality and foaming.	(Chao et al., 2018)
	Pressure: 200, 400, 600 MPa	-	Increase up to 600 MPa.	Decrease at 600 MPa	WHC increased at 600 MPa	Increase of ES, foam expansion, and stability	(Hall & Moraru, 2021)
US	**Frequency:** 20 kHz; **Power levels:** 30 % (195 W), 60 % (390 W), 90 % (585 W); **Time:** 10 and 20 min.	-	Increase with time reduction and increase with power level increase	Increase with time and power level	WHC increases with time, and power level increase	-	(G. Xu et al., 2025)
	Sequential ultrasound power: 0, 100, 150, 200, 300, and 400 W	-	Increase up to 150 W	Increase up to 200 W	-	-	(Gao et al., 2022)
	Amplitude: 25, 50, 75%; **Time:** 5, 10 or 20 min	Increased up to 75% with time,	Increase up to 75% and 20 min	Increase up to 75% and with time,	-	EA and ES increased as a function of time and amplitude	(Mozafarpour et al., 2022)

		depending on the amplitude		depending on the amplitude			
	Frequency: 20 kHz, **acoustic intensity:** 17.12 W/cm2, **amplitude:** 25 % **Time:** 0, 10, 20, 30 min	-	-	Solubility increases up to 30 min.	-	FA, FC, EA, and ES increase with US application.	(Ozkan et al., 2024)
CP	**Reactive species:** O_3, NxOy, H_2O_2 and OH; **pH:** 2 and 7	-	At pH 2, an increase occurred after treatment with O_3 and OH	At pH 2, an increase occurs when treated with O_3, NxOy, and OH	-	O_3, NxOy/O_3, and OH formed gel at 15% protein with a higher strength at 20% protein. O_3 and OH had high EC.	(Bu et al., 2022)
	Voltages: 25, 30 and 35 kV; **Time:** 2, 4, 6, and 8 min	At 30 kV, the increase occurred until 4 min	At 30 kV, the increase occurred until 4 min	Increase with voltage and time	WHC and OHC increase up to 6 min for all voltages	EC, ES, G(Rout & Srivastav, 2024)l 6 min at 30 kV	(Rout & Srivastav, 2024)
	Frequency: 3500 Hz; **Voltage Output:**	Decrease after CP	Increase after CP	-	Increase in WHC	CP improved gelling capacity at 10% protein	(Zhang et al., 2021)

	0–30 kV; **Current output:** 0–1 A					concentration and low temperatures (70°C)	
	Power settings: 90 W, 130 W, and 170 W; **Time:** 3, 6, 12, and 20 min	-	-	The increase was higher at 90W and 12 min	Higher WHC and OHC at 90 and 130 W for 12 min and 170 W for 6 min;	Higher EA and FA at 90W and 12 min; Increase in gel hardness after CP	(Chuang et al., 2025)
PEF	**Intensity:** 5, 7.5, 10, 12.5 kV/cm; **Time:** 1, 2, 3, 5 min.	Decrease up to 10 kV/cm and 3 min	Increased up to 7.5 kV/cm	-	WHC increases up to 10 kV/cm and 3 min OHC decrease up to 10 kV/cm and 3 min	Decrease EA compared to control Enhancement of ES	(Guo et al., 2024)
	Frequency: 50 Hz and 20 kHz; **Strength:** 5, 10, 20 V/cm.	Higher decrease at 20 kHz and 5 V/cm	Highest increase at 50 Hz and 20 V/cm	-	Decreasing frequency and increasing decrease WHC	-	(Y. Chen et al., 2022)
	Frequency: 400 Hz, pH: 5 and 6, and **pulse number:** 20,000 and 60,000	Decrease by PEF	-	Decrease by PEF application.	At pH 6.0, WHC and OHC increase by PEF application	PEF increased foam ability	(Melchior et al., 2020)

	Intensity: 0, 5, 10, 15, 20, 25 kV/cm.	-	Increase after PEF. Higher at 10 kV/cm.	-	-	PEF increased PPI binding with EGCG	(Z. L. Chen et al., 2023)

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5. Conclusion and perspectives

This literature review highlights the potential of NTTs, such as US, HPP, CP, and PEF, to modify the structure of pea protein fractions. These technologies induce protein unfolding and expose hydrophobic side chains, thereby altering protein solubility, WHC, and OHC and enhancing key techno-functional properties such as gelation, emulsification, and foaming (**Table 3**). These modifications not only improve the techno-functional properties of pea protein but also offer new opportunities for its application in the food industry, whether as an additive in existing products or in the creation of innovative plant-based alternatives. However, as discussed, further research is essential to optimize the use of NTTs, particularly in PEF and HPP, where studies remain limited. A critical area for future investigation is the exploration of individual protein fractions, as they exhibit distinct techno-functional characteristics that can be leveraged for specific food applications. By gaining a deeper understanding of the structural modifications induced by NTTs, it will be possible to enhance the versatility of pea protein fractions and expand their techno-functionality for a wider range of food formulations. Ultimately, the continued innovation and optimization of these technologies not only promise to enhance the applicability of pea protein fractions small but also contribute to the development of more sustainable food products. By reducing the environmental impact of food production and providing healthier, more accessible options, these advancements align with the growing demand for sustainable and plant-based food solutions in response to global dietary shifts.

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CHAPTER V.

GENERAL CONCLUSION AND PERSPECTIVES

General conclusions

This work was driven by the hypothesis that modifications in pea protein structure and formulation parameters can alter the structure of hybrid gels. Through this work, it was concluded that the protein ratio and the modification of pea protein structure via pH shifting could modulate the characteristics of the hybrid gel.

The structure of hybrid gels is strongly governed by the protein ratio, with pronounced non-monotonic effects upon partial replacement of casein by pea protein. While casein gels form heterogeneous networks with larger cavities that promote higher water mobility, pea protein gels exhibit more homogeneous and compact structures that restrict water dynamics. Regarding the ratio, even small amounts of casein significantly disrupt the compact pea protein network, demonstrating that structural evolution is not linear with composition. Multi-scale analysis revealed that these differences are primarily associated with large-scale organization rather than molecular-scale rearrangements, supporting the hypothesis that casein and pea proteins form independent network domains without co-aggregation. Rheological measurements further confirmed that casein-rich gels are more sensitive to temperature increases, whereas pea-rich systems exhibit greater structural stability due to their smaller pore size and denser network. Overall, large-scale structural organizations emerge as the dominant factor controlling gel stiffness and water mobility.

To increase the interaction between casein and pea protein, the pH shifting approach was conducted. After the treatment, the pea protein structure has changed, increasing the intrinsic hydrophobicity, reducing the particle size, and consequently increasing the solubility. Because of those changes, when the modified pea protein was used to formulate hybrid gels with casein micelles, the gel's behavior changed. The effect was greater in the gels with a high amount of pea protein. Overall, the findings confirm that structural modification of pea protein via pH-shifting can partially overcome the thermodynamic incompatibility between plant and dairy proteins, although the final functionality depends on the relative proportion of each protein in the system.

Similar to pH shifting, non-thermal technologies such as ultrasound, pulsed electric field, high-pressure processing, and cold plasma represent effective strategies to modify pea protein structure. These approaches have the potential to modulate the

formation and properties of hybrid gels, providing opportunities to optimize hybrid protein systems for tailored functionality.

Perspectives

Modulation of pea protein fractions structure by non-thermal technologies

Non-thermal technologies' effect on pea protein has been demonstrated by several articles. However, it is still unclear how each pea protein fraction can be modulated by the different non-thermal technologies. Understanding how the structure of each fraction can respond to non-thermal technologies can bring insights into how they can be better used in industry.

Hybrid gels formed with proteins modified by non-thermal technologies

Since non-thermal technologies can be used to modify protein structure, the interaction between proteins from two sources may increase, though modifying the hybrid gel characteristics, creating a new structure more similar to the conventional gel (animal-protein gel).

Supplementary material

Supplementary material

Multi-scale organizations and rheology of casein and pea protein mixed hydrogel formed by acidification: ratio and temperature impact

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Table S.1. Fits of the Bouchoux model to the SAXS data at 20 °C. The ratios means 100:0 – 100% casein and 80:20 – 80% casein and 20% pea protein

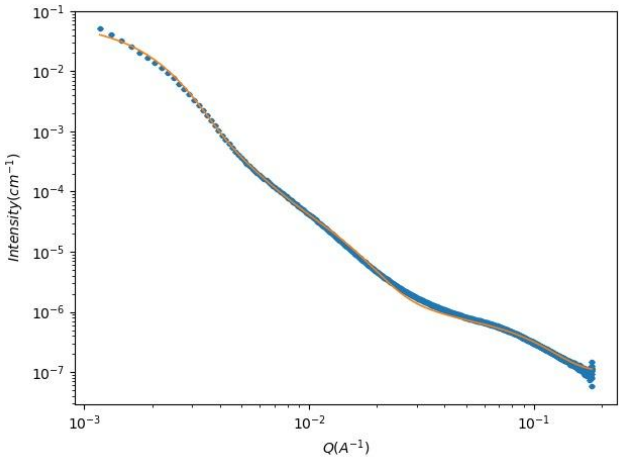
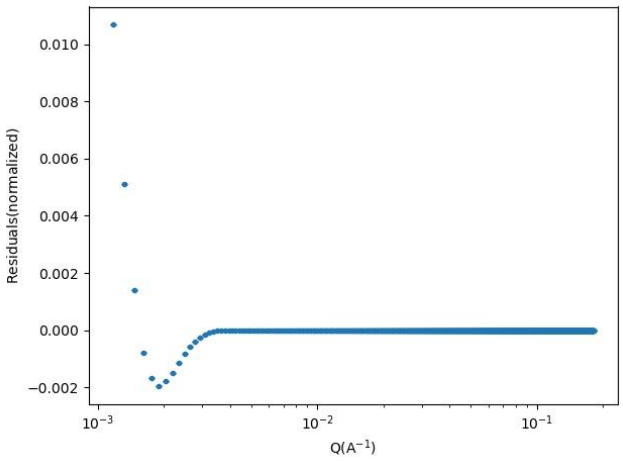
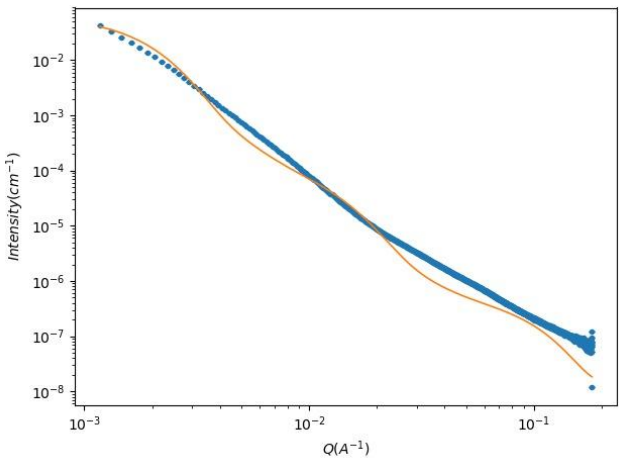
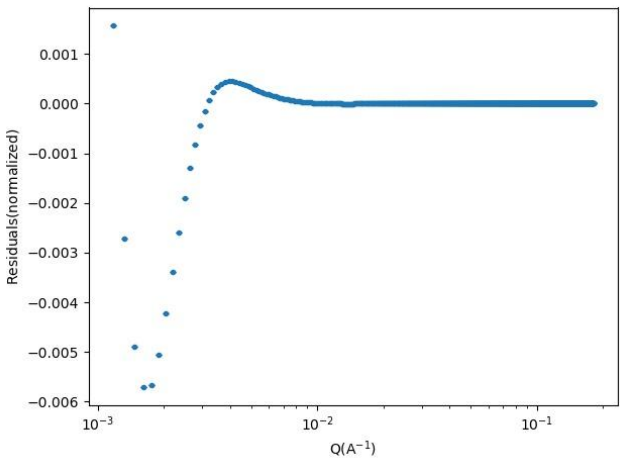
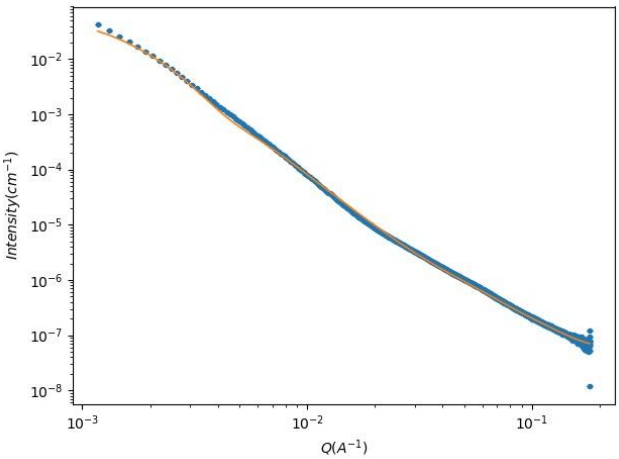
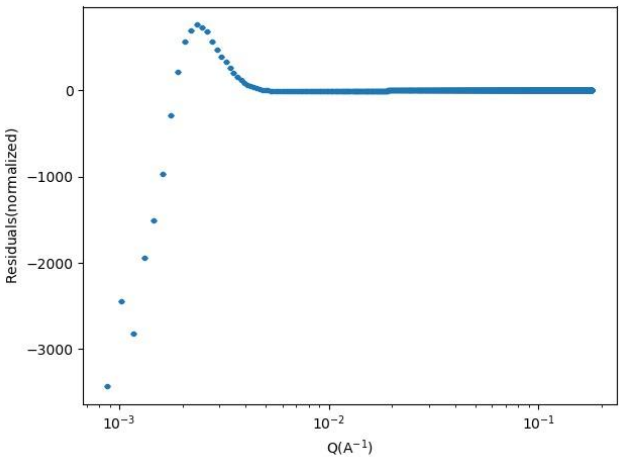
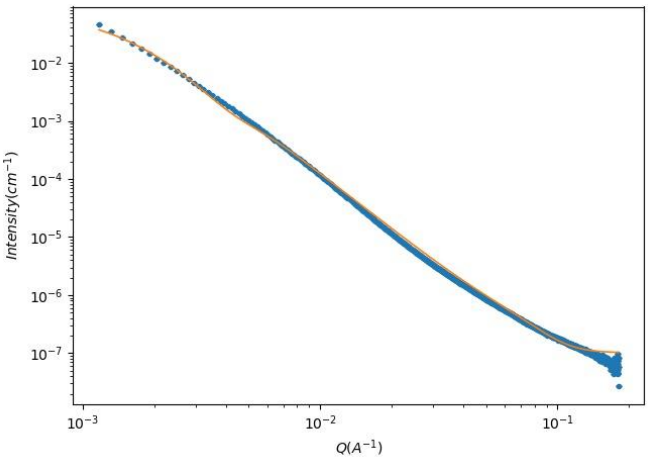
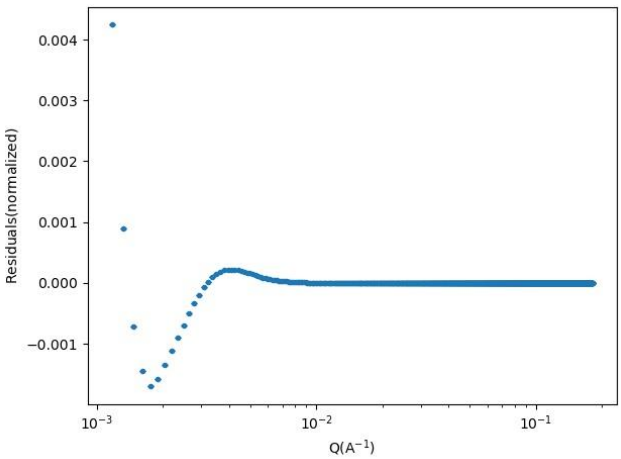
20 °C	Intensity (cm^{-1}) x Q (A^{-1})	Residual x Q (A^{-1})
100:0		
80:20		

Table S.2. Fits of the Unified model to the SAXS data at 20°C. The ratios means 80:20 – 80% casein and 20% pea protein, 20:80 – 20% casein and 80% pea protein and 0:100 – 100% pea protein.

20 °C	Intensity (cm^{-1}) x Q (A^{-1})	Residual x Q (A^{-1})
80:20		
20:80		
0:100