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ABSTRACT

This study aimed to investigate the suitability of the application of high-intensity ultrasounds (HIUS) to improve the acid induced gelation of mixed protein systems formed by casein micelles (CMs) and pea. The protein suspensions were prepared in different protein ratios CMs: pea (100:0, 80:20, 50:50, 20:80, 0:100) at 8% (w/w) total protein concentration. In the suspensions, the ultrasound treatment produced an increase in solubility, surface hydrophobicity, and a decrease in the samples' viscosity, with more remarkable differences in protein blends in which pea protein was the major component. However, the replacement of 20% of CMs for pea proteins highly affected the gel elasticity. Hence, the creation of smaller and more hydrophobic building blocks before acidification due to the HIUS treatment increased the elasticity of the gels up to 10 times. Therefore, high-intensity ultrasounds are a suitable green technique to increase the gelling properties of CMs: pea systems.

1. Introduction

The expected population growth of 2 billion people in the next 30 years will increase the worldwide demand for edible proteins (United Nations, 2015). Only for the proteins from animal sources such as milk and meat, the demand is expected to increase by 58 and 73%, respectively (Fasolin et al., 2019). However, the planet's limited resources combined with climate changes require more sustainable protein production. Thus, the wider utilization of proteins from vegetable origins such as soybean, lentils, chickpea, and pea can diversify protein production and make it more sustainable (Aiking & de Boer, 2020).

Pea is one of the largest legumes produced worldwide, in over 84 countries. Its annual production is estimated at 35 million meters tons, comprising 36% of total pulse production (Lu et al., 2020; Burger & Zhang, 2019). Pea presents lower protein content compared to soy; however, pea proteins stand out due to their non-allergenic status and a good balance of essential amino acids, being rich in lysine. The main

drawbacks of pea proteins are their reduced techno-functional properties (emulsifying, foaming, and gelling capability) and their beany flavor when compared to milk proteins (Ge et al., 2020).

Milk is worldwide produced and consumed. Besides the fluid milk, the formulation of different dairy products is possible due to milk proteins' techno-functional properties (Walstra et al., 2006). Milk presents an average of 3.2% of proteins, 80% caseins, and 20% serum proteins. There are four fractions of caseins, α-s1, α-s2, β, and κ that self-assemble in supramolecular structures called casein micelles (CMs) (Goulding, Fox, & O'Mahony, 2020). The gelation of milk is usually achieved by the destabilization of the κ-casein, the fraction that confers electrostatic and steric repulsion for the CMs. Thus, the fabrication of milk products such as yogurt and cheese depends on the gelling properties of CMs (Li & Zhao, 2019).

In this way, the creation of mixed systems, which can incorporate two complementary protein sources gained attention (Alves & Tavares, 2019). The combination of milk proteins with pea proteins can minimize

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the undesirable effects of pea proteins, and at the same time increase the versatility of dairy products (Guyomarc'h et al., 2021). However, a better comprehension of the protein behavior in these systems must be gained. The few studies focusing on the acidification of mixed CMs: pea protein resulted in gels with reduced stiffness when compared to pure systems (Oliveira et al., 2022; Ben-Harb et al., 2018). The reduction in the gel elasticity is caused by the competitive behavior of the proteins during the gelation, and frequently two distinct protein networks are formed (Roesch et al., 2004). Thus, the application of pre-treatments such as sonication, prior to gelation may be an alternative to improve the gelling properties of mixed systems.

Beghdadi et al. (Beghdadi et al. 2022) evaluated the effectiveness of heat as a pre-treatment, on the interaction between pea protein and casein micelles. The work stated that the heat treatment promoted higher hydrophobic interactions between casein and pea proteins which greatly affected the acid gelation functionalities of casein micelles. The mixture of pea and casein proteins led to the formation of gel networks with interesting stiffness and firmness when compared to the casein proteins alone. A previous work evaluated the transglutaminase-induced gelation of soy protein isolate (SPI) and wheat gluten (WG) using high intensity ultrasonic as pretreatment. Ultrasound was able to reduce the particle size of the mixture (SPI/WG) of proteins and improved the gel strength, water holding capacity, and storage modulus. The protein mixture of SPI and WG pretreated by ultrasound generated denser gels with a more homogeneous network of protein (Qin et al., 2016). Another study evaluated the gel properties of soybean-whey mixed protein (SPI-WPI) under microbial transglutaminase cross-linking, after ultrasound treatment. Ultrasound treatment reduced the SPI-WPI particle size and the mixing system was more uniform and stable. The gel hardness and water holding capacity of the SPI-WPI hybrid system reached the highest value after 30 min of sonication. The study suggested the promising application of ultrasound in order to induce protein interaction and significant improvements in gelling properties of mixed protein systems (Cui et al., 2020). Therefore, it is possible that ultrasonic treatment may have analogical function on pea protein mixed with casein micelles. This was the first time ultrasound was implemented in pea/casein system to improve the gelling properties which might bring new knowledge in the field.

The high-intensity ultrasound (HIUS) treatment consists of the application of acoustic waves in series of compression and rarefaction cycles with frequencies higher than 20 kHz. At sufficient energy input, the formation of small gas bubbles that eventually violently implode and generates a punctual increase in temperature, pressure, and shear forces (Chemat & Khan, 2011). In protein suspensions, HIUS usually impacts their tertiary and secondary structures, generating a decrease in the size of the aggregates, and an increase in the hydrophobicity and solubility, which impacts directly the techno-functional properties such as gelling, foaming, and emulsion stability (Gallo, Ferrara, & Naviglio, 2018). The use of HIUS shows promising results in increasing the gelling properties of a diversity of proteins such as sunflower, soy, chickpea, lentils, and pea (Bernardi et al., 2021).

Despite the promising results in pure suspensions, the effects of HIUS have not been studied for the acid induced gelation of CMs mixed with pea proteins. Thus, the aim of this study was to apply HIUS in mixed suspensions composed of CMs and pea proteins and evaluate the effect of the modifications in the improvement of the gelling properties.

2. Materials and methods

2.1. Materials

Casein micelle powder (Promilk 852B) containing 81% w/w protein, 7.5% ash, 5.5% lactose, and 1% fat was provided by Ingredia S.A (Arras, France). The pea protein powder (F85F) containing 85% protein, 5% ash, and 5% fibers was provided by Roquette (Lestrem, France). No additional purification step was applied to the protein powders. All the

other reagents used in this study were of analytical grade.

2.2. Sample preparation

The protein powders were separately rehydrated in deionized water at 8% (w/w) and stirred overnight at 25 °C. To prevent microbial growth, sodium azide was added at 0.03% (w/w). Then two routes of ultrasound processing were applied. In route 1, the pure protein dispersions were mixed in three ratios of casein micelles (CMs): pea protein (80:20, 50:50, 20:80). The blends were mixed for additional 2 h under the same stirring conditions used to rehydrate the powders. After that, the samples were submitted to high-intensity ultrasound treatment (HIUS) described in detail in section 2.3. In route 2, the CMs and pea protein suspensions were ultrasonicated individually, in the same conditions described in section 2.3, and then mixed in the three protein ratios 80:20, 50:50, and 20:80. The pure systems before and after ultrasound were also analyzed.

2.3. High-intensity ultrasound treatment (HIUS)

The samples (60 mL) were put in a beaker which was inserted in an ice bath to keep the sample's temperature always below 35℃, therefore, avoiding any temperature effects. HIUS treatment was performed using a sonifier apparatus operating at a constant frequency of 20 kHz (Emerson, St. Louis, MO, USA), using the same parameters as Kumar et al. (2022). Briefly, the ultrasound probe was inserted in the center of the beaker at 2 cm distance from its bottom. Then, 495 W of power was applied for 15 min in pulsed mode with 5 s ON and 5 s OFF. The real energy input was calculated based on the calorimetry method described by Arzeni et al. (2012). The samples' temperature in the first 30 s of ultrasound treatment was recorded using a thermocouple (Pico Technology, St Neots, UK). Then, Eq. (1) was used to determine the acoustic power (P) in Watts (W) applied in the sample, and Eq. (2) was applied to calculate the acoustic intensity (I) in W/cm². The power and intensities for all protein suspensions are shown in the supplemented material (Table 1 supplemented material).

$$P = mCp\frac{dT}{dt} \tag{1}$$

$$I = \frac{P}{Sa} \tag{2}$$

where m (g) is the mass of the treated suspension, Cp (J/g °C) is the specific heat of the suspension, dT/dt is the change in temperature as a function of time, and Sa (cm²) is the area of the emitting ultrasound surface.

2.4. Suspension analysis

2.4.1. Particle size and ζ- potential

Dynamic light scattering using a Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, UK) was used to determine particle size and ζ-potential of particles according to Nascimento et al. (2020). Briefly, the samples were diluted 100 times in deionized water and put in capillary cells (Malvern Instruments, Worcestershire, UK). The samples were allowed to equilibrate for 5 min and the analysis was recorded at 30 °C.

2.4.2. SDS-Page electrophoresis

SDS- Page electrophoresis in polyacrylamide gels was used to determine if the HIUS application would impact the primary structures of the proteins. A 12% polyacrylamide gel was formulated according to Queiroz et al. (2021). The gels were loaded with 10 μL of each sample, previously diluted in a buffer solution containing 125 mM Tris HCl (pH 6.8), 2.4% SDS, 50 mM DTT, 10% v/v glycerol, 0.5 mM EDTA, and bromophenol blue. The gels were placed in a Mighty Small (Hoefer) and

100 V was applied for the first 15 min of running, after, the voltage was increased to 150 V and kept until the lower band achieve the last 1:4 of the gel height. Then, the gels were dyed by immersion in a solution containing Coomassie brilliant blue for 4 h, followed by discoloration in ethanol: water solution. The gels were scanned and the images were analyzed using ImageJ software to calculate the protein band intensities.

2.4.3. Intrinsic fluorescence

The intrinsic fluorescence of tryptophan (Trp) in the samples was accessed using a SPECTRAmax GEMINI spectrophotometer (Molecular Devices, CA, USA) according to Yerramilli, Longmore, and Ghosh (2017). The samples were diluted in deionized water at 0.1 mg/mL and placed in a 96-well plate. The samples were excited at 280 nm wavelength and the emission was recorded between 340 and 400 nm wavelength.

2.4.4. Surface hydrophobicity (H_0)

The surface hydrophobicity (H_0) was determined using 1-anilino-8-naphthalenesulfonate (ANS) at 8 mM as described by Kumar et al. (2022) with slight modifications. In test tubes, the samples were diluted in four distinct concentrations (1, 0.75, 0.5, 0.25 mg.ml^{-1}) using deionized water as solvent. Then, 20 μL of ANS was added to 4 mL of each protein dilution. Then, the test tubes were vortex and kept in the dark for 15 min to allow reaction. After the reaction time, 200 μL were placed in a 96-well plate and put in a SPECTRAmax GEMINI spectrophotometer (Molecular Devices, CA, USA). The blanks were composed of deionized water with ANS and protein dilutions without ANS. The excitation wavelength was set to 390 nm, and the emission intensity at 468 nm was recorded. The fluorescence intensity results were plotted against the protein concentrations and a linear regression curve was calculated. The slope of the curve can be understood as the sample's surface hydrophobicity (H_0).

2.4.5. Flow properties

The samples were put in a stress-controlled rheometer (Discovery HR-2, TA Instruments, USA) equipped with a concentric cylinder geometry with the temperature set to 30℃. Then, a shear rate ramp varying from 10 to 320 s^{-1} was recorded to determine flow properties. To verify the presence of time-depend behavior (thixotropy), the experiment was conducted with three sweeps (up-down-up). The apparent viscosity at 60 s^{-1} was used to compare the samples since it is a shear rate value that correlates with the shear rate found in the mouth. The data of the third curve was fitted using the power law model (Eq. (3)) to determine the consistency index and the behavior index.

$$\sigma = k.y^n \tag{3}$$

where $\sigma(Pa)$ is the shear stress, k (Pa.s^n) is the consistency index, $y(s^{-1})$ is the shear rate, and n (dimensionless) is the behavior index.

2.4.6. Solubility

The solubility assay was performed according to Silventoinen and Sozer (2020), with slight modifications. The suspensions were centrifuged at 10,000 g for 15 min at 4℃. Then, the supernatant was withdrawn. The protein dispersion before centrifugation and the supernatant were analyzed for the nitrogen content using the Dumas. The solubility was calculated according to Eq. (4).

$$Solubility(\%) = \frac{Ns}{Nt} x100 \tag{4}$$

where *Ns* is the protein found in the supernatant and *Nt* is the total protein before centrifugation.

2.5. Gelling properties

2.5.1. Small amplitude oscillatory shear (SAOS) test

The gel formation was followed by SAOS test with the test parameters within the linear viscoelastic region (LVR). Glucono-δ-lactone (GDL) was added to the samples, followed by 1 min stirring to allow complete GDL solubilization. Then, the samples were placed in a stress-controlled rheometer (Discovery HR-2, TA Instruments, USA) equipped with a stainless steel parallel-plate geometry (40 mm diameter, with a 1 mm gap). Then, a time sweep test was performed for 5 h at 1 Hz frequency and 1 % of amplitude at 30℃. The edges of the geometry were covered with silicon oil to avoid water loss during the experiment. After the 5 h, without disturbing the formed gel, a frequency sweep test was performed by recording the elastic modulus (G') over a frequency (f) range varying from 0.1 to 50 Hz. The frequency dependence was determined by calculating the curve slope of the double logarithmic plot of elastic modulus against frequency (*d logG'/d logf*) (Klost, M., Brzeski, C., & Drusch, S., 2020). After that, a strain sweep was performed by varying the applied oscillation strain from 0.1 to 500 %.

In the conditions used in this experiment, the GDL was added in enough quantity to allow the samples to reach pH 4.6 at the same time (in 5 h). The first point in the rheograms occurred 5 min after GDL addition for all samples. The delay is due to the equilibration step performed before starting the measurements. The gelation time (Tgel) was defined as the time where G* reached 1 Pa.

2.5.2. Water holding capacity (WHC)

The WHC was determined according to Nascimento et al. (2020). The gels were allowed to form in 50 mL centrifuge tubes. The centrifugation was performed at 4000*g* at 4 °C for 10 min. The supernatant was carefully removed from the tube and weight. The percentage of the water entrapped in the gel was calculated according to Eq. (5).

$$WHC(\%) = \frac{mi - ms}{mi} x100 \tag{5}$$

where *mi* is the initial mass and *ms* is the supernatant mass.

2.5.3. Confocal laser scanner microscopy (CLSM)

CSLM was performed according to Andoyo, Guyomarc'h, Cauty, and Famelart (2014) with slight modifications. 0.2 g.kg^{-1} of rhodamine B isothiocyanate (RITC) was added to the samples to label the proteins (Sigma Sigma-Aldrich). The suspensions were stirred for 5 min at room temperature to ensure RITC solubilization. Then, the required amount of GDL was added to the samples and stirred for 1 min to allow GDL solubilization. Then, the samples were carefully placed into an 8- well chamber slide (Ibidi GmbH, Germany), which was placed in a water bath at 30℃ to acidify. After acidification, the samples were visualized using an inverted microscope (Nikon Ti2) equipped with a 100x/1.45 objective, a spinning disc module (CSU-W1, Yokogawa) and an sCMOS camera (Photometrics Prime95b). The sample was illuminated at 561 nm and imaged through a bandpass filter (600/50 nm).

2.6. Statistical analysis

The samples were compared by Analysis of variance (ANOVA). The effect of protein ratio was verified before and after HIUS treatment. Then, the effect of the HIUS application in each protein ratio was verified. When a significant difference (p < 0.05) was found, the Tukey HSD test with 5% significance was applied to differentiate means. All the experiments were performed, at least, two independent times, and the data was evaluated utilizing *SAS* software student edition.

3. Results and discussion

3.1. Particle size and ζ-potential

Before HIUS treatment, it was observed a distinct particle distribution comparing CMs dispersion and pea protein dispersion. The CMs dispersion was characterized by a unique population with the main hydrodynamic diameter of 206 ± 1.2 nm (Fig. 1A), while the pea protein dispersion presented two populations, the first peak at 157.0 ± 2 nm and the second at 973.5 ± 7.7 nm (Fig. 1B).

The observed particle distributions are due to pea protein aggregates, probably formed due to the harsh processing condition applied to extract the pea proteins from the bean (Tanger et al., 2020). The pea protein aggregates sizes can vary depending on the extraction method, and the values found here agree with the results found by who found aggregates as bigger as 1 μm.

For the protein blends, it was observed a gradual increase in the particle sizes of the dispersions when more CMs were substituted for pea protein (S1 supplemented material). However, at the ratio of 20:80 (CMs:pea) the particle size population mean was 680.4 ± 4.6 nm, which was higher than the mean for the pea protein alone (535.6 ± 12 nm). This increase is due to the increase of the second peak population, indicating that the presence of CMs in this particular ratio increases the

size of the larger pea aggregates (Fig. 1E).

After the HIUS treatment, the particle size decreased for all the studied suspensions, being the more remarkable difference in the ratios where the pea was the major protein component. Showing that the extent of the size reduction is directly related to the initial size of the particles. The CMs dispersion particle size decreased by around 21% compared to CMs before ultrasounds application, at the time that the 0:100 sample decreased to 68.9 %, and the 20:80 ratio to 76.61%. As can be seen in Fig. 1B, 1D, and 1E, the ultrasound treatment broke the larger aggregates in pea protein dispersion, making disappear the second particle peak. In addition, the 0:100 system presented a higher particle size mean than the 20:80 ratio, 157.1 ± 0.2 nm, and 175.1 ± 1.47 nm respectively, confirming that the previous aggregates were formed by interactions among the pea proteins. The breakdown of protein aggregates is probably due to the frontal and tangential collisions among them caused by the turbulence originating from HIUS application (Arzeni, Martínez, Zema, Arias, Pérez, & Pilosof, 2012). No significative differences were observed regarding the HIUS process routes.

The ζ-potential measures the resultant surface charge of the particles, which is an indication of suspension electrostatic stability (Larsson, Hill, & Duffy, 2012). All samples presented negative ζ-potential which means that the surface of the particle contained a higher amount of negatively charged amino acids than positively charged ones. The
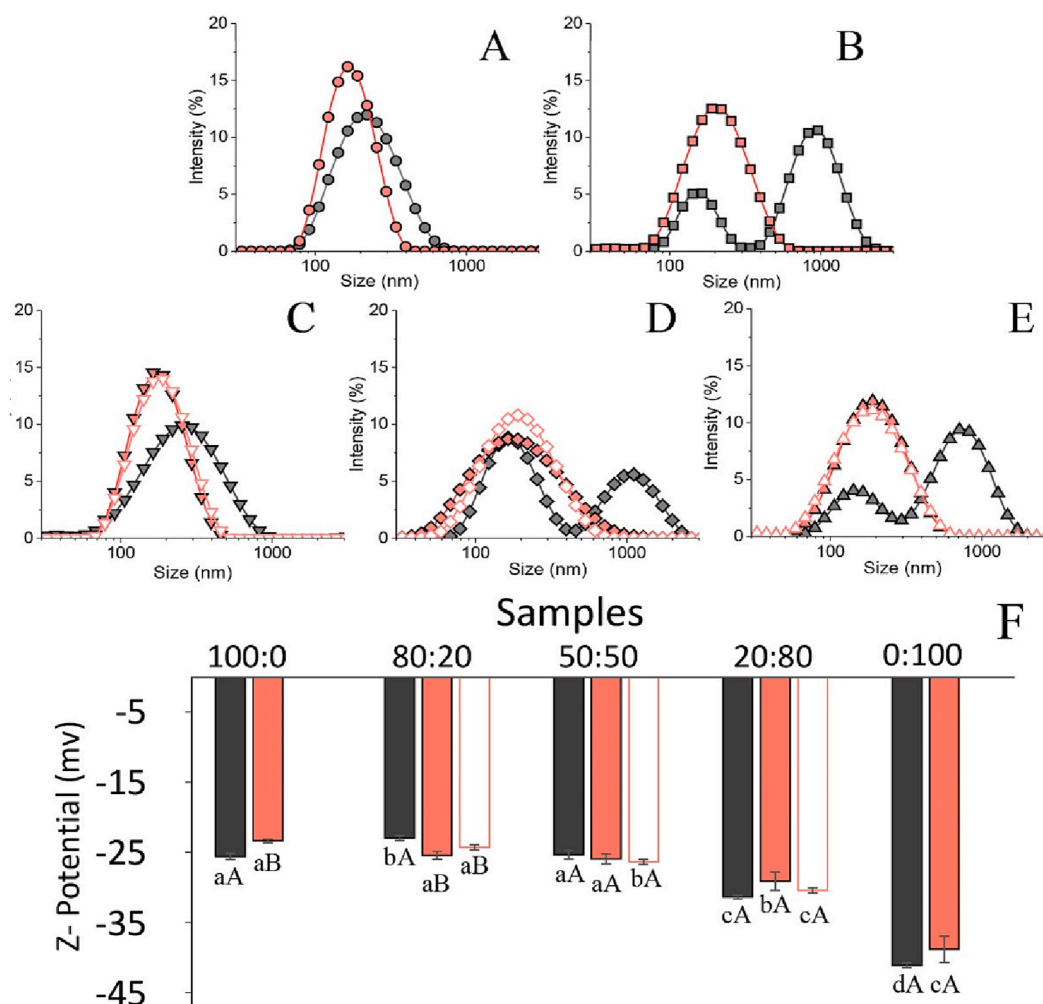
Fig. 1. Black symbols and columns (■) stand for samples before HIUS application, reddish full symbols and columns stands for samples after HIUS application. for route 1 (■), and empty reddish symbols and columns stands for samples after HIUS application. by route 2 (□). (A) Protein particle size distributions B- 100:0, C-0:100, D- 80:20, E- 50:50, F- 20:80. (B) ζ- potential of protein particles. Different lower-case letters mark a significant difference between the protein ratios in the same sonication stage. Different upper-case letters mark a significant difference between the HUIS treatments at the same protein ratio. The significance of the Tukey test was 5%.

results found for the pure CMs and pea are in agreement with other authors (Nascimento et al, 2020; Shevkani, Singh, Kaur, & Rana, 2015; Dalgleish, 2011). Comparing the effect of the ultrasound treatment in each ratio, it was observed that the blends 80:20 and 20:80 showed a significant difference (p < 0.05), which is an indication of specific protein interaction in these particular ratios. Nevertheless, after ultrasound treatment, ζ -potential slightly increased for the 80:20 mixture, and slightly decreased for the 20:80 mixture, this indicates clear effect of ultrasound on the protein structural conformation and surface composition which will change surface charge according to the exposed amino acid groups. Cheng and Cui (2021) also observed a decrease in the ζ -potential of pea proteins after sonication, with a higher decrease when more intense treatments were applied. The authors explained their observation based on the increase in the protein interactions, which would decrease the ζ -potential.

3.2. Electrophoresis

The HIUS treatment was responsible for the breakdown of protein aggregates, thus, the electrophoresis technique was employed to evaluate the impact of sonication on proteins' primary structure. The electrophorese results were plotted in terms of band intensity (S2 supplemented material). The pea proteins present several protein fractions including α- and β- legumin, α-, β- and γ – vicilin, and con-vicilin. The legumin fractions are associated by S—S bonds and appear in ~ 65 KDa in electrophorese analysis (Jiang et al., 2017), however, the use of 2-mercaptoethanol cleavages the disulfide bonds, for this reason, legumin- αβ cannot be observed in our results (Shand, Ya, Pietrasik, & Wanasundara, 2007). In the pure CMs systems, the four casein fractions present in the CMs appear in the electrophorese results. It was observed that the presence of a small amount of whey proteins in the studied gels, which remain in the powder after the purification process. Concerning the HIUS treatment, it was not observed the presence of new bands, nor the disappearance of previous bands. Similar results were found by Xiong et al. (2018) where even in higher HIUS intensities, no disruption in the amino acids backbone was observed. Despite the disappearance or formation of bands, it was noted an increase in the band's intensity after the HIUS application. The increased intensity is probably due to the disruption of protein aggregates and the increase in their solubility, which facilitated the entering of the protein in the polyacrylamide gel. Therefore, the HIUS applied did not cause modification in the primary structures of proteins, only disruption of the aggregates.

3.3. Intrinsic fluorescence

The intrinsic fluorescence intensity of all the samples increased with
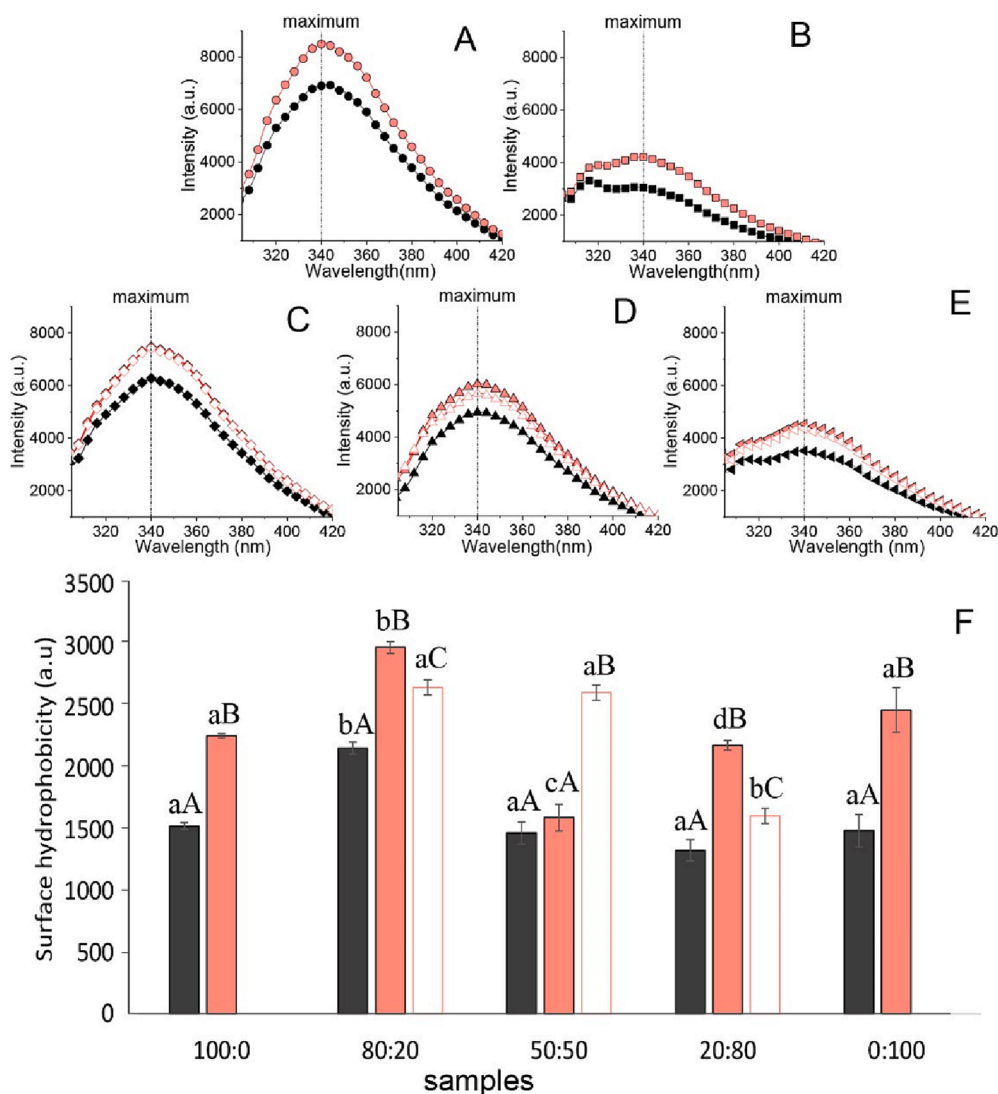
Fig. 2. Black symbols and columns (■) stand for samples before HIUS application, reddish full symbols and columns stands for samples after HIUS application. for route 1 (■), and empty reddish symbols and columns stands for samples after HIUS application. by route 2 (□). (A) Intrinsic fluorescence and (B) surface hydrophobicity of protein particles. Different lower-case letters mark a significant difference between the protein ratios in the same sonication stage. Different upper-case letters mark a significant difference between the HUIS treatments at the same protein ratio. The significance of the Tukey test was 5%.

the ultrasound application (Fig. 2A–E). The highest intensity was at 340 nm wavelength for all samples, without red or blue shifts. The applied wave-length excites mainly the Trp residue. Thus, the increase in fluorescence intensity can be explained by the modification of the Trp position in relation to the neighbor environment. It is known that the exposition of Trp to the solvent causes fluorescence quenching, which reduces the fluorescence intensity (Cheng & Cui, 2021). However, it was observed the opposite result. Thus, it is hypothesized that before ultrasound treatment, the Trp residue was buried inside the protein aggregate. After HIUS treatment, the aggregates were disrupted and the proteins suffered structural modifications which exposed more Trp to the solvent, but at the same time increased the distance from other quenching species, increasing the fluoresce intensity. Similar results were reported by Wang, Zhang, Xu, and Ma (2020), the authors observed an increase in fluorescence intensity in pea proteins extracted using HUIS, where the intensity increased with the prolonged sonication time until a maximum after 15 min of treatment. It is remarkable the difference in the fluorescence intensity for the systems composed mainly of CMs and pea proteins (Fig. 1A and B), which is explained by the quantity of Trp, while CMs present $\cong$ 1.4 %, pea proteins have $\cong$ 0.9 %. Thus, the differences in the intrinsic fluorescence among the protein ratios may come from a simple additive effect caused by unbalance of Trp content. Thus, the surface hydrophobicity was evaluated to have a more complete insight into the difference in the systems before and after HIUS application.

3.4. Surface hydrophobicity (H_0)

In H_0 analysis, a fluorophore (ANS) is added to the protein suspensions and it interacts with the hydrophobic regions of the molecules (Kato & Nakai, 1980). The HIUS treatment promoted an increase in the H_0 for all the studied ratios. The turbulence applied in the systems due to the cavitation disturbed the protein aggregates, breaking them and promoting the exposure of hydrophobic regions that was before buried (Xiong et al., 2018). Similar results were found by Wang et al. (2020), where the HIUS increased the H_0 of chickpea protein suspensions, with higher H_0 after 20 min HIUS processing.

Concerning the processing routes, the observed result depends on the studied protein ratio. In unbalanced dispersions, *i.e.*, when the amount of protein source was much higher than the other (80:20 or 20:80) route 1 presented higher H_0 than route 2 (Fig. 2F). The inverse was observed in protein ratios of equal amounts of both proteins (50:50), the route 2 producing slightly higher H_0. It is hypothesized that when a small amount of one protein is dispersed in a higher amount of another, better homogeneity and particle distribution are achieved, and some protein association can arise (Krentz et al., 2022). In addition, the turbulence caused by cavitation may turn easier the CMs- pea interactions, and some of the pea protein, mainly the vicilin fraction, may be entrapped in the CMs structure (Krentz et al., 2022). In the 50:50 ratio, the thermodynamic incompatibility of the proteins generates a stronger protein separation, and the HIUS imputed energy increased it.

3.5. Flow properties and solubility

The consistent index and behavior index was calculated by regression using the power law model and showed r^2 superior to 0.99 for all studied samples (supplemented material S3). Observing the effect of the protein ratio before the HIUS treatment, the viscosity values increased with the increase of pea protein, being the dispersion of 0:100 presenting the highest value (Fig. 3A). The same tendency was observed by Oliveira et al. (2022) in mixed milk: pea systems at concentrations higher than 7% (v/v). The authors argued that the presence of insoluble protein aggregates may be responsible for the increased viscosity. With respect to the replacement of more CMs for pea proteins, it is interesting to note that viscosity values did not change significantly (p > 0.05), comparing 100:0 and 50:50 samples. Comparing the effect of HIUS for
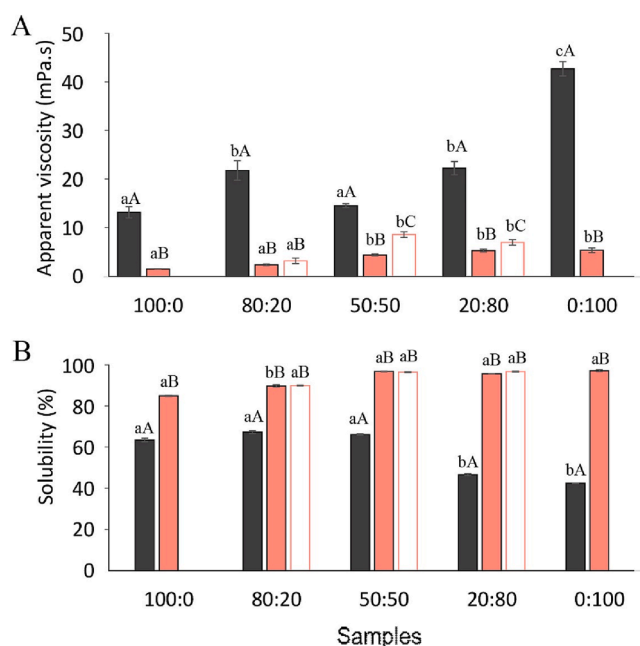

Fig. 3. (A) Apparent viscosity. (B) solubility of protein suspensions. (■) before HIUS application, HIUS application in route 1 (■) and route 2 (□). Different lower-case letters mark a significant difference between the protein ratios in the same sonication stage. Different upper-case letters mark a significant difference between the HUIS treatments at the same protein ratio. The significance of the Tukey test was 5%.

each protein ratio, it was observed a significant decrease in the viscosity (p < 0.05), for all samples. However, the effect of different processing routes was observed only for 50:50 and 20:80 ratios, where route 1 led to lower viscosity values than route 2. The same reduction in viscosity after HUIS treatment was observed by O'Sullivan et al. (2016) (O'sullivan et al., 2016) when evaluating four different animal and plant protein suspensions. In protein suspension, the size and surface properties of the particle play an important role in the final suspension viscosity (Kornet et al., 2020). The larger differences in the viscosity values among the protein ratios before HIUS application may be due to the differences in the size of the protein particles in the systems. In fact, the decrease of the hydrodynamic diameter (as observed in section 3.1) is related to a reduction in the volume fraction of proteins in the suspensions, and consequently, the viscosity is also reduced (McPhie, Daivis, & Snook, 2006).

The changes in the solubility of the samples are shown in Fig. 3B. Initially, the CMs presented solubility around 64.0 ± 0.9 %, which was more soluble than pea protein (42.8 ± 0.6 %). The low solubility of pea proteins is an industrial challenge that compromises their application in more food products (Alves & Tavares, 2019). Regarding the solubility of the protein mixtures before ultrasound treatment, it is interesting to note that the solubility did not change with the addition of pea protein until it became the major protein present. After the ultrasound treatment, the solubility increased until reaching 85.6 ± 0.2 % for pure CMs dispersions and 97.5 ± 0.4 % for systems formed solely by pea proteins (Fig. 3B). An increase in solubility of rapeseed proteins was also reported by Li et al. (2020), where sonication increased almost 6 times their solubility. The cavitation disrupts the insoluble protein aggregates, which after dissociation, re-aggregates forming soluble ones, also the formation of soluble complex between pea and fibers present in the initial pea protein powder may contribute to the increase in solubility as observed by Gao, Rao, and Chen, 2022. The HIUS was also efficient in increasing protein solubility in mixed systems, the 50:50, and 20:80 samples presented comparable solubility to 0:100 after HIUS application (p < 0.05).

Thus, the modifications caused by HIUS treatment are a tool to increase protein solubility in mixed systems, however, no differences were detected concerning the processing routes. The modification in the proteins also can impact direct the gelling, emulsion, and foam properties of these proteins. Thus, the gelling properties were studied to understand the effect of the HIUS application.

3.6. Gelling properties

3.6.1. Gel formation

The gel formation after GDL addition of the pure systems before and after ultrasound treatment is shown in Fig. 4A. The first minutes of acidification were marked by a fast increase in G* for both pure systems. After 55 min, the pure CMs reached a maximum G*, however, in the next minutes of acidification, the G* decreased and only started to increase again in minute 155. This phenomenon can be also observed in terms of loss tangent (inserted graph), in which the peak took place in minute 155 after acidification. The observed decrease in G*, as well as the increase in loss tangent, can be explained by the solubilization of calcium phosphate (Andoyo et al., 2014). The CMs are supramolecular structures formed by four protein fractions. These fractions interact mainly by hydrophobic interaction and nanoclusters of calcium phosphate (Walstra et al., 2006). When the pH decreases, the calcium phosphate increases its solubility, causing the weakening of casein fractions interactions, which is noted by the decrease in G*. After, the pH kept decreasing, and the degree of protein interactions increased, which caused the re-increasing in G*. For pure systems after sonication, a decrease in gelation time (Tgel) occurred from 21.67 to 18.56 min for the 100:0 sample and from 16.53 min to 13.88 min for the 0:100 sample. Arzeni et al. (2012) also observed a decrease in the Tgel of whey protein

systems treated with HIUS. The faster tridimensional network formation may be related to the higher surface hydrophobicity of the proteins, which increased the formation of hydrophobic interaction among them. The HIUS treatment increased the final G* for the CMs gel by more than 10 times and reduced the decrease in the G* during the protein rearrangement period. Chandrapala, Zisu, Kentish, and Ashokkumar (2013) demonstrated the HIUS also increased the strength of CMs gels. However, Chandrapala et al. (2014) showed that in natural pH conditions, the HIUS treatment only disrupts the protein aggregates and does not interfere with the integrity of the CMs. The pure pea system (0:100) also presented a step increase in the G* in the first minutes of acidification, however, the G* reaches a stable value around 105 min without any strong changes, only with protein network reinforcement, as can be also visualized in the constant decrease in loss tangent values (inserted graph). The HIUS application also increased the final G* for pure pea systems probably due to the decrease in the protein particle sizes and the increase in their surface hydrophobicity (Figs. S1 and 2F), which created a more homogeneous and interconnected network.

It was observed an effect caused by the protein ratios before the HIUS application (Fig. 4B, C, D). The sample 80:20 (Fig. 4D) presented the highest Tgel (32.74 min), an increase in 11 min compared with the 100:0 sample. Thus, the replacement of 20% of CMs for pea proteins impacted the initial aggregation steps of the CMs. In another hand, Tgel decreased in 9.5 min, comparing pure pea protein gels (0:100) with 20:80. This behavior supports the observation made in section 3.2, where the presence of small amounts of CMs intensifies the attractive interactions among the pea proteins which increased the number of bigger aggregates at the begging of acidification, hence decreasing the Tgel in the 20:80 system. Thus, the pea protein seems to retard aggregation of CMs, while CMs seem to tune aggregation in pea systems. In
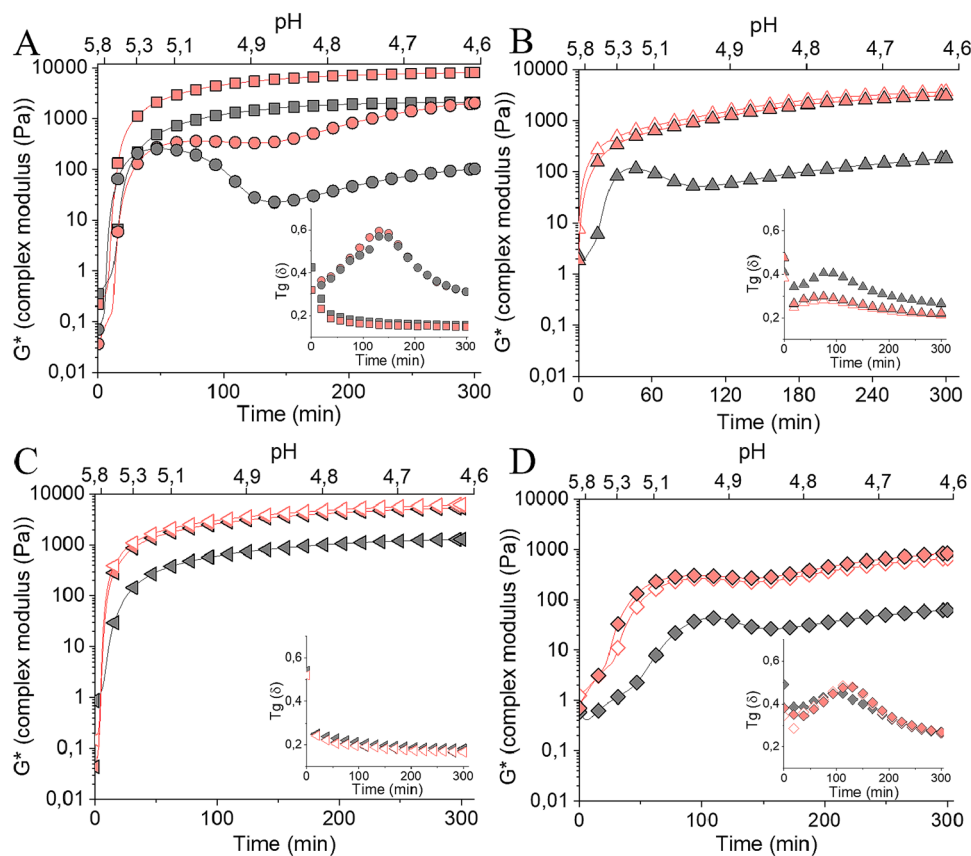
Fig. 4. Complex modulus G* as a function of time after addition of GDL. (A) pure CMs (●. ●) and pure pea protein (■. ■). (B) 50:50, (C) 20:80, and (D) 80:20. The inserted graphs are the loss tangent plots in the function of time. Black symbols stand for samples before HIUS application, reddish full symbols and columns stands for samples after HIUS application. for route 1, and empty reddish symbols and columns stands for samples after HIUS application. by route 2.

the 50:50 ratio, where the protein of different sources is balanced, the G* was higher than 1 Pa before 5 min, but it was observed a slower G* development compared to the 100:0 sample. Thus, even if the presence of pea proteins disturbs the CMs network formation, the concomitant formation of the pea protein network contra-balances this effect, resulting in a gel stronger than 100:0 gel, but weaker than 0:100 gel. Grygorczyk, Alexander & Corredig (2013) studied the formation of an acid gel composed of a combination of cow and soy milk, the authors found that the differences between aggregation times led to the formation of independent protein networks. The formation of independent networks also was observed by Silva et al. (2019) in thermal gels formed between CMs and different plant proteins.

During acidification, the gel formation curve of the 20:80 sample was similar to 0:100, with a slightly lower G* caused probably by the presence of CMs. However, in the 80:20 ratio, it was observed an antagonist effect in the final G*, which means that G* for 80:20 is lower than the lowest pure gel G* (100:0). As observed in Tgel, the presence of a small amount of pea seems to interfere strongly with the CMs gel formation. The CMs gel has probably a lower amount of junction zones compared to the 0:100 gel, for this reason, the presence of a different protein source caused a higher impact in Tgel and in the final G*.

The application of HUIS in the protein suspensions before the acidification increased the final G* for all studied protein blends, with the more remarkable change observed in the 50:50 ratio. It was observed an increase proximately 4, 13, and 20 times in the final G* for the samples 20:80, 80:20, and 50:50 respectively, showing the efficiency of the sonication in improving the gelling properties of mixed protein systems. Before HUIS treatment, the 50:50 sample acidification curve was similar to the pure CMs system, showing a pronounced decrease in G* around pH 5.0. However, after sonication, the curves are closer to those observed in pea pure systems with a lower reduction in G* during the calcium solubilization. This difference can be confirmed by observing the loss tangent plot (inserted graph), where was observed a smaller peak in the loss tangent values for the samples treated with HIUS. It can be explained by the reduction of the protein aggregates and the increase in their surface hydrophobicity before gel formation, which could increase the interaction between the pea proteins during acidification. Thus, the modifications in CMs internal structure were less remarked.

3.6.2. Frequency dependence and amplitude sweep

The pure pea gel (0:100) presented the lowest frequency dependence and the pure CMs gel (100:0) the highest (Fig. 5A). These results agree with those discussed in section 3.7.1, where it was concluded that the interactions formed in CMs gels were more sensible to disturbing compared to the interactions in 0:100. In general, it was observed a decrease in frequency dependence when more pea protein was added to the samples, except for the 80:20 blend. These results corroborate with gel formation data, discussed in section 3.7.1. The presence of a different protein source disturbs the network formation of CMs and 20% of pea protein is not enough to establish a strong network by itself. It was also noticed that the presence of a small amount of CMs in the pea protein gel also impacted its frequency dependence, but it did not decrease severally the gel elasticity once a higher degree of disturbance is necessary to change the pea gel rheological properties, probably due to its higher connected network.

Amplitude sweep tests were performed to characterize the gels after their complete formation. The region where the G' of the samples shows non-significant deviation from a constant value is denoted as the linear viscoelasticity region (LVR) (Tunick, 2011). In LVR, the applied strain is not strong enough to break irreversibly the bonds within the gel structure (Tunick, 2011). Thus, the end of LVR can be used as a structural parameter, where a stronger gel presents a larger LVR (Bong and Moraru, 2014). The application of HIUS increased the size of the LVR region for all the studied samples (Fig. 5B1–B4). Despite the pea gels being less frequency-dependent and possessing a more interconnected network, the pure CMs gel presented a higher LVR (end in 35% deformation) than
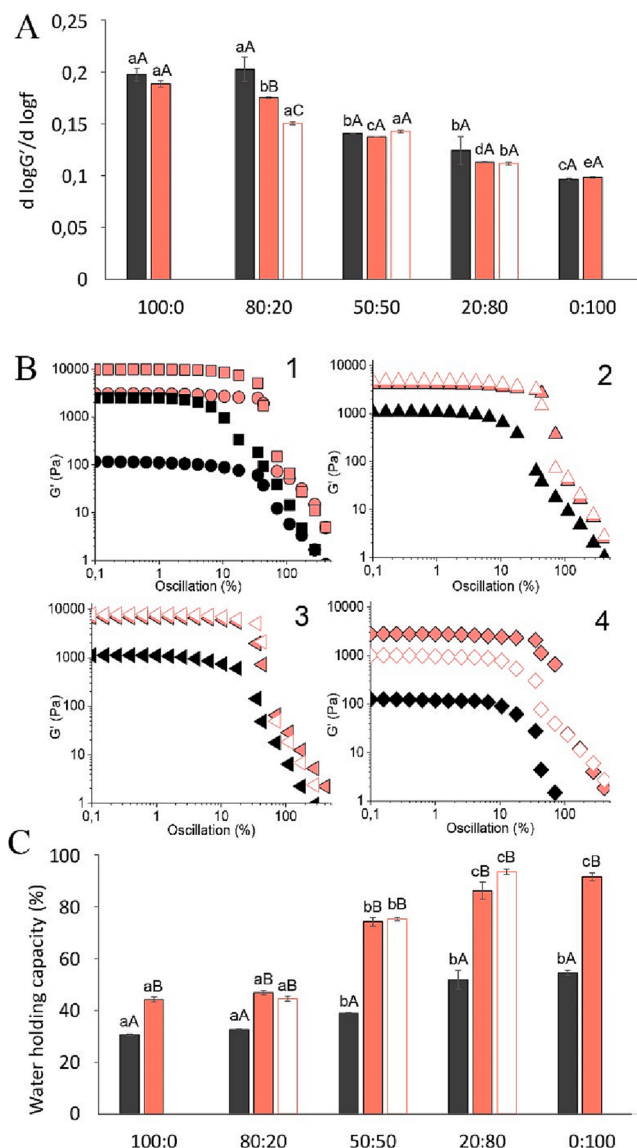
Fig. 5. Black symbols and columns (■) stand for samples before HIUS application, reddish full symbols and columns stands for samples after HIUS application. for route 1 (■), and empty reddish symbols and columns stands for samples after HIUS application. by route 2 (□). (A) Frequency dependence of the gels. (B) Strain sweep test of protein gel: B1- pure CMs (●, ●) and pure pea protein (■, ■), B2- 50:50, B3- 20:80, B4- 80:20, and (C) water holding capacity of the gels. Different lower-case letters mark a significant difference between the protein ratios in the same sonication stage. Different upper-case letters mark a significant difference between the HUIS treatments at the same protein ratio. The significance of the Tukey test was 5%.

pure pea gels (end in 10% deformation). Thus, despite the more sensible aggregation, the CMs gels are more resistant to breaking when a stress is applied than pea gels, probably because the lower junction zones in CMs are stronger. Thus, the increase in pea protein content in the samples narrowed the LVR compared with the 100:0 sample. However, the decrease in LVR was not linear, once it was at 24, 33, and 13% deformation for samples 80:20, 50:50, and 20:80 respectively. As observed during the gel formation, the small amount of pea protein disturbs the initial aggregation of the CMs, which interferes in the Tgel and final G*, thus, the size of the LVR was also affected. In the 20:80 ratio, the amount of CMs is very small and gel behavior is close to the 0:100 sample. However, at the 50:50 sample the decrease in LVR was small compared to the 100:0 gel, showing that this specific ratio allows the formation of a

stable pea and CMs network. The HIUS treatment did not have a substantial effect on the sample frequency dependence, however, it increased the LVR for all samples, which indicates a reinforcement of the protein network, but with the same kind of intermolecular interactions.

3.6.3. Water holding capacity (WHC)

The WHC before the HIUS application increased when more pea protein was present in the samples, once the pea proteins presented a higher WHC as can be verified by comparing the WHC of both pure systems (Fig. 5C). The WHC of all samples increased significantly after the application of HIUS, in a higher degree in the samples containing more pea proteins. The results found in the WHC of the gels are directly linked to the increase in protein solubility before gel formation. It was noticed that despite the higher WHC of pea proteins, the 80:20 mixed gel did not show a significant increase in the WHC compared to 100:0, reinforcing the finds that the small amount of pea is not able to contrabalance the disturbing effect caused in CMs network. It shows the high potential of HUIS treatment in conferring desirable features to mixed systems, once the ability to retain water inside the gel network, avoiding syneresis, is one of the stability problems found in yogurt.

3.6.4. Confocal laser scanner microscopy (CLSM)

The gel images obtained by CLSM are shown in Fig. 6. Before the HIUS treatment, it is noted the presence of large protein aggregates and open and low connected structures in both pure CMs and pea gels. The same gel aspect was observed for the samples 80:20 and 20:80. Interestingly, the 50:50 gel presented a more homogeneous structure with smaller protein aggregates compared to all other samples before HIUS, showing that the presence of both proteins in the same amount led to better protein distribution by breaking down the previous big aggregates. It was observed a remarkable change in the gel microstructure after applying sonication before suspension gelation. The CMs gels after HUIS were more homogeneous and without the presence of large protein aggregates. The same was observed for pure pea protein gels, where it is visualized as a highly connected network. The results of CLSM aggress with the rheological results, where the pea gels showed higher final G* and lower frequency dependence. It can be noted that the 80:20 gel presents a coarser structure compared to 100:0, which also aggress with the rheological and WHC results. The 20:80 gel microstructure is similar to 0:100, but presents a slightly bigger pore size, showing that the CMs also disturb the pea protein aggregation, but the results are less pronounced in the rheological analysis since the network still rests highly connected. The 50:50 gel presents a structure similar to pure CMs, which aggress with the strain sweep results. It shows that in this specific ratio, the formation of an independent network occurs, but both proteins are in sufficient amounts to form stable networks. No visual differences were observed in the gel's microstructure concerning the process routes.

4. Conclusion

The HIUS breaks down the protein aggregates and increases their solubility and surface hydrophobicity without changing particle charges, which decreases the suspension's viscosities. This knowledge can be easily transferred to application in the production of protein beverages that can possess higher protein content with lower viscosity. At the same time, the protein modifications caused by HIUS decrease the suspension's viscosities, while increased the elasticity of all studied gels. Showing that the previously weak gels formed by the mixed systems can be more elastic after HIUS application. As a practical application, higher elastic acid gels can be produced using the same amount of protein. The process routes showed slight differences in the suspension analysis. However, it did not interfere with the gel rheological properties, showing that any of the routes are suitable for the application. Nevertheless, the variation of HIUS parameters such as processing time, wave frequency, input energy, and pulse time can be employed to optime the gel rheological properties.
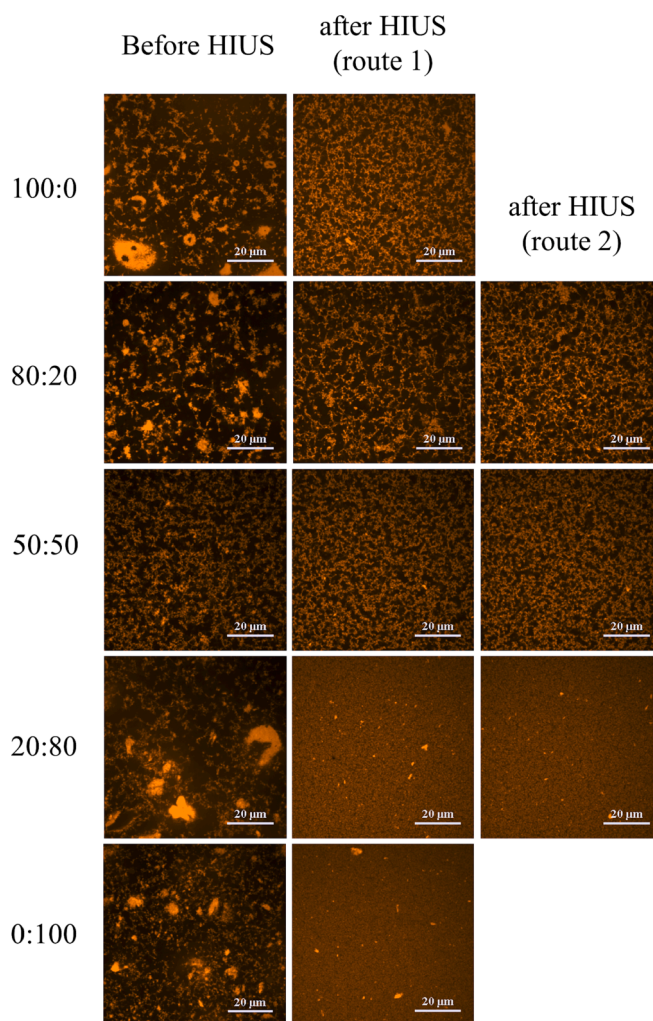

Fig. 6. CLSM images of protein gels.

CRediT authorship contribution statement

Luis Gustavo Lima Nascimento: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing. **Lucas Sales Queiroz:** Conceptualization, Methodology, Validation, Writing – review & editing. **Heidi Olander Petersen:** Formal analysis. **Rodolphe Marie:** Formal analysis. **Naaman Francisco Nogueira Silva:** Writing – review & editing. **Mohammed Amin Mohammadifar:** Writing – review & editing. **Paulo Peres de Sá Peixoto Júnior:** Writing – review & editing. **Guillaume Delaplace:** Writing – review & editing. **Antônio Fernandes de Carvalho:** Supervision, Project administration. **Federico Casanova:** Supervision, Project administration.

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.

Data availability

No data was used for the research described in the article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.136178.

References

Aiking, H., & de Boer, J. (2020). The next protein transition. *Trends in Food Science & Technology, 105*, 515–522.

Alves, A. C., & Tavares, G. M. (2019). Mixing animal and plant proteins: Is this a way to improve protein techno-functionalities? *Food Hydrocolloids, 97*, Article 105171.

Andoyo, R., Guyomarc'h, F., Cauty, C., & Famelart, M. H. (2014). Model mixtures evidence the respective roles of whey protein particles and casein micelles during acid gelation. *Food Hydrocolloids, 37*, 203–212.

Arzeni, C., Martínez, K., Zema, P., Arias, A., Pérez, O. E., & Pilosof, A. M. R. (2012). Comparative study of high intensity ultrasound effects on food proteins functionality. *Journal of Food Engineering, 108*(3), 463–472.

Beghdadi, A., Picart-Palmade, L., Cunault, C., Marchesseau, S., & Chevalier-Lucia, D. (2022). Impact of two thermal processing routes on protein interactions and acid gelation properties of casein micelle-pea protein mixture compared to casein micelle-whey protein one. *Food Research International, 155*, Article 111060.

Ben-Harb, S., Panouille, M., Huc-Mathis, D., Moulin, G., Saint-Eve, A., Irlinger, F., … Souchon, I. (2018). The rheological and microstructural properties of pea, milk, mixed pea/milk gels and gelled emulsions designed by thermal, acid, and enzyme treatments. *Food Hydrocolloids, 77*, 75–84.

Bernardi, S., Lupatini-Menegotto, A. L., Kalschne, D. L., Moraes Flores, É. L., Bittencourt, P. R. S., Colla, E., & Canan, C. (2021). Ultrasound: A suitable technology to improve the extraction and techno-functional properties of vegetable food proteins. *Plant Foods for Human Nutrition, 76*(1), 1–11.

Bong, D. D., & Moraru, C. I. (2014). Use of micellar casein concentrate for Greek-style yogurt manufacturing: Effects on processing and product properties. *Journal of Dairy Science, 97*(3), 1259–1269.

Burger, T. G., & Zhang, Y. (2019). Recent progress in the utilization of pea protein as an emulsifier for food applications. *Trends in Food Science & Technology, 86*, 25–33.

Chandrapala, J., Zisu, B., Kentish, S., & Ashokkumar, M. (2013). Influence of ultrasound on chemically induced gelation of micellar casein systems. *Journal of dairy research, 80*(2), 138–143.

Chandrapala, J., Zisu, B., Palmer, M., Kentish, S. E., & Ashokkumar, M. (2014). Sonication of milk protein solutions prior to spray drying and the subsequent effects on powders during storage. *Journal of Food Engineering, 141*, 122–127.

Chemat, F., & Khan, M. K. (2011). Applications of ultrasound in food technology: Processing, preservation and extraction. *Ultrasonics Sonochemistry, 18*(4), 813–835.

Cheng, J., & Cui, L. (2021). Effects of high-intensity ultrasound on the structural, optical, mechanical and physicochemical properties of pea protein isolate-based edible film. *Ultrasonics Sonochemistry, 80*, Article 105809.

Cui, Qiang, Wang, Guorong, Gao, Da, Wang, Lin, Zhang, Anqi, Wang, Xibo, Xu, Ning, Jiang, Lianzhou. Improving the gel properties of transgenic microbial transglutaminase cross-linked soybean-whey mixed protein by ultrasonic pretreatment. Process Biochemistry,91, 104-112.

Dalgleish, D. G. (2011). On the structural models of bovine casein micelles—review and possible improvements. *Soft matter, 7*(6), 2265–2272.

Fasolin, L. H., Pereira, R. N., Pinheiro, A. C., Martins, J. T., Andrade, C. C. P., Ramos, O. L., & Vicente, A. A. (2019). Emergent food proteins–Towards sustainability, health and innovation. *Food Research International, 125*, Article 108586.

Gallo, M., Ferrara, L., & Naviglio, D. (2018). Application of ultrasound in food science and technology: A perspective. *Foods, 7*(10), 164.

Gao, K., Rao, J., & Chen, B. (2022). Unraveling the mechanism by which high intensity ultrasound improves the solubility of commercial pea protein isolates. *Food Hydrocolloids, 131*, Article 107823. https://doi.org/10.1016/j.foodhyd.2022.107823

Ge, J., Sun, C. X., Corke, H., Gul, K., Gan, R. Y., & Fang, Y. (2020). The health benefits, functional properties, modifications, and applications of pea (Pisum sativum L.) protein: Current status, challenges, and perspectives. *Comprehensive Reviews in Food Science and Food Safety, 19*(4), 1835–1876.

Goulding, D. A., Fox, P. F., & O'Mahony, J. A. (2020). Milk proteins: An overview. *Milk proteins*, 21–98.

Grygorczyk, A., Alexander, M., & Corredig, M. (2013). Combined acid-and rennet-induced gelation of a mixed soya milk–cow's milk system. *International journal of food science & technology, 48*(11), 2306–2314.

Guyomarc'h, F., Arvisenet, G., Bouhallab, S., Canon, F., Deutsch, S. M., Drigon, V., … Gagnaire, V. (2021). Mixing milk, egg and plant resources to obtain safe and tasty foods with environmental and health benefits. *Trends in Food Science & Technology, 108*, 119–132.

Jiang, S., Ding, J., Andrade, J., Rababah, T. M., Almajwal, A., Abulmeaty, M. M., & Feng, H. (2017). Modifying the physicochemical properties of pea protein by pH-

shifting and ultrasound combined treatments. *Ultrasonics Sonochemistry, 38*, 835–842.

Kato, A., & Nakai, S. (1980). Hydrophobicity determined by a fluorescence probe method and its correlation with surface properties of proteins. *Biochimica et biophysica acta (BBA)-Protein structure, 624*(1), 13–20.

Klost, M., Brzeski, C., & Drusch, S. (2020). Effect of protein aggregation on rheological properties of pea protein gels. *Food Hydrocolloids, 108*, Article 106036.

Kornet, C., Venema, P., Nijsse, J., van der Linden, E., van der Goot, A. J., & Meinders, M. (2020). Yellow pea aqueous fractionation increases the specific volume fraction and viscosity of its dispersions. *Food Hydrocolloids, 99*, Article 105332.

Krentz, A., García-Cano, I., Ortega-Anaya, J., & Jiménez-Flores, R. (2022). Use of casein micelles to improve the solubility of hydrophobic pea proteins in aqueous solutions via low-temperature homogenization. *Journal of Dairy Science, 105*(1), 22–31.

Kumar, S., Queiroz, L. S., Marie, R., Nascimento, L. G. L., Mohammadifar, M. A., de Carvalho, A. F., … Casanova, F. (2022). Gelling properties of black soldier fly (Hermetia illucens) larvae protein after ultrasound treatment. *Food Chemistry, 386*, Article 132826.

Larsson, M., Hill, A., & Duffy, J. (2012). Suspension stability; why particle size, zeta potential and rheology are important. *Annual Transactions of the Nordic Rheology Society, 20*(2012), 6.

Li, Q., & Zhao, Z. (2019). Acid and rennet-induced coagulation behavior of casein micelles with modified structure. *Food chemistry, 291*, 231–238.

Li, Y., Cheng, Y., Zhang, Z., Wang, Y., Mintah, B. K., Dabbour, M., … Ma, H. (2020). Modification of rapeseed protein by ultrasound-assisted pH shift treatment: Ultrasonic mode and frequency screening, changes in protein solubility and structural characteristics. *Ultrasonics Sonochemistry, 69*, Article 105240.

Lu, Z. X., He, J. F., Zhang, Y. C., & Bing, D. J. (2020). Composition, physicochemical properties of pea protein and its application in functional foods. *Critical Reviews in Food Science and Nutrition, 60*(15), 2593–2605.

McPhie, M. G., Daivis, P. J., & Snook, I. K. (2006). Viscosity of a binary mixture: Approach to the hydrodynamic limit. *Physical Review E, 74*(3), Article 031201.

Nascimento, L. G. L., Casanova, F., Silva, N. F. N., de Carvalho Teixeira, Á. V. N., Júnior, P. P. D. S. P., Vidigal, M. C. T. R., … de Carvalho, A. F. (2020). Use of a crosslinked casein micelle hydrogel as a carrier for jaboticaba (Myrciaria cauliflora) extract. *Food Hydrocolloids, 106*, Article 105872.

Oliveira, I. C., de Paula Ferreira, I. E., Casanova, F., Cavallieri, A. L. F., Lima Nascimento, L. G., de Carvalho, A. F., & Nogueira Silva, N. F. (2022). Colloidal and Acid Gelling Properties of Mixed Milk and Pea Protein Suspensions. *Foods, 11*(10), 1383.

O'sullivan, J., Murray, B., Flynn, C., & Norton, I. (2016). The effect of ultrasound treatment on the structural, physical and emulsifying properties of animal and vegetable proteins. *Food hydrocolloids, 53*, 141–154.

Queiroz, L. S., Regnard, M., Jessen, F., Mohammadifar, M. A., Sloth, J. J., Petersen, H. O., … Casanova, F. (2021). Physico-chemical and colloidal properties of protein extracted from black soldier fly (Hermetia illucens) larvae. *International Journal of Biological Macromolecules, 186*, 714–723.

Qin, Xin Sheng, Luo, Shui Zhong, Cai, Jing, Zhong, Xi Yang, Jiang, Shao Tong, Zhao, Yan Yan, Zheng, Zhi. Transglutaminase-induced gelation properties of soy protein isolate and wheat gluten mixtures with high intensity ultrasonic pretreatment. Ultrasonics Sonochemistry, 31, 590-597.

Roesch, R., Juneja, M., Monagle, C., & Corredig, M. (2004). Aggregation of soy/milk mixes during acidification. *Food Research International, 37*(3), 209–215.

Shand, P., Ya, H., Pietrasik, Z., & Wanasundara, P. (2007). Physicochemical and textural properties of heat-induced pea protein isolate gels. *Food Chemistry, 102*(4), 1119–1130. https://doi.org/10.1016/j.foodchem.2006.06.060

Shevkani, K., Singh, N., Kaur, A., & Rana, J. C. (2015). Structural and functional characterization of kidney bean and field pea protein isolates: A comparative study. *Food Hydrocolloids, 43*, 679–689.

Silva, J. V., Cochereau, R., Schmitt, C., Chassenieux, C., & Nicolai, T. (2019). Heat-induced gelation of mixtures of micellar caseins and plant proteins in aqueous solution. *Food research international, 116*, 1135–1143.

Silventoinen, P., & Sozer, N. (2020). Impact of ultrasound treatment and pH-shifting on physicochemical properties of protein-enriched barley fraction and barley protein isolate. *Foods, 9*(8), 1055.

Tanger, C., Engel, J., & Kulozik, U. (2020). Influence of extraction conditions on the conformational alteration of pea protein extracted from pea flour. *Food Hydrocolloids, 107*, Article 105949.

Tunick, M. H. (2011). Small-strain dynamic rheology of food protein networks. *Journal of Agricultural and Food Chemistry, 59*(5), 1481–1486.

Yerramilli, M., Longmore, N., & Ghosh, S. (2017). Improved stabilization of nanoemulsions by partial replacement of sodium caseinate with pea protein isolate. *Food Hydrocolloids, 64*, 99–111.

Wang, Y., Wang, Y., Li, K., Bai, Y., Li, B., & Xu, W. (2020). Effect of high intensity ultrasound on physicochemical, interfacial and gel properties of chickpea protein isolate. *Lwt, 129*, Article 109563.

Wang, F., Zhang, Y., Xu, L., & Ma, H. (2020). An efficient ultrasound-assisted extraction method of pea protein and its effect on protein functional properties and biological activities. *LWT, 127*, Article 109348.

Walstra, P., Wouters, J. T. M., & Geurts, T. J. (2006). Dairy Science and Technology (Second Edi., Vol. 4). Taylor & Francis. doi:9780824727635.

Xiong, T., Xiong, W., Ge, M., Xia, J., Li, B., & Chen, Y. (2018). Effect of high intensity ultrasound on structure and foaming properties of pea protein isolate. *Food Research International, 109*, 260–267.