



Physicochemical properties of high-pressure treated lentil protein-based nanoemulsions

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ARTICLE INFO

Keywords:

Nanoemulsion
Lentil protein
High-pressure homogenization
Particle size distribution
Interfacial tension

ABSTRACT

The emulsifying properties of pulse proteins offer the possibility to provide functionality to colloidal food systems like nanoemulsions. The objective was to obtain stable lentil nanoemulsions through High-Pressure Homogenization (HPH) (50–300 MPa) using different number of homogenization passes (1–3) and emulsifier:oil ratios (1:1, 1:2, 2:1). Oil-in-water nanoemulsions were characterized through particle size distribution, mean droplet size, polydispersity index (PDI), ζ -potential, interfacial tension (IT), stability against creaming, and flow behavior. HPH > 100 MPa changed particle size distribution of 1:1 lentil nanoemulsion from multimodal to bimodal, regardless of homogenization passes number. Two homogenization passes and ratios of 1:1 and 2:1 improved reduction of droplet size (587–149 nm) and PDI (0.821–0.168), with no difference between those ratios. ζ -potential values were lower than -30 mV, which can be considered as a stable emulsion. As emulsifiers, lentil proteins decreased IT of water-oil interphase from 16.5 to < 7.0 mN/m, regardless pressure treatment applied. Nanoemulsions showed pseudoplastic characteristics at all experimental conditions evaluated. HPH decreased η_{app} , and higher η_{app} were observed with 2:1 ratio. Stable lentil nanoemulsions can be obtained by using 1:1 emulsifier:oil ratio and HPH above 200 MPa with two homogenization passes, which could be used as novel food systems.

1. Introduction

Pulses are an important source of protein, dietary fiber, minerals and vitamins required for human health, especially in countries where animal proteins are not available or are limited by self-imposed habits (Boye, Zare, & Pletch, 2010). Pulse proteins are of special interest because of their nutritional value, availability, low cost and health benefits (Karaca, Low, & Nickerson, 2011). Among pulses, lentil (*Lens culinaris*) seems a promising protein because of its high protein content, between 20.6 and 31.4 g/100 g, being especially rich in essential amino acids like leucine and lysine (Jarpa-Parra et al., 2014). Also, lentil proteins have good functional properties, including foaming, emulsifying, fat/water binding and gelling properties, making them good candidates for its application to food emulsions (Toews & Wang, 2013). Oil-in-water (O/W) emulsions can be defined as systems comprised of oil and water in which oil is dispersed as droplets (the dispersed phase) throughout the water phase (the continuous or external phase). O/W emulsions are an important constituent of food, beverages, and drug delivery systems. As emulsions are in a thermodynamically unstable state, the addition of an emulsifier is critical to decreasing the droplet size as

the emulsifier decreases the interfacial tension between water and oil phases of the emulsion (Gupta, Eral, Hatton, & Doyle, 2016). In general, proteins are effective in stabilizing O/W emulsions due to their amphiphilic nature, being able to create an interfacial layer that prevents aggregation and reduces interfacial tension between the two phases during homogenization (Joshi et al., 2012). The effectiveness of proteins as emulsifiers depends on their ability to denature, migrate to the oil-water interface and create a film around the oil droplets (Boye et al., 2010). The most used proteins to form emulsions have been dairy-based proteins like casein and whey proteins, however, vegetable proteins, especially pulses, are becoming more attractive for their use in food applications.

For the production of food emulsions HPH is a commonly used method which provides good texture properties and greater stability. During homogenization, the combination of intense shear, cavitation, and turbulent flow disrupts the oil droplets, creating droplets of smaller size (Kuhn & Cunha, 2012). In the HPH, an increase in homogenization pressure and/or in the number of passes reduces the particle size of the emulsion, improving its stability, but at high pressures an increase in the number of passes can promote droplet coalescence (Kuhn & Cunha,

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<https://doi.org/10.1016/j.lwt.2018.11.070>

Received 12 September 2018; Received in revised form 16 November 2018; Accepted 21 November 2018

Available online 22 November 2018

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2012). HPH can also change the surface activity of some proteins, enhancing their emulsifying properties by improving the efficiency to form interfacial layers (bib_Lee_et_al_2009Lee, Lefèvre, Subirade, & Paquin, 2009). Each level of pressure applied could induce different changes in protein conformation. Pressure up to 300 MPa usually result in reversible partial denaturation while pressures over 500 MPa result in irreversible and extensive effects on proteins (Balny, Masson, & Travers, 1989).

Previous studies on lentil protein emulsions have used pressures up to 137 MPa (Primožic et al., 2017, 2018bib_Primozic_et_al_2017bib_Primozic_et_al_2018), but no further studies have been published with the application of higher pressures. As proteins are affected in different ways by pressure level, the objective of this work was to evaluate the effect of different treatments of HPH (50–300 MPa) with different number of passes (1, 2, and 3) and emulsifier:oil ratios (1:1, 1:2 and 2:1) on the stability and physicochemical properties of lentil nanoemulsions. Stability was evaluated using physical properties like particle size distribution (droplet size, polydispersity index), surface charge (ζ -potential), rheology behavior, interfacial tension, stability against creaming and molecular size distribution.

2. Materials and methods

2.1. Raw materials

Green lentils (*Lens culinaris* Medik) produced in Chile were provided by the *Instituto de Investigaciones Agropecuarias* Quilamapu (INIA- Chile). Lentils were harvested on 2017 and the moisture, protein, lipid, fiber and carbohydrate content were found as 12.5 ± 0.1 , 22.1 ± 0.8 , 2.4 ± 0.2 , 3.8 ± 0.8 , and 57.0 ± 1.1 g/100 g, respectively. The grains were milled using miller with an aperture size of 1 mm to obtain lentil flour. Lentil flour was packed in plastic bags and stored at 4 °C until its use. 100% soy oil with citric acid and TBHQ was purchased from the local market. All chemicals were analytical-grade.

2.2. Protein extraction

Protein extraction was performed according to a modified Jarpa-Parra et al. (2014) protocol. Lentil flour (400 g) was mixed in distilled water at 1:10 ratio and pH was adjusted to 9.0 using 1 mol/L NaOH. After extraction insoluble solids were precipitated by a centrifuge (4500 $\times$ g, 15 min, 20 °C). The supernatant was adjusted to pH 4.2, allowed to precipitate overnight at 4 °C and then the proteins were precipitated by centrifuge (4500 $\times$ g, 15 min, 20 °C). Precipitated proteins were freeze-dried and dry powder was stored in plastic bags at 4 °C until use. Protein concentration in the extract was determined by Dumas analysis with an NA 2100 Nitrogen and Protein Analyser (ThermoQuest-CE Instruments, Italy). Methionine was used as a standard during the analysis. All samples were measured in triplicate. A conversion factor of 6.25 was used to convert nitrogen values to protein content. The protein content of freeze-dried lentil protein extract was 74.9 g/100 g, indicating that other components like carbohydrates or minerals could be co-precipitated with proteins.

2.3. Preparation of lentil-protein solution

Lentil protein solution was prepared by dispersing lentil protein extract in ultrapure water at emulsifier:oil ratios of 1:2, 1:1, 2:1, which corresponds to protein concentrations of 2.5, 5.0, and 10 g/100 g. The pH solution was adjusted to 7.0 using NaOH 1 mol/L and the solution was kept overnight with magnetic stirring at room temperature to allow complete protein dissolution. This pH was selected to simulate food and beverage conditions that possess pH around neutral. To prevent microbiological spoilage, 0.02 g/100 g sodium azide was added to each emulsion prior homogenization steps.

2.4. Emulsion formulation

Oil-in-water emulsions were prepared at 20 °C using 5 g/100 g soy

oil as dispersed phase and lentil protein solution as continuous phase using two sequential homogenization methods. First, emulsions were pre-homogenized using a rotor-stator homogenizer (Ultra Turrax® T25 digital, IKA, Germany) at 10000 rpm for 5 min. Secondly, the pre-homogenized emulsion was subjected to homogenization in a high-pressure homogenizer (NG12500, Stansted Fluid Power Ltd., UK) connected to a water circulation unit at 1 °C (WiseCircu WCR-P8, Witeg, Germany). Pressures of 50, 100, 200, and 300 MPa were applied with 1, 2 and 3 passes through the homogenizer. For each composition, three separate batches of emulsions were prepared.

2.5. Droplet size, polydispersity index (PDI) and droplet charge (ζ -potential)

Average emulsion droplet size and ζ -potential of soy oil droplets in the emulsion were determined by dynamic light scattering with a Zetasizer Nano ZS laser diffractometer (Malvern Instruments Ltd., UK). The Zetasizer operates at 633 nm and is equipped with a backscatter detector (173) that is used to measure sub-micron particles. Emulsions were diluted 100 times with ultra-pure water to prevent the multiple-scattering effect. The dispersant phase refractive index was measured with a manual refractometer (RX-5000 CX, ATAGO, Japan), and dispersant phase absorbance was measured with a spectrophotometer (Spectroquant® Pharo 300, Merck, Germany). Droplet size measurements were performed immediately after preparing the emulsion at 15 °C; the average particle size was reported as z-average values. The PDI was also measured. To measure ζ -potential samples were diluted 100 times and placed in a disposable zeta cuvette (DTS1070); the electrophoretic mobility of the droplet was transformed into ζ -potential values using the Smoluchowski model with the instrument's software (Zeta Sizer software v.7.11). Three diluted samples from each batch of an emulsion of identical composition were used to determine their z-average, PDI, and ζ -potential.

2.6. Rheological behavior

Emulsion rheological behavior was analyzed at 15 °C using a controlled stress rheometer (Physica MCR300, Anton Paar, Germany). The instrument was fitted with parallel plate (50 mm diameter; smooth) geometry. Samples leak out onto the center of the base plate and the gap was set up to 0.4 mm between the two plates. The sample temperature was kept constant at 15 °C and internally controlled by a peltier plate system attached to a water circulation unit.

Flow curves of emulsions samples were measured by varying the shear rate from 0 to 1000 s⁻¹. Samples were changed before each measurement. The resulting data were fit to the Herschel-Bulkley and Ostwald de Waele models with the instrument's software (US200 Physica version 2.01, USA) to obtain the emulsion rheological parameters; consistency index (k), flow behavior index (n) and apparent viscosities (η_{app}). Seven repetitions for sample from each batch of emulsions with the same composition were done.

2.7. Interfacial tension

Interfacial tension was measured by the du Noüy ring method with an Attention Force Tensiometer (Sigma 700/701, Biolin Scientific AB, Sweden). Ten measurements were taken for sample from each bath of emulsions with identical composition and all measurements were made at 15 °C.

2.8. Protein profile (SDS-PAGE)

Native electrophoresis and Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions were used to evaluate the modification on lentil proteins with a Mini-PROTEAN® Tetra (BIORAD). Native electrophoresis was performed using pre-cast gels (4–20%, Mini-PROTEAN® TGX™, BIORAD) at 150 mV with the NativeMark Unstained Protein Standard, ThermoFisher Scientific. SDS-

PAGE was performed according to Laemmli (1970). Homogenized protein solutions were diluted to obtain a protein concentration equal to 20 mg mL^{-1} , and mixed with SDS sample loading buffer in 1:1 ratio. Samples were heated at 95°C during 5 min, cooled in ice, centrifuged ($1520 \times g$, 5 min) and loaded into a 12 g/100 mL polyacrylamide gel along with protein marker standard Precision Plus Protein™all Blue (BIORAD). Running conditions were 120 mV and 3 h using Tris-glycine running buffer. Gels were stained with Brilliant Blue R (0.25 g/100 mL Brilliant Blue R, 40 mL/dL methanol and 7 mL/dL acetic acid, Sigma-Aldrich) overnight and de-stained in destaining solution (50 mL/dL methanol, 10 mL/dL acetic acid, and 40 mL/dL deionized water) during 5 h.

2.9. Emulsion stability

Immediately after preparation, 10 mL of each emulsion were placed in transparent glass tubes (height 12 cm and internal diameter 1.5 cm) and sealed with plastic cap. The tubes were kept at room temperature and emulsions were photographed until destabilization. Additionally, 10 mL of each emulsion were placed in 15 mL centrifuge tubes to be centrifuged at 3500 g during 20 min at 15°C (Restu, Sampora, Meliana, & Haryono, 2015). Emulsion stability was evaluated according to the volume of cream on the top of the tube following the equation:

$$\text{Emulsion stability (\%)} = \frac{\text{cream volume}}{\text{total emulsion volume}} * 100$$

2.10. Statistical analysis

Results were analyzed by one-way analysis of variance (ANOVA) using OriginPro 2015 software (©OriginLab Corporation, USA) to determine significant differences among treatments. Significance testing was performed by Tukey's test; and the differences were taken to be statistically significant when the p -value was < 0.05 .

3. Results and discussion

3.1. Particle size distribution

Many emulsion characteristics like stability, rheology, appearance, color, and texture depend on droplet mean size and size distribution (McClements, 2015). Emulsions with particle size 20–500 nm are considered as nanoemulsions, which are more stable due to the lower droplet size which reduces creaming velocity as stated in Stokes' law so they are preferred over conventional macroemulsions (particle size $> 500 \text{ nm}$) (Gupta et al., 2016; Kuhn & Cunha, 2012). The effects of pressure and number of passes on particle size distribution of lentil emulsions are shown in Fig. 1. Unpressurized emulsion showed a multimodal distribution with peaks at 220, 2670 and 5500 nm. HPH changed the distribution of 1:1 emulsion from multimodal to bimodal regardless of pressure level and number of passes used, with peaks at 250 and 5500 nm. As homogenization pressure increased up to 200 MPa, the peak $> 1 \mu\text{m}$ decreased while the peak $< 1 \mu\text{m}$ increased in volume, furthermore pressure up to 300 MPa created a shoulder of smaller size, indicating a rupture of the bigger or aggregated droplets with the increasing pressure. Probably these multimodal and bimodal distributions are originated by the aggregation of proteins and the flocculation of oil droplets which creates particles of size $> 1 \mu\text{m}$ (Puppo et al., 2005). The change in particle size distribution could also be observed through the mean droplet size (Fig. 2a) and polydispersity index (Pdl) (Fig. 2b), which is an estimate of the width distribution, where HPH significantly decreased both parameters as the pressure increased. Homogenization at 50 MPa reduced the initial droplet size of $607 \pm 86 \text{ nm}$ to $350 \pm 24 \text{ nm}$, and a pressure dependent reduction effect was observed as pressures of 100, 200 and 300 MPa produced emulsions with a mean size of 271 ± 25 , 236 ± 25 and $186 \pm 12 \text{ nm}$, respectively. In terms of Pdl, the increase of pressure produced a significant decrease at 50, 100, and 200 MPa, but there was

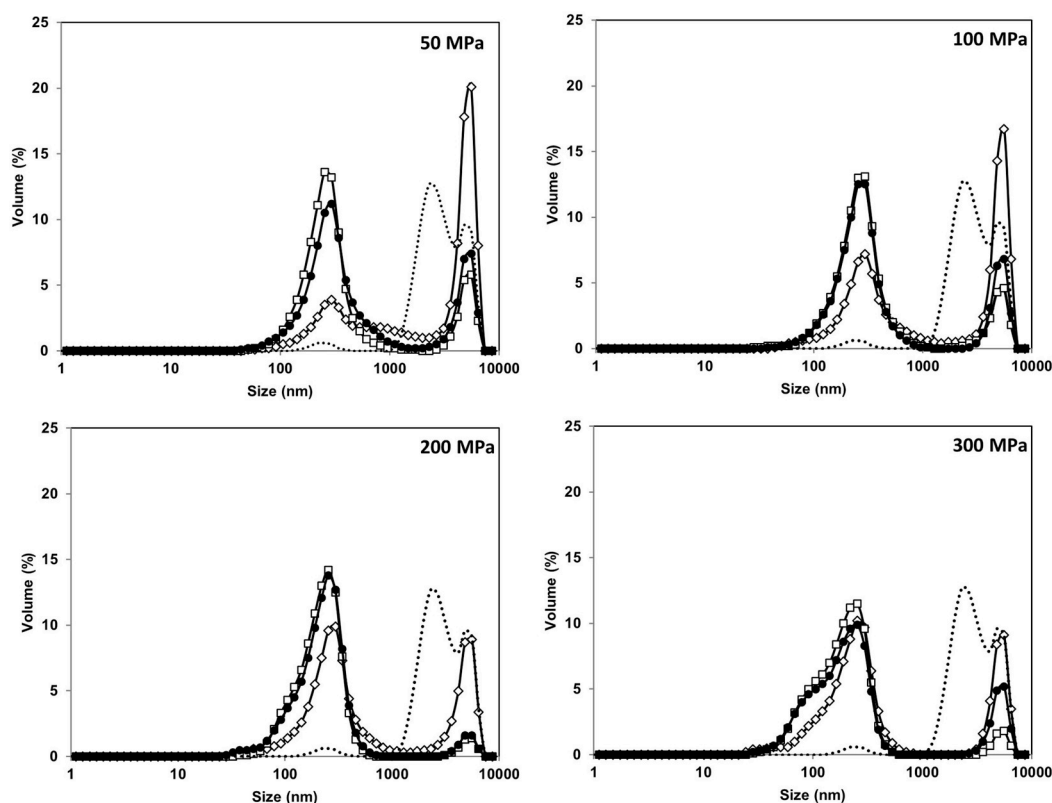


Fig. 1. Effect of number of passes on particle size distribution of lentil nanoemulsions with emulsifier:oil ratio of 1:1 homogenized at different pressures. (---) Control 0 MPa; ($\diamond$) 1 homogenization pass; ($\square$) 2 homogenization passes; ($\bullet$) 3 homogenization passes.

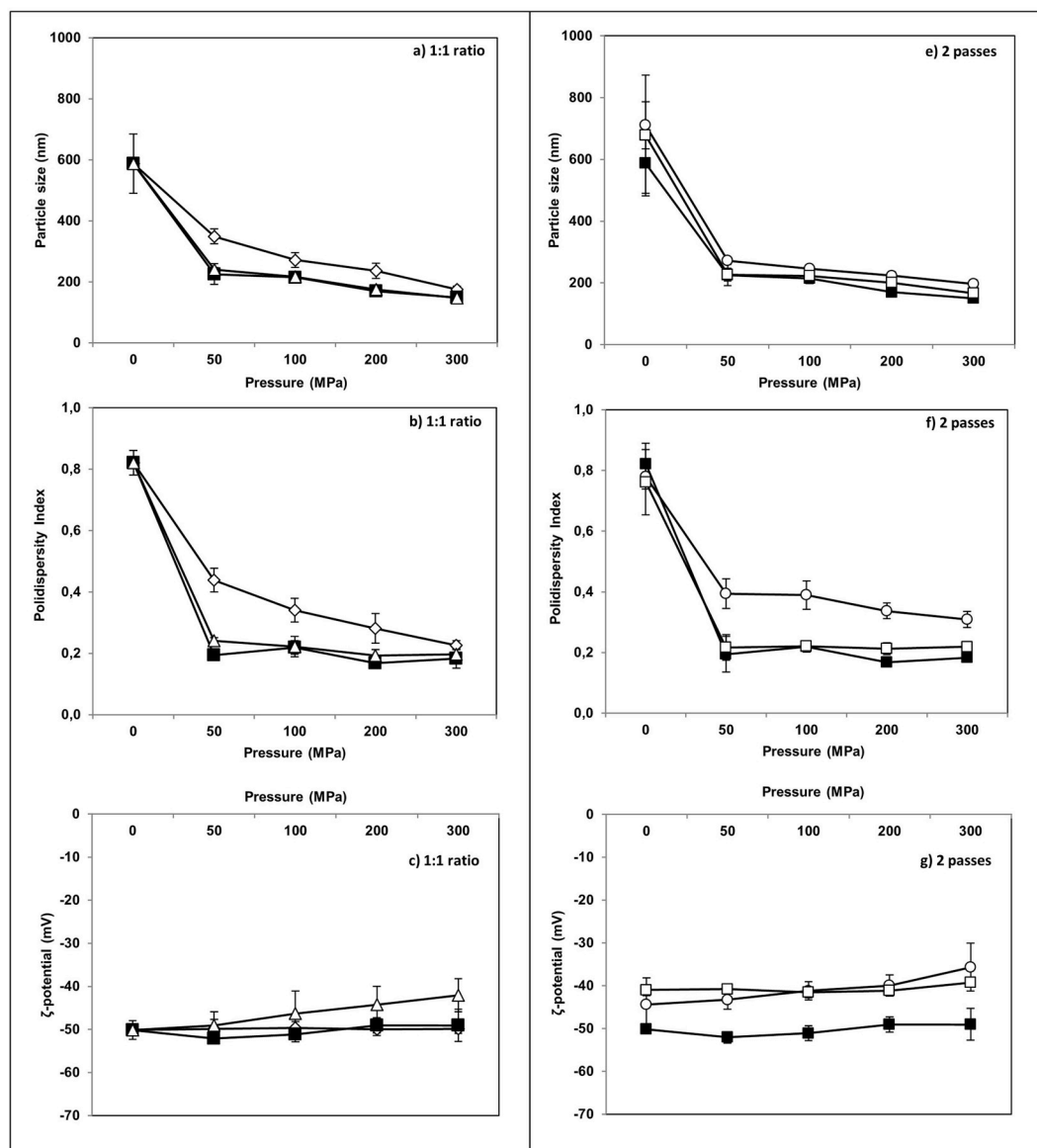


Fig. 2. Effect of HPH on particle size, polydispersity index and ζ -potential of lentil nanoemulsion. a-c) effect of pressure level and number of passes on 1:1 lentil nanoemulsions; ($\circ$) 1 homogenization pass; ($\blacksquare$) 2 homogenization passes; ($\triangle$) 3 homogenization passes. d-f) Effect of pressure level and emulsifier:oil ratio on nanoemulsions with 2 homogenization passes; ($\circ$) 1:2 ratio (2.5 g/100 g); ($\blacksquare$) 1:1 ratio (5 g/100 g); ($\square$) 2:1 ratio (10 g/100 g).

no significant difference between samples treated at 200 MPa and 300 MPa with one homogenization pass (Fig. 2b).

To improve particle size distribution and obtain better mean droplet size and PDI, which are related to improved stability, lentil nanoemulsions were subjected to 1, 2, and 3 passes through the HPH (Fig. 1) (Piorkowski & McClements, 2014). The increase in number of passes produced a notorious increase in the peak < 1 μ m and a decrease in the second peak, indicating the rupture of big droplets to smaller ones. By obtaining a less disperse particle size distribution when doubling the number of passes, droplet size significantly decreased at all pressures evaluated ($p < 0.05$) but the use of 3 passes did not further decrease droplet size. HPH also decreased the PDI, indicating an improvement on particle size distribution, finding a significant reduction when doubling the number of passes, but using 3 passes did not further improve PDI. This is in agreement to the results obtained by Qian and McClements (2011) who observed that after certain number of passes the decrease in emulsion droplet size becomes not significant.

According to the results obtained on particle size distribution, two homogenization passes were used to evaluate the emulsifier:oil ratio

effect on nanoemulsions physicochemical properties (Fig. 3). The ratio of emulsifier to oil phase strongly influences mean droplet size, so emulsifier:oil ratios of 1:2; 1:1 and 2:1 were used to evaluate the ratio effect. Unpressurized lentil emulsions with 1:2 and 2:1 ratios also showed multimodal distribution with 3 peaks < 1 μ m and one peak > 1 μ m. HPH in 1:2 ratio nanoemulsions decreased the number of peaks to 3, with a mean size of 91, 350, and 5600 nm. The homogenization increased the volume of the peak > 1 μ m, and as pressure increased, the peaks of smaller size got closer together, showing a large number of particles with sizes between 70 and 500 nm. At ratio 2:1, homogenization at 50 MPa produced a monomodal distribution with a single peak at 255 nm, however, higher pressures induced droplet flocculation (peak > 1 μ m) with peaks size < 300 nm.

The effect of pressure level on particle size at different emulsifier:oil ratios is shown in Fig. 2d and e. Nanoemulsions prepared with ratio of 1:2 had bigger droplet size and PDI than nanoemulsions with ratios of 1:1 and 2:1, at all pressures evaluated. The increase in protein concentration to achieve a ratio of 1:1 decreased particle size and PDI at all pressures but 100 MPa, however, increasing protein ratio from 1:1 to

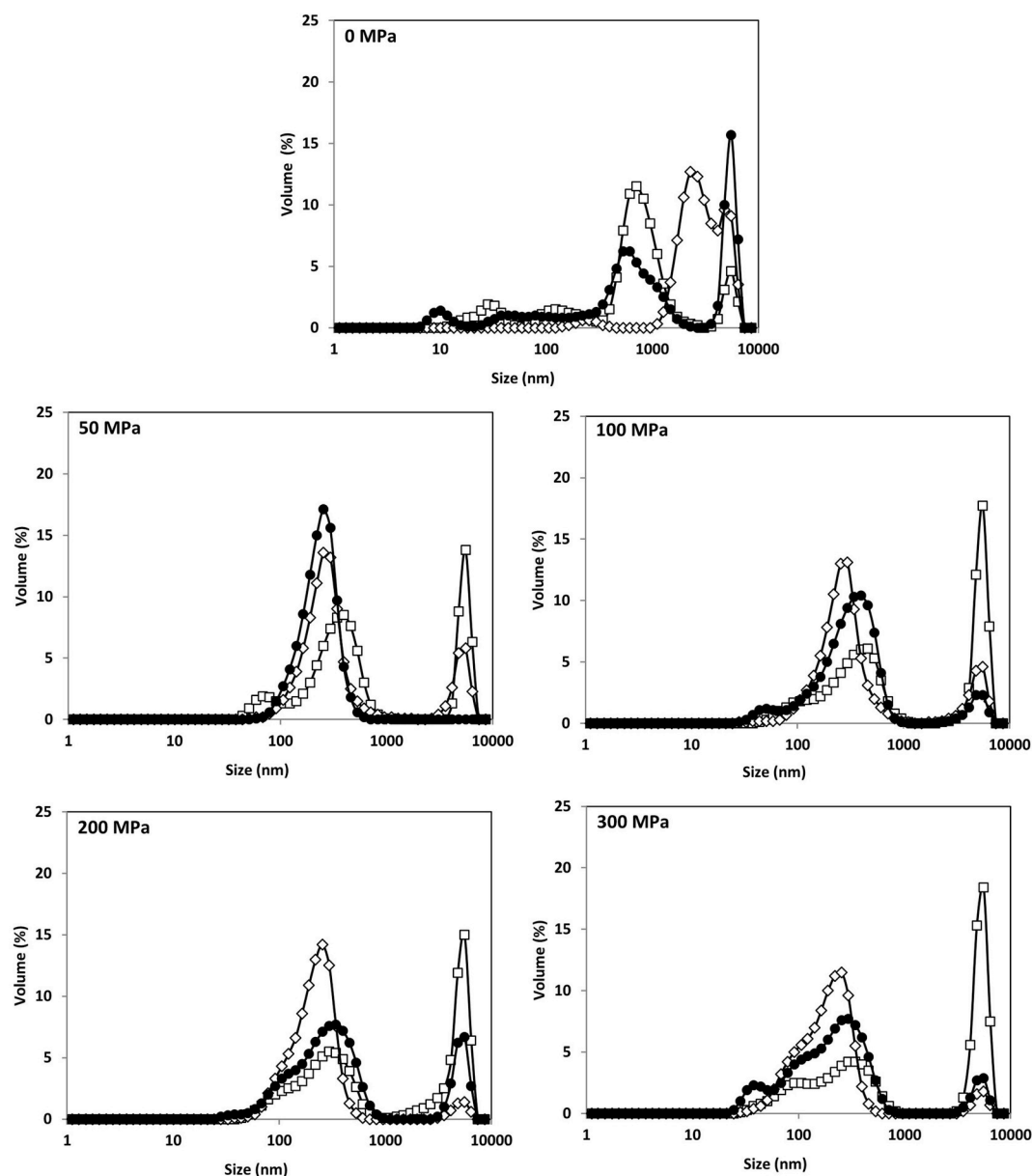


Fig. 3. Effect of emulsifier:oil ratio on particle size distribution of lentil nanoemulsions homogenized at different pressures with two homogenization passes. (□) 1:2 ratio (2.5 g/100 g); (◇) 1:1 ratio (5 g/100 g); (●) 2:1 ratio (10 g/100 g).

2:1 did not significantly improve these parameters ($p > 0.05$). Probably, with a ratio of 1:2 there is not enough protein to cover the surface of oil droplets, and this promotes coalescence of small droplets to bigger ones, increasing nanoemulsion dispersity and PDI. With the increase in protein concentration to achieve a 1:1 ratio the oil-water interphase probably saturates and increasing protein concentration does not contribute to further decrease droplet size (Joshi et al., 2012).

3.2. Surface charge

Fig. 2c shows the ζ -potential for lentil nanoemulsions as a function of pressure and number of passes applied. Nanoemulsions ζ -potential were negative because pH used is over the lentil protein isoelectric point and thus the charge of proteins is negative. HPH did not affect ζ -potential when applying 1 or 2 homogenization passes at any pressure level, however, when applying 3 homogenization passes ζ -potential increased with increasing pressures, becoming less negative above 100 MPa. A more positive charge means a decrease in the repulsive

forces formed between oil droplets, which could indicate less effectiveness in the stabilization of nanoemulsions through physical mechanisms (Ralla et al., 2017). The electrostatic repulsion plays an important role in the stability against aggregation of charged oil droplets that generates short range steric repulsions such as globular proteins. For these type of emulsions the magnitude of ζ -potential should be > 20 mV to produce long term stable systems (Piorkowski & McClements, 2014), which was accomplished in our lentil nanoemulsions.

Nanoemulsions with 1:1 ratio had ζ -potential values significantly ($P < 0.05$) more negative than ratios 1:2 or 2:1 (Fig. 2f). This suggests that using the same concentration of oil and lentil protein increases the electric repulsive forces between oil droplets, which could help to prevent coalescence and thus increase the stability of the system (Ng et al., 2017). There was no significant difference between nanoemulsions prepared with ratios of 1:2 and 2:1, and different pressure levels did not affect surface charge of lentil nanoemulsions independently of emulsifier:oil ratio used but the nanoemulsion with 1:2 ratio homogenized at 300 MPa, where ζ -potential became less negative. Is it

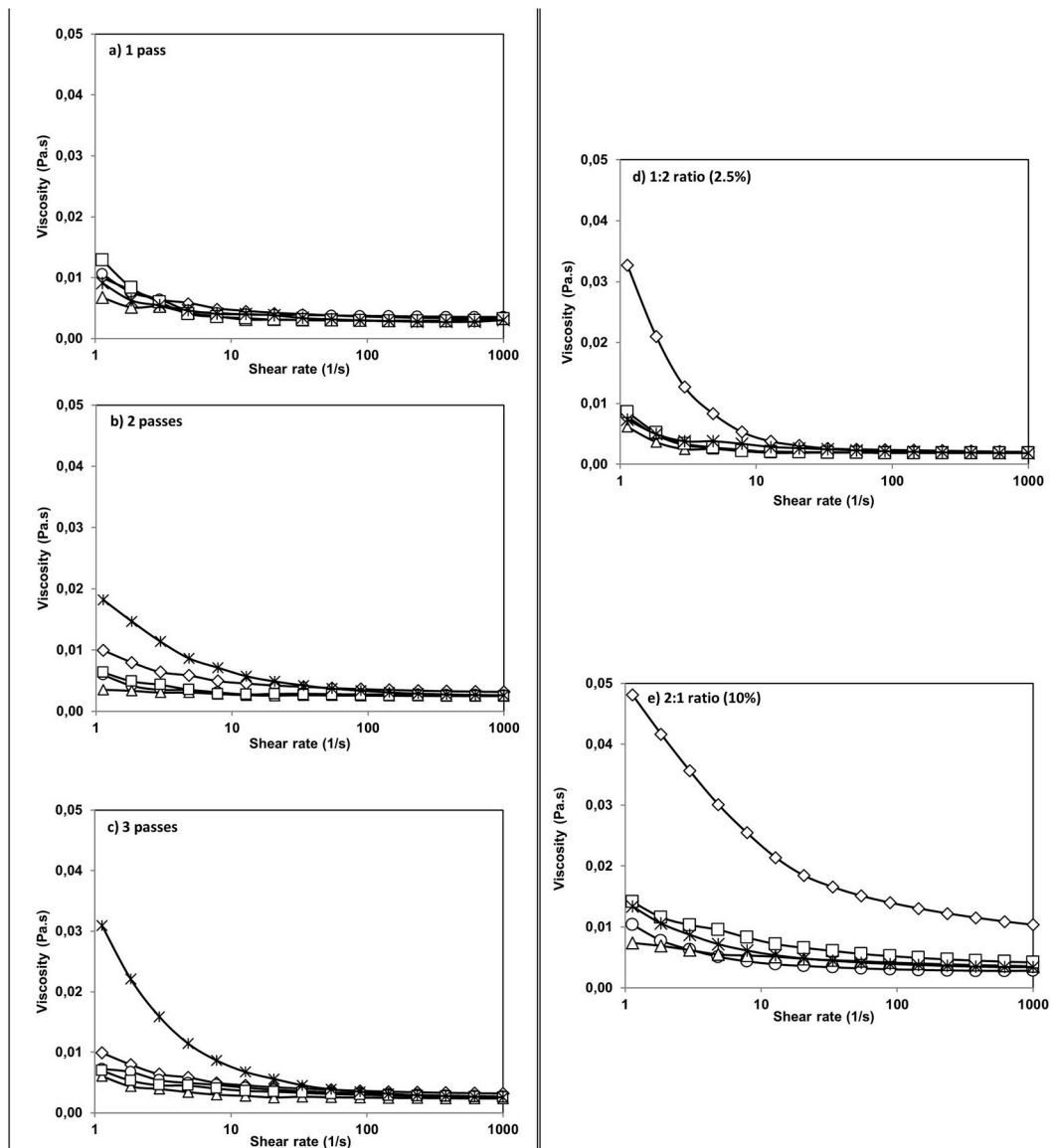


Fig. 4. Effect of HPH on viscosity of lentil nanoemulsions. a-c) effect of pressure level and number of passes; d-e) effect of pressure level and emulsifier:oil ratio; ($\diamond$) 0 MPa; ($\circ$) 50 MPa; ($\triangle$) 100 MPa; ($\square$) 200 MPa; ($*$) 300 MPa.

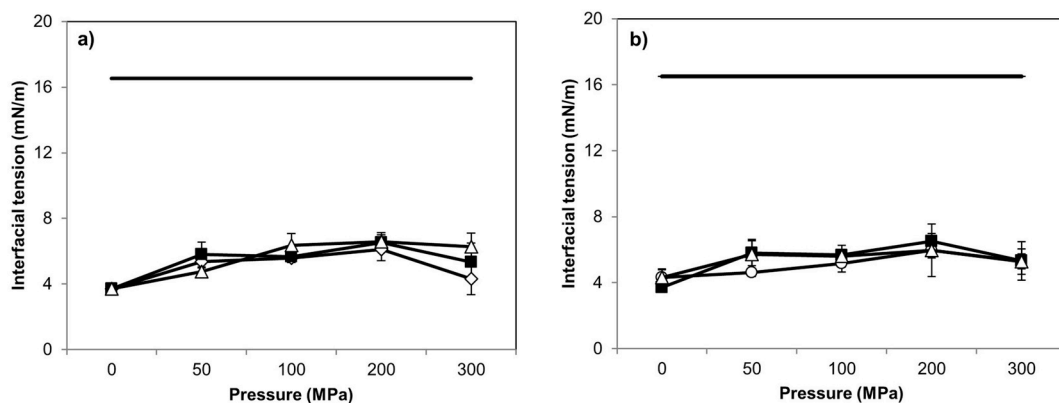


Fig. 5. Interfacial tension of lentil nanoemulsions. a) Effect of number of passes and pressure level on 1:1 ratio lentil nanoemulsions; b) Effect of emulsifier:oil ratio and pressure level on lentil nanoemulsions with 2 homogenization passes. (—) Water-oil interphase; ($\diamond$) 1 homogenization pass; ($\blacksquare$) 2 homogenization passes; ($\triangle$) 3 homogenization passes.

possible that the higher stress applied to this nanoemulsion could expose charged residues, decreasing the net charge of the droplet surface and thus decreasing the magnitude of ζ -potential.

3.3. Viscosity

The apparent viscosity (η_{app}) curves for lentil nanoemulsions are shown in Fig. 4. Curves fitted Herschel Bulkley and Ostwald de Waele models, since the yield stress was not significant (< 0.01 Pa) Ostwald de Waele was chosen as the fitting model. Lentil nanoemulsions showed pseudoplastic behavior ($n < 1$) and low consistency index ($k < 0.05$) as η_{app} decreased with increasing shear rates. Viscosity was evaluated at moderate shear rate, 90 s^{-1} , as this is frequently used to evaluate food processes (Kuhn & Cunha, 2012). HPH > 100 MPa of 1:1 ratio nanoemulsions decreased η_{app} (Fig. 4a–c), which could be caused by the disruption of the structure network when higher pressures are applied to nanoemulsions, because the particle size is reduced and leads to lower internal resistance than the generated by large droplets or linear chains of droplets (Ng et al., 2017). The pseudoplastic behavior has been attributed to a deflocculation of oil droplets produced by the high shear stress applied. At low shear rate oil flocs move as one with fixed shape and size, but as shear rate increases oil flocs start to deform and align with the shear field producing a reduction in viscosity (Floury, Desrumaux, & Lardières, 2000).

There was no difference between using ratios of 1:2 and 1:1 (2.5 and 5 g/100 g protein) but nanoemulsions with 2:1 ratio (10 g/100 g protein) had higher η_{app} (Fig. 4d and e). HPH caused a reduction in η_{app} at ratios of 1:2 and 2:1 with no difference between applying 50, 100, 200 or 300 MPa, indicating that the decrease in particle size obtained at pressures < 200 MPa has no significant influence on emulsion η_{app} .

3.4. Interfacial tension

The interfacial tension (IT) values for lentil nanoemulsions are shown in Fig. 5. Lentil protein was able to adsorb to oil-water interface as it decreased oil-water IT from 16.5 mN/m to < 7.0 mN/m when proteins were added to samples. The use of HPH in nanoemulsions with emulsifier:oil ratio of 1:1 increased IT from 3.7 to 4.3–6.6 mN/m, finding no difference between applying 2 and 3 homogenization passes. The effect of emulsifier:oil ratio can be observed in Fig. 5b. At low pressures (50–100 MPa) the 1:2 ratio decreases IT, however this difference disappears at higher pressures, always with IT values < 6.0 mN/m. There is no difference between ratio of 1:1 and 2:1, which indicates that increasing protein concentration does not further decrease IT. These results are in accordance to droplet size and PDI of lentil emulsions, were no difference was found between applying 2 and 3 passes or between using emulsifier:oil ratio of 1:1 and 2:1.

3.5. Stability

Emulsions are thermodynamically unstable systems as given sufficient time the phases will separate. The stability of lentil nanoemulsions was evaluated through two different methods. An accelerated stability test was performed using centrifugation to determine creaming stability immediately after nanoemulsions were prepared and the stability along time was determined as the days the nanoemulsion maintained their original physical appearance (Fig. 6). Control emulsions, those not subjected to HPH, were the most unstable systems with creaming volumes between 6 and 9%, forming a cream layer on the top of the tube in the first day. Creaming values decreased even to non-detectable levels as pressure and protein concentration increased, finding no creaming in nanoemulsions with 1:1 ratio homogenized at 300 MPa and 2:1 ratio homogenized at 200 and 300 MPa (Fig. 6). Along time, nanoemulsions homogenized at 50–100 MPa changed faster than those homogenized at 200 and 300 MPa. Nanoemulsions with 1:2 ratio showed a maximum stability of 5 days for nanoemulsion homogenized

at 300 MPa meanwhile nanoemulsions with excess emulsifier (ratio 2:1) achieved a maximum stability of 8 days when homogenized at 200 and 300 MPa. Regardless that all pressurized nanoemulsions gelled, the best stability was achieved with 1:1 ratio and 300 MPa, retaining its physical appearance up to 21 days. Emulsion gelling phenomena can be caused by flocculation of oil droplets, that frequently occurs when proteins are used as an emulsifier (Piorkowski & McClements, 2014). Furthermore, gelled nanoemulsions presented lower pH than fresh nanoemulsions (data not shown) which decreases the electric charge of oil droplets, which could decrease the repulsion between them and thus promote flocculation. As there was no significant difference in surface charge between nanoemulsions prepared with ratios of 1:2 and 2:1, the higher stability of 2:1 ratio in comparison to 1:2 is not related to electric

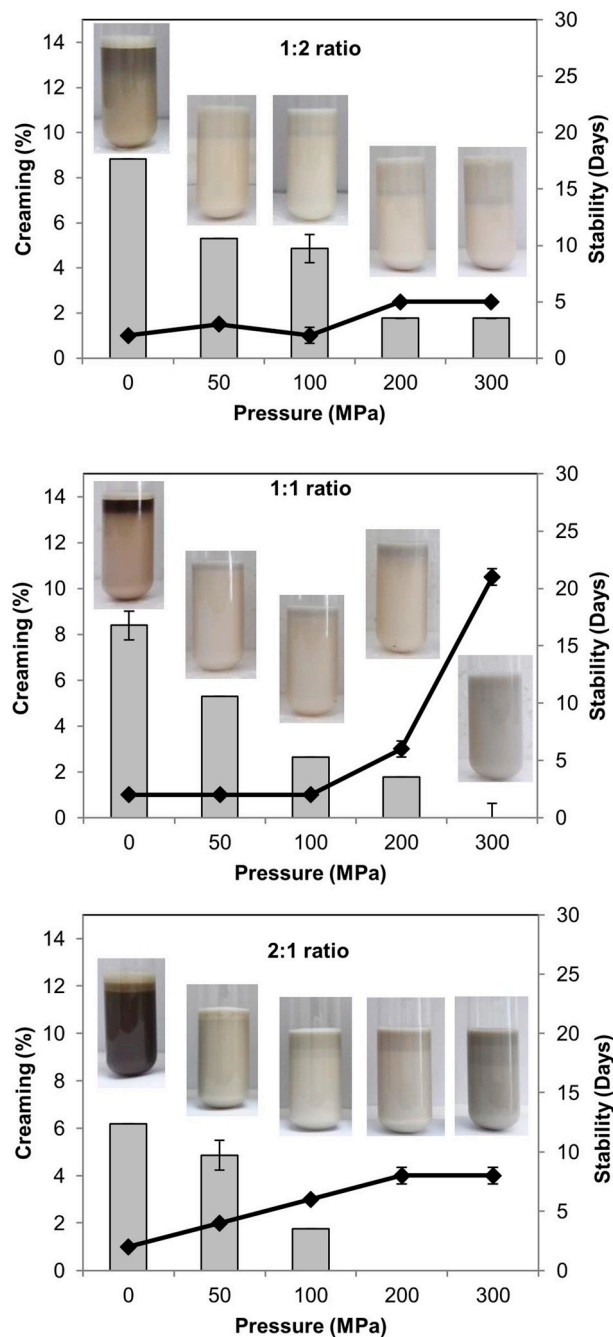


Fig. 6. Stability of lentil nanoemulsions formulated with different emulsifier:oil ratios. Photographs correspond to emulsions in their destabilization day. (■) Creaming; (◆) Stability.

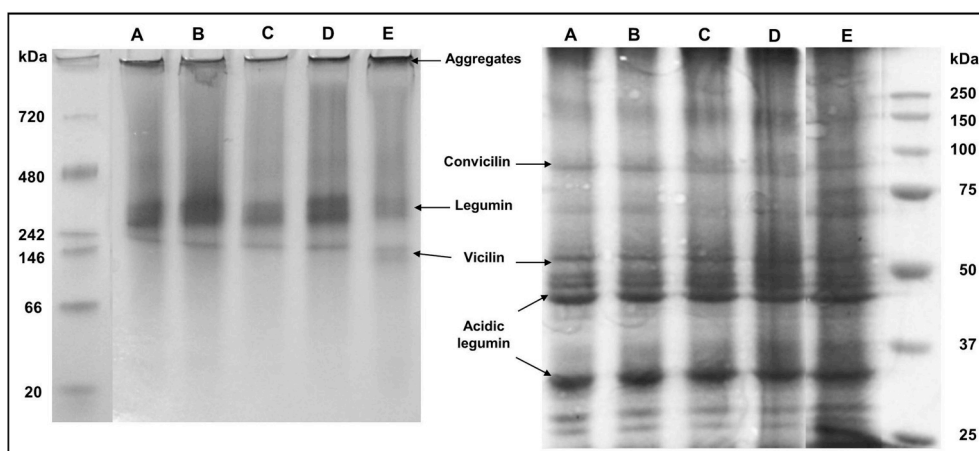


Fig. 7. Native electrophoresis (a) and SDS-PAGE under denaturing conditions (b) of lentil protein solution homogenized at different pressures. A, 0 MPa; B, 50 MPa; C, 100 MPa; D, 200 MPa; E, 300 MPa.

repulsion but a steric repulsion mechanism due to an excess of protein on the interfacial layer (Joshi et al., 2012). According to the PDI, mean droplet size, surface charge and IT values obtained, the higher stability of 1:1 nanoemulsion homogenized at 300 MPa in comparison to its equivalent at 2:1 ratio could be attributed to an electric repulsion between droplets and not a steric repulsion as no significant difference was found in droplet size and IT between ratios 1:1 and 2:1, but surface charge at 1:1 is significantly more negative than at 2:1.

3.6. Electrophoresis

Gel electrophoresis was performed to determine if there was a protein modification caused by HPH. Native and SDS-PAGE electrophoresis are shown in Fig. 7. In native electrophoresis there were two dominant bands, one between 242 and 480 KDa that probably corresponds to the hexamer of legumin (six monomers of approximately ~60 KDa) and the second band that could be attributed to vicilin (trimeric protein with monomeric chains of 40–70 KDa) (Jarpa-Parra et al., 2015; Sanchez-Monge et al., 2004). At the top of the gel it can be observed that some proteins did not enter the gel, probably aggregated proteins with molecular size > 720 KDa. At 300 MPa the bands become wider and less intense, meanwhile the aggregates on the top of the gel become more intense, which could account for a pressure-induce aggregation of proteins at this pressure. Under reducing conditions there are bands that can be identified as convicillin (~95), vicilin (~50 KDa), and acidic subunits of legumin (~32 KDa and ~40 KDa), finding no significant effect of pressure. It could be that aggregates observed at 300 MPa in native electrophoresis are disaggregated by the SDS in the SDS-PAGE.

4. Conclusion

The results of this work show that stable lentil nanoemulsions can be obtained by combining an increased number of passes through the homogenizer at 1:1 emulsifier:oil ratio. The use of HPH with 2 homogenization passes allowed to obtain a bimodal size distribution with droplet size < 150 nm and PDI < 0.20; however, the application of a third homogenization pass did not further improve these parameters. Smaller PDI and droplet sizes were obtained using ratios of 1:1 and 2:1, with no difference between them, indicating that the interphase is already saturated with protein at 1:1 ratio and the increase in protein concentration does not further contribute to improve dispersity and size decrease. Nanoemulsions homogenized at 300 MPa were more stable against cremation, especially at 1:1 and 2:1 ratios, however best stability along time was achieved with 1:1 ratio. In conclusion, stable lentil nanoemulsions can be obtained by using HPH at pressures > 200 MPa with two homogenization passes at 1:1 emulsifier:oil ratio. These lentil

nanoemulsions could be a promising source of vegetable proteins to incorporate into novel colloidal food systems.

Funding

This work was funded by CONICYT [FONDECYT Project 1161531].

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