



Impact of environmental stresses on the stability of acidic oil-in-water emulsions prepared with tofu whey concentrates

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ABSTRACT

The emulsifying properties of tofu-whey concentrates (TWCs) at pH 3.0, 4.0, and 5.0, and the stability of the resultant oil-in-water emulsions against freeze-thawing (24 h, $-20\text{ }^{\circ}\text{C}$) and controlled or mechanical stress (orbital stirring at 275 rpm, 40 min) were addressed. TWCs were prepared from tofu-whey by heating at $50\text{ }^{\circ}\text{C}$ (8.0 kPa) or $80\text{ }^{\circ}\text{C}$ (24.0 kPa), dialysis ($4\text{ }^{\circ}\text{C}$, 48 h), and freeze-drying, giving the samples TWC50 and TWC80, respectively. The particle size and interfacial properties at the oil/water interface were measured. Emulsions were prepared by mixing the TWC aqueous dispersions (1.0% protein w/w) and refined sunflower oil (25.0% w/w) by high-speed and ultrasound homogenization. The preparation of TWCs at higher temperatures ($80\text{ }^{\circ}\text{C}$) promoted the formation of species of larger particle size, a slight decrease of interfacial activity, and the adsorption of more rigid biopolymer structures associated with an increase of film viscoelasticity in interfacial rheology measurements. The emulsifying properties of both concentrates were enhanced with decreasing pH (5.0–3.0), through a significant decrease of particle size ($D_{4,3}$) and flocculation degree (FD), but only those prepared with TWC80 exhibited higher stability to freeze-thawing and mechanical stress at pH 3.0. This could be ascribed to a combination of low initial $D_{4,3}$ and FD values, high protein load, and the presence of rigid species that impart high viscoelasticity to the oil/water interface. These results would be of great importance for the utilization of TWCs as food emulsifiers in acidic systems to impart high stability to environmental stresses.

1. Introduction

Tofu is the main processed soybean product. It is produced from soymilk by addition of calcium salts or acidulants such as glucono- δ -lactone. The soy curd is broken and pressed to make the tofu, and a liquid by-product called tofu-whey (TW) is generated (Benedetti et al., 2015; Chua and Liu, 2019; Matemu et al., 2009; Shen and Kuo, 2017). TW has a high water content and also contains proteins, peptides, simple sugars, non-starch soluble polysaccharides, and phytochemicals such as isoflavones (Corzo-Martínez et al., 2016). Due to high processing costs associated with the TW wastewater treatment before its disposal, this liquid is often discarded causing industrial, economic, and environmental problems (Belén et al., 2013; Chua and Liu, 2019). However, TW should not be considered a residual liquid because it contains a substantial number of valuable compounds that can be exploited for various uses (Belén et al., 2013). According to Chua and Liu (2019), tofu-whey valorization can be classified into two main categories: recovery of

nutrients through the use of physical methods and biotransformation through the application of microbiological and/or enzymatic methods. Physical methods can be applied for the extraction of the protein and polysaccharides present in TW to prepare tofu-whey concentrates (TWCs) (Henao Ossa et al., 2020).

When tofu curd is obtained by coagulation with calcium salts, the polypeptide composition of TW is highly dependent on the salt concentration (Kao et al., 2003). The Kunitz trypsin inhibitor (KTI), lectin, and some subunits of β -conglycinin are present in TWCs (Henao Ossa et al., 2020). Although KTI and lectin are known antinutritional bioactive compounds, they are totally denatured and inactivated during the hydrothermal treatments of tofu manufacturing (Vagadia et al., 2017). As was observed in a previous work, tofu-whey proteins obtained by precipitation with organic solvents exhibited good emulsifying properties in model oil-in-water emulsions (Sobral et al., 2018). In another article, the emulsifying properties of tofu-whey proteins were improved by conjugation with soy polysaccharides through Maillard-type

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glycosylation (Matemu et al., 2009). In the mentioned papers, the study of emulsifying properties was performed at neutral pH, but the behavior of tofu-whey proteins as emulsifier in acidic medium was scarcely assessed. In this regard, acidulants are often incorporated into food emulsions to improve their sensory characteristics as well as to ensure microbiological safety. Therefore it is necessary to evaluate the emulsifying properties of tofu-whey proteins in acidic conditions. Moreover, in practice, the emulsion-based products tend to experience a variety of environmental stresses such as thermal processing, chilling, freeze-thawing, drying, and mechanical stirring during their production, storage, and utilization. All these processes often produce irreversible changes in the emulsion microstructure, thus promoting destabilization (McClements, 2016).

TWCs were obtained by heating under reduced pressure at two different pressure and temperature conditions. In a previous paper, where the structural and foaming properties of TWCs were evaluated, the increase of temperature during their preparation promoted protein aggregation by hydrophobic effect, thiol-disulfide exchange reactions and incipient glycosylation, the decrease of protein structural flexibility and the carbohydrate solubilization (Henao Ossa et al., 2020). On the basis of the above considerations, the aim of the current article was to investigate the emulsifying properties of TWCs and the influence of freeze-thawing and controlled mechanical stirring on the stability of the resultant emulsions. The freeze-thaw stability is an important attribute for food emulsions that need to be frozen prior to consumption (Ghosh and Coupland, 2008). Likewise, the study of the impact of mechanical stirring on the stability of emulsions is relevant because these systems are often subjected to applied shear forces during their production, transport, and storage (McClements, 2016; Mitidieri and Wagner, 2002). The emulsifying properties of TWCs were evaluated at pH 3.0, 4.0, and 5.0, where the ζ -potential values of particles in aqueous dispersion were positive, close to zero, and negative, respectively (Henao Ossa et al., 2020). Then, the impact of environmental stresses on emulsion stability was assessed.

2. Materials and methods

2.1. Materials

The tofu whey wastewater (TW, pH 5.6 ± 0.1) was provided by Soyana S.H. (San Martín, Buenos Aires, Argentina). This liquid was filtered using a cotton mesh (~ US 50 mesh) to remove the coarse tofu curd particles. The chemical composition of filtered TW (g/L) was: total solids, 2.1 ± 0.1 (oven drying at 103 ± 1.0 °C until constant weight); crude protein (Kjeldahl, $N \times 6.25$), 0.32 ± 0.05 ; total carbohydrates (phenol-sulfuric acid method), 0.87 ± 0.04 ; ash (dry ashing at 550.0 ± 5.0 °C), 0.44 ± 0.04 . Refined sunflower oil was purchased in a local supermarket. Bovine serum albumin (99.0%, fatty acid free) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). All the other chemicals were analytical grade reagents purchased from Anedra (Research AG; Buenos Aires, Argentina).

2.2. Preparation of tofu whey concentrates

The detailed experimental procedure of preparation of TWCs was described elsewhere (Henao Ossa et al., 2020). Briefly, TW samples previously filtered (48 Tyler mesh) were concentrated using a rotary evaporator (Heidolph Laborota OB 4000, Heidolph Instruments, GmbH & Co. KG; Schwabach, Germany) at two different combinations of temperature and pressure for 120 min: 50 ± 2 °C (8.0 kPa) and 80 ± 2 °C (24.0 kPa). The initial TW volume was reduced up to 80% during the concentration stage. The resultant liquids were dialyzed against distilled water (48 h, 4 °C) using a 3.5 kDa cutoff membrane (Thermo Scientific; Rockford, IL, USA). The dialyzed liquids were finally freeze-dried (-45 °C, 2.0 Pa, 85 h, L-I-2E260 device, Rificor S.A; Buenos Aires, Argentina) giving the samples TWC50 and TWC80. The crude

protein contents ($N \times 6.25$, on dry basis) of TWC50 and TWC80 were $37.3 \pm 0.1\%$ and $47.4 \pm 0.1\%$ w/w, respectively. Moreover, the total carbohydrate contents (phenol-sulfuric acid method, on dry basis) of TWC80 and TWC50 were $43.5 \pm 0.4\%$ and $42.1 \pm 0.6\%$ w/w, respectively. Finally, the calcium contents of TWC80 and TWC50, determined on ash, were $4.6 \pm 0.1\%$ and $6.5 \pm 0.1\%$ w/w, respectively (Henao Ossa et al., 2020).

The freeze-dried powders were dispersed in distilled water at equivalent protein content (0.1, 0.25 or 1.0% w/w) by mild magnetic stirring for at least 2 h to ensure complete hydration. The pH of the resultant TWC50 and TWC80 aqueous dispersions, ranging from 6.6 to 6.8, was then adjusted to 3.0, 4.0 or 5.0 with 1.0 M HCl solution. This process was conducted in triplicate for each concentrate at different pH values.

2.3. Characterization of aqueous dispersions

2.3.1. Particle size measurements

The particle size distribution (PSD) was obtained by static light scattering (SLS) using a Malvern Mastersizer 2000E analyzer equipped with a Hydro 2000MU wet dispersion unit (Malvern Panalytical Ltd; Worcestershire, UK). Aqueous dispersions (pH 3.0, 4.0 or 5.0) were previously diluted with distilled water adjusted to the same pH with 0.1 M HCl solution. The stirring speed was set at 2,000 rpm in the dispersion unit. The optical parameters used to obtain the PSD were: refractive index of dispersant, 1.33; refractive index and adsorption coefficient of the particles, 1.52 and 0.1, respectively. Particle sizes were expressed as the Sauter, surface-weighted mean diameter ($D_{3,2}$), De Brouckere, volume-weighted mean diameter ($D_{4,3}$), and the 90th volume and surface percentiles ($D_{v,9}$ and $D_{s,9}$, respectively).

2.3.2. Interfacial behavior

The interfacial behavior of particles in tofu whey concentrates (TWC50 and TWC80 at pH 3.0, 4.0 or 5.0) was evaluated through both the equilibrium interfacial tension and interfacial rheology measurements at the oil/water interface, using a platinum-iridium Du Noüy ring. The equilibrium interfacial tension was determined at 25 °C (Lauda TD3 LMT 850 automatic tensiometer; Lauda Königshofen, Germany). The interfacial tension values of water (adjusted to pH 3.0, 4.0 or 5.0 with 1.0 M HCl solution) and aqueous dispersions (0.25% protein w/w) at the same pH ($\gamma_{int, w}$ and $\gamma_{int, D}$ respectively) were obtained. Then, the interfacial pressure (π_{int}) was calculated as:

$$\pi_{int} = \gamma_{int, w} - \gamma_{int, D} \quad (1)$$

On the other hand, the interfacial rheology was evaluated on aqueous dispersions (1.0% w/w; pH 3.0, 4.0, 5.0; TA AR-G2 rheometer, TA Instruments, Waters, L.L.C.; Newcastle, USA). Oscillatory shear measurements were performed at 25 °C at a constant frequency of 0.3 Hz. The strain was set to 3.0% within the linear viscoelastic range. The complex viscosity (η^*) of layer, which includes both the elastic and the viscous responses, was calculated as:

$$\eta^* = [(G')^2 + (G'')^2]^{0.5} / \omega \quad (2)$$

where G' is the interfacial elastic module, G'' the interfacial viscous module, and ω the angular frequency (Baldursdottir et al., 2010). The involvement of adsorption layer was monitored through the variation of η^* as a function of time; when this parameter leveled off at 30 min, the corresponding value (η^*_{30}) was recorded.

2.4. Preparation of o/w emulsions

First, aqueous dispersions (1.0% w/w) were prepared by mild magnetic stirring in distilled water; the pH was adjusted to 3.0, 4.0 or 5.0 with 1.0 M HCl solution. To prepare the pre-emulsions, 15.0 g of aqueous dispersions and 5.0 g of refined sunflower oil (oil mass fraction,

$\phi_m = 0.25$) were mixed by high-speed homogenization (Ultra-Turrax T-25 rotor/stator device, S25N-8G dispersing tool, IKA Labortechnik; Staufen, Germany) at 20,000 rpm for 1 min. Then, the pre-emulsions were poured into cylindrical beakers (height, 45 mm; internal diameter, 30 mm) and sonicated for 3 min (on/off sequences of 20 s), at 20 kHz and 40% amplitude (Sonics Vibracell VCX750, Sonics & Materials Inc.; Newtown, CT, USA). The increase of temperature ($<30^\circ\text{C}$) during the sonication was avoided by putting the beakers in a water-ice bath. For each concentrate at different pH values, the homogenization process was conducted in triplicate.

2.5. Emulsion treatments

2.5.1. Freeze-thawing

Emulsions (10.0 mL) were transferred to cylindrical beakers (height, 55 mm; internal diameter, 28 mm) with plastic lids and were isothermally stored in still air at $-18.0 \pm 2.0^\circ\text{C}$ for 24 h. After storage at subzero temperature, frozen emulsions were thawed in a water bath at $30.0 \pm 2.0^\circ\text{C}$ for 1 h and immediately characterized (section 2.6).

2.5.2. Mechanical stirring

Emulsions (20.0 mL) were transferred to cylindrical beakers (height, 55 mm; internal diameter, 28 mm) with plastic lids, and subjected to controlled mechanical stirring, following a similar procedure previously reported by Mitidieri and Wagner (2002). The beakers were placed in a cabinet (Ferca TT400, Ferca Equipamientos; Buenos Aires, Argentina) that provided orbital stirring at room temperature (25°C). The emulsions were stirred at 275 rpm for 40 min. Aliquots of emulsions (1.0 mL) were withdrawn every 10 min and immediately characterized (section 2.6).

2.6. Characterization of emulsions

2.6.1. Flow behavior

The flow behavior of unfrozen emulsions was evaluated in the range $0.01\text{--}200\text{ s}^{-1}$ using a TA AR-G2 rheometer (TA Instruments, Waters L.L.C.; Newcastle, USA) equipped with a cone-and-plate geometry (gap, 50 μm ; cone diameter, 40 mm; cone angle, 2°). The temperature was set at $25.0 \pm 0.5^\circ\text{C}$ with a water bath associated with the rheometer (Julabo ACW100, Julabo Labortechnik; Seelbach, Germany). All emulsions were allowed to rest for 2 min after loading to allow temperature equilibration. Flow curves were fitted to the power law equation:

$$\tau = K \cdot \gamma^n \quad (3)$$

where τ is the shear stress (Pa), γ is the shear rate (s^{-1}), K is the consistency index ($\text{Pa} \cdot \text{s}^n$), and n is the flow behavior index (dimensionless).

2.6.2. Particle size distribution (PSD)

The PSD of unfrozen, freeze-thawed, and mechanically stirred emulsions was obtained by SLS using a Malvern Mastersizer 2000E analyzer equipped with a Hydro 2000MU wet dispersion unit (Malvern Panalytical Ltd; Worcestershire, United Kingdom). Aliquots of emulsions were poured into the dispersion unit and stirred continuously (2,000 rpm) to ensure the sample homogeneity. The traditional method using sodium dodecyl sulfate to dissociate the flocs was not used to evaluate the flocculation degree (FD). Instead, PSD was measured after 1 min of sonication at 40 W using the ultrasonic probe of the dispersion unit. This method was more appropriate for emulsions prepared with samples rich in polysaccharides (Porfiri et al., 2016). The optical parameters used to obtain the PSD were: refractive index of dispersant, 1.33; refractive index and adsorption coefficient of the particles, 1.47 and 0.01, respectively. Particle sizes were expressed as the $D_{3,2}$ and $D_{4,3}$ values; FD was calculated as:

$$\text{FD} (\%) = [(D_{4,3-S} - D_{4,3+S})/D_{4,3+S}] \times 100 \quad (4)$$

where $D_{4,3-S}$ and $D_{4,3+S}$ are $D_{4,3}$ values obtained from PSD measured without and with sonication, respectively.

2.6.3. Adsorbed protein and protein load contents

To determine the adsorbed protein and protein load contents, the experimental procedure reported by Puppo et al. (2005) was followed with some modifications. Initial emulsions were poured into plastic centrifuge tubes, subsequently centrifuged at $5,000 \times g$ for 30 min at 20°C (Sigma 3–18 KS centrifuge, Sigma Laborzentrifugen GmbH; Germany) and finally frozen at $-18 \pm 2^\circ\text{C}$ for 4 h. The tubes were cut to separate the phases. The frozen serum phase was thawed at 20°C , and the protein concentration was determined by the modified Lowry method (Markwell et al., 1978) using bovine serum albumin as standard protein. The protein concentration was also determined in the aqueous dispersions. The adsorbed protein percentage ($\% P_A$) was calculated as:

$$\% P_A = [(C_d - C_s)/C_d] \times 100 = [\Delta C/C_s] \times 100 \quad (5)$$

where C_d and C_s are the protein concentration of dispersions and serum phase, respectively.

Moreover, the protein load (Γ) was obtained as follows (Li et al., 2011; Puppo et al., 2005):

$$\Gamma = \Delta C/\text{SSA} \quad (6)$$

where SSA is the specific surface area (Walstra, 1983):

$$\text{SSA} = 6\phi/D_{3,2} \quad (7)$$

$D_{3,2}$ is the Sauter mean diameter measured after sonication in the wet dispersion unit (section 2.6.2), ϕ the oil volume fraction, which was calculated from ϕ_m using the density values of sunflower oil and aqueous dispersions at 25°C . In turn, the density values were determined using a density bottle (McClements, 2016).

2.6.4. Emulsion microstructure

Emulsions were diluted 1:10 (v/v) with distilled water adjusted at the same pH of that emulsion. Then, aliquots of diluted emulsions were placed on glass slides and covered with 22×22 mm cover slips. Micrographs were obtained using a Cytation 5 cell imaging multi-mode reader (BioTek Instruments, Inc.; Winooski, VT, USA), operating at $200 \times$ magnification.

2.7. Statistical analysis

All assays were performed at least in triplicate ($n=3$), and the results were reported as the mean and standard deviation. Data were analyzed by analysis of variance (ANOVA), and differences between mean values were determined by the Fisher's least significant differences (LSD) at $p < 0.05$ significance level. Statistical analysis was carried out using Statgraphics Centurion XV software (Statpoint Technologies Inc.; Warrenton, VA, USA).

3. Results and discussion

3.1. Characterization of aqueous dispersions of tofu whey concentrates

The PSD of TWC50 and TWC80 aqueous dispersions is shown in Fig. 1. At all pH values, the PSD, expressed as volume frequency, exhibited a high degree of polydispersity: a minor particle population with diameters lower than $2\ \mu\text{m}$ and the main population distributed in a broad range of particle sizes ($2\text{--}1000\ \mu\text{m}$). Heating at higher temperatures (80°C) during the concentration stage promoted the formation of larger particles, evidenced by both an increase in the mode of the main particle population and an increase of all particle size parameters ($D_{4,3}$, $D_{3,2}$, $D_{v0.9}$, and $D_{s0.9}$) for TWC80 dispersions whatever the pH (Table 1, $p < 0.05$). As was expected, the minor population was the main one when the PSD was expressed as surface frequency; the main peaks were

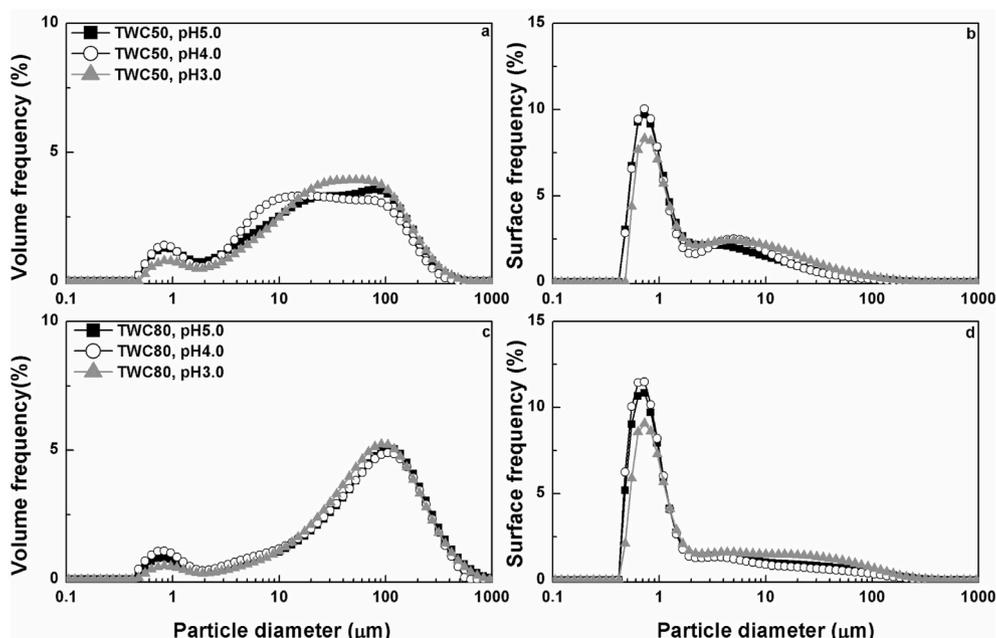


Fig. 1. Particle size distributions (PSDs) of aqueous dispersions (pH 3.0, 4.0 and 5.0) prepared with tofu whey concentrates obtained by thermal treatment of tofu whey at 50 °C (TWC50) and 80 °C (TWC80). PSDs were expressed as volume (a, c) and surface (b, d) frequency.

Table 1

Sauter, surface-weighted, mean diameter ($D_{3,2}$), De Brouckere, volume-weighted mean diameter ($D_{4,3}$), 90th volume and surface percentiles ($D_{v0,9}, D_{s0,9}$, respectively), interfacial pressure (π_{int} , oil/water interface), and interfacial complex viscosity values (at 30 min, η^*_{30} , oil/water interface) of aqueous dispersions prepared with tofu whey concentrates (TWC50 and TWC80; pH 3.0, 4.0 and 5.0). For particle size, interfacial pressure and interfacial rheology measurements the protein concentration was 0.1% w/w, 0.25% w/w and 1.0% w/w, respectively.

Sample	pH	$D_{3,2}$ (μm)	$D_{4,3}$ (μm)	$D_{v0,9}$ (μm)	$D_{s0,9}$ (μm)	π_{int} (mN/m)	η^*_{30} (Pa·s)
TWC50	3.0	7.9 ± 0.2 ^c _A	55.9 ± 0.5 ^c _A	140.4 ± 1.0 ^c _A	19.5 ± 0.2 ^c _A	8.9 ± 0.2 ^{a,A}	1.1·10 ⁻⁵ ± 2.0·10 ^{-6a,A}
	4.0	5.1 ± 0.1 ^a _A	41.3 ± 0.3 ^a _A	112.2 ± 0.7 ^a _A	11.3 ± 0.1 ^a _A	9.6 ± 0.2 ^{b,A}	1.1·10 ⁻⁵ ± 3.0·10 ^{-6a,A}
	5.0	5.5 ± 0.2 ^b _A	49.9 ± 0.4 ^b _A	131.4 ± 0.9 ^b _A	12.1 ± 0.3 ^b _A	9.6 ± 0.3 ^{b,A}	1.3·10 ⁻⁵ ± 1.5·10 ^{-6a,A}
TWC80	3.0	12.8 ± 0.5 ^c _B	95.4 ± 0.4 ^b _B	218.8 ± 1.3 ^b _B	36.2 ± 0.7 ^c _A	8.9 ± 0.1 ^{a,A}	2.6·10 ⁻⁵ ± 2.1·10 ^{-6c,B}
	4.0	7.2 ± 0.4 ^a _B	86.3 ± 0.2 ^b _B	207.3 ± 1.1 ^a _B	14.2 ± 0.3 ^a _A	9.7 ± 0.1 ^{b,A}	2.2·10 ⁻⁵ ± 3.0·10 ^{-7b,B}
	5.0	9.1 ± 0.1 ^b _B	99.4 ± 0.5 ^c _B	233.4 ± 1.2 ^c _B	20.8 ± 0.2 ^b _A	9.4 ± 0.2 ^{b,A}	1.6·10 ⁻⁵ ± 1.0·10 ^{-6a,B}

Values were expressed as the mean of three independent determinations ($n = 3$) ± standard deviation. Mean values in the same column followed by different lowercase letters are significantly different ($p < 0.05$) as determined by the Fisher's test. Mean values in the same column followed by different capital letters are significantly different between concentrates ($p < 0.05$) as determined by the Fisher's test.

overlapped whatever the pH, and 90% of the total area, evaluated through $D_{s0,9}$, was distributed into particles of diameters smaller than 40 μm (Fig. 1b and d, Table 1). As was mentioned above, both concentrates are mainly composed of proteins and polysaccharides. According to Henao Ossa et al. (2020), the thermal treatment at 80 °C

promoted the protein aggregation by hydrophobic effect, incipient glycosylation, and thiol-disulfide exchange reactions and, at the same time, the increase of polysaccharide solubility in acid medium. In fact, in the pH range 3.0–5.0, the protein solubility of TWC80 was 20% lower than that of TWC50. Moreover, at pH 4.0, both concentrates exhibited the lowest protein solubility and ζ -potential values close to zero. At this pH, the weakening of electrostatic repulsion would mainly promote the protein self-association and the formation of a new population of insoluble particles in the range 2.0–10.0 μm, which was detected by SLS, especially for TWC50. The presence of this new population at pH 4.0 induced a decrease of all size parameters with respect to those at pH 3.0 and 5.0 (Table 1, $p < 0.05$). In this regard, the formation of a new particle population by protein aggregation was also observed in aqueous dispersions of defatted soy flour by addition of calcium chloride (Márquez et al., 2018).

On the other hand, the interfacial properties of TWCs at the oil/water interface were also evaluated through the determination of equilibrium interfacial pressure (π_{int}) and complex interfacial viscosity, which were measured in aqueous dispersions at equivalent protein concentration (section 2.3.2). Although both concentrates showed different degrees of biopolymer aggregation, clearly evidenced through the PSD expressed as volume frequency (Fig. 1), no significant differences were observed between π_{int} values at each pH ($p > 0.05$). Moreover, for each TWC sample, a slightly smaller interfacial activity was evidenced at pH 3.0 ($p < 0.05$, Table 1). However, the interfacial rheology measurements showed marked differences between both concentrates, especially at pH 4.0 and 3.0. The species present in TWC80 formed a more viscoelastic interfacial film, which was evidenced through a significant increase of η^*_{30} with respect to those of TWC50 ($p < 0.05$, Table 1). According to McClements (2016), the rheology of the interfaces depends on the concentration, size, and interactions between the adsorbed species that they contain. For both concentrates, when PSD was expressed as surface frequency, most of the total area was distributed in particles smaller than 2 μm (Fig. 1b–d), so the differences observed for η^*_{30} would be ascribed to the presence of larger particles. Moreover, the viscoelasticity of interfacial films is also associated with the structural characteristics of adsorbed biopolymers and the intensity of the intermolecular interactions between these molecules at interfacial level (Baldursdottir et al., 2010). Thus for TWC80, the presence of more rigid structures at

the oil/water interface would also lead to the increase of η^*_{30} , especially at pH 3.0 where the particles would be more effectively wetted by both the aqueous and oil phase.

3.2. Characterization of initial emulsions

The PSD of initial emulsions prepared with TWC50 and measured by SLS with or without previous sonication in the wet dispersion unit is shown in Fig. 2. At pH 3.0 and 4.0, TWC50 and TWC80 emulsions exhibited a monomodal PSD whatever the ultrasound treatment. Conversely, for TWC50 emulsions at pH 5.0 the PSD was bimodal, with a main population with diameters higher than 2.0 μm . The presence of large particles in the aqueous dispersions (Fig. 1) was not observed in the PSD of emulsions. In this regard, the ultrasound devices can produce acoustic cavitation, which means the formation and the collapse of air bubbles during the homogenization (Taha et al., 2021). The oscillation and collapse of bubbles could generate several physical effects such as high temperature, pressures, shock waves, turbulence, and extreme physical shearing (Ashokkumar, 2011). In these conditions, the biopolymer aggregates could partially dissociate, decreasing their particle sizes (Niroula et al., 2021; Taha et al., 2021). Moreover, the oil proportion (25.0% w/w) is fairly higher than that of the biopolymer molecules, so the PSD of emulsions would be governed by the presence of oil droplets.

For emulsions prepared with both concentrates, as pH increased from 3.0 to 5.0, the mode of the main population was displaced towards higher particle sizes. This result was consistent with a significant increase of $D_{3,2}$ and $D_{4,3}$. In addition, a comparative analysis between the mean diameters of TWC50 and TWC80 emulsions at each pH value

revealed that those prepared with the latter sample exhibited lower values ($p < 0.05$). At the same time, the flocculation degree (FD) showed the lowest value at pH 3.0 for the emulsions prepared with both concentrates ($p < 0.05$, Table 2). It is worth noting that FD values should be treated with some caution, due to their dependence on the time and intensity of sonication during the particle size measurements and hence, the mentioned values are apparent. Despite this limitation, this strategy has been used in previous works to evaluate the FD of o/w emulsions prepared with samples of complex chemical composition and rich in polysaccharides (Colletti et al., 2020; Porfiri et al., 2016). In addition, the FD had a direct influence on the flow behavior of emulsions. When the power law model was used, all emulsions were well fitted to it with high determination coefficients ($R^2 > 0.99$). The emulsions showed a fluid-like behavior; although in all cases a shear-thinning behavior was observed, the consistency index (K) showed the following increasing order as a function of pH: 3.0 < 4.0 < 5.0. Moreover, at each pH, K showed lower values for TWC80. At the same time, the pseudoplastic character, evaluated through the flow behavior index (n), was enhanced with increasing pH ($p < 0.05$, Table 2). In preparing the concentrates, most of the simple sugars are removed during the dialysis step (Henao Ossa et al., 2020). Although TWC50 and TWC80 samples are rich in polysaccharides (section 2.2), they have little effect on the viscosity of the continuous phase, so the variation of K as a function of pH was mainly attributed to a variation of FD (Tables 1 and 2).

Broadly, the results showed a better behavior of TWC80 as emulsifier whatever the pH, and also the highest emulsifying ability at pH 3.0. According to Jafari et al. (2008) the emulsifying process includes two steps: (I) the increase of interfacial area due to droplet deformation and breakup and (II) stabilization of the fresh interface by the emulsifier to

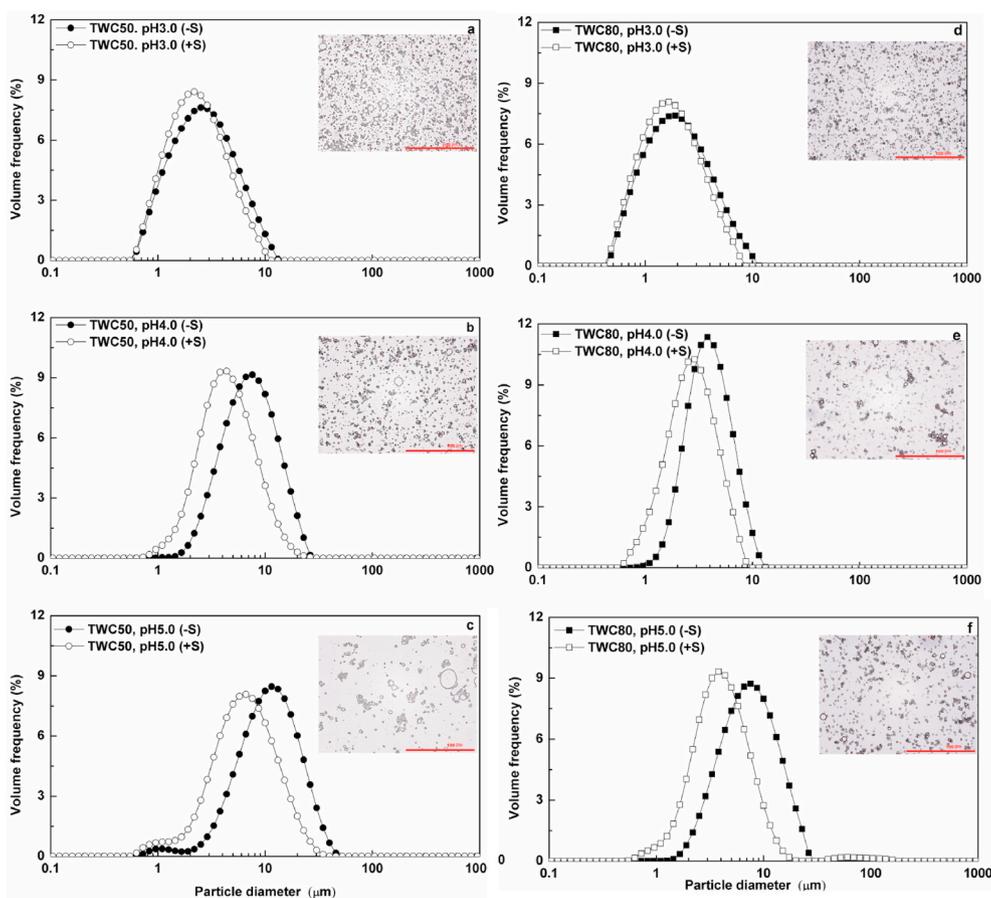


Fig. 2. Particle size distributions (PSDs, expressed as volume frequency) and microstructure (200 \times magnification) of o/w emulsions prepared with aqueous dispersions of tofu whey concentrates and refined sunflower oil as dispersed phase (oil mass fraction, $\phi_m = 0.25$): a, b and c) TWC50 at pH 3.0, 4.0 and 5.0, respectively; d, e and f) TWC80 at pH 3.0, 4.0 and 5.0, respectively. PSDs were measured without (–S) and with previous sonication (+S); for micrographs, scale bar = 100 μm .

Table 2

Sauter, surface-weighted, mean diameter ($D_{3,2}$), De Brouckere, volume-weighted, mean diameter ($D_{4,3}$), flocculation degree (FD), adsorbed protein percentage (% P_A), protein load (Γ), consistency index (K), and flow behavior index (n) of o/w emulsions prepared with aqueous dispersions of tofu whey concentrates (TWC50 or TWC80, 1.0 protein % w/w, pH 3.0, 4.0 or 5.0) and refined sunflower oil as dispersed phase (oil mass fraction, $\phi_m = 0.25$).

Sample	pH	$D_{3,2}$ (μm)	$D_{4,3}$ (μm)	FD (%)	% P_A	Γ (mg/m^2)	K (Pa·s)	n
TWC50	3.0	2.0 ± 0.3 ^{aA}	3.0 ± 0.1 ^{aB}	17.1 ± 1.2 ^{aB}	39.5 ± 0.1 ^{aA}	3.1 ± 0.2 ^{cA}	0.10 ± 0.01 ^{aB}	0.90 ± 0.01 ^{cA}
	4.0	5.6 ± 0.2 ^{bb}	7.6 ± 0.3 ^{bb}	56.1 ± 5.4 ^{bb}	37.4 ± 0.2 ^{cA}	1.5 ± 0.1 ^{bA}	0.13 ± 0.02 ^{aB}	0.80 ± 0.03 ^{bb}
	5.0	6.7 ± 0.4 ^{cA}	11.8 ± 0.1 ^{cb}	65.0 ± 1.4 ^{cb}	35.8 ± 0.2 ^{bA}	1.2 ± 0.1 ^{aA}	0.30 ± 0.08 ^{bb}	0.70 ± 0.04 ^{cb}
TWC80	3.0	1.7 ± 0.1 ^{aA}	2.4 ± 0.1 ^{aA}	7.5 ± 1.0 ^{aA}	50.1 ± 0.5 ^{cb}	4.1 ± 0.2 ^{cb}	0.03 ± 0.01 ^{aA}	0.90 ± 0.03 ^{cA}
	4.0	5.1 ± 0.1 ^{bA}	4.6 ± 0.1 ^{bA}	43.4 ± 0.6 ^{bA}	46.4 ± 0.3 ^{bb}	2.6 ± 0.1 ^{bb}	0.08 ± 0.02 ^{bA}	0.70 ± 0.06 ^{bA}
	5.0	6.5 ± 0.1 ^{cA}	9.0 ± 0.2 ^{cA}	62.5 ± 1.2 ^{cA}	41.1 ± 0.2 ^{ab}	1.7 ± 0.1 ^{ab}	0.12 ± 0.02 ^{cA}	0.50 ± 0.06 ^{aA}

Values were expressed as the mean of three independent determinations ($n = 3$) ± standard deviation. Mean values in the same column followed by different lowercase letters are significantly different ($p < 0.05$) as determined by the Fisher's test. Mean values in the same column followed by different capital letters are significantly different between concentrates ($p < 0.05$) as determined by the Fisher's test.

prevent the re-coalescence of newly formed droplets. As was mentioned above, heating at 80 °C during the preparation of the concentrates promoted protein aggregation and the formation of species of larger particle size (Fig. 1). Moreover, the structural flexibility of TWC80 species was lower than that of TWC50 (Henao Ossa et al., 2020). Under the conditions occurring during homogenization, the convection movements favor the displacement of more dense materials and hence, the adsorption of aggregates predominates (Walstra, 1983; Puppo et al., 2005). In this regard, the stabilization of the oil/water interface through a Pickering mechanism cannot be discarded due to the presence of insoluble particles in the aqueous dispersions. In preparing Pickering emulsions, the size of particles should be noticeably smaller than that of emulsion droplets; nevertheless, Pickering emulsions with droplet sizes lower than those of particles have been occasionally reported, due to the dissociation of particles during homogenization (Niroula et al., 2021). In this process, the species present in TWCs should be quickly adsorbed to cover the oil/water interface. According to McClements (2016), if the adsorption rate is faster than unfolding at the interface, the surface load of the emulsifier will be higher, and the interface will be thicker. The lower structural flexibility and higher particle size of TWC80 species in aqueous dispersions would slow down any conformational change during the adsorption, thereby increasing the surface load. In fact, the adsorbed protein percentages (% P_A) of TWC80 emulsions were higher than those prepared with TWC50 whatever the pH. At the same time, for emulsions prepared with the same concentrate, % P_A increased with decreasing pH ($p < 0.05$). Moreover, protein load (Γ) values exhibited the same tendency with regard to pH and sample type, but the values were noticeably high at pH 3.0 (Table 2, $p < 0.05$). This latter result is directly associated with the low values of $D_{3,2}$ (Table 2) because there is a relationship between the surface load and the increase of interfacial area (Li et al., 2011; Puppo et al., 2005). The better emulsifying properties of both concentrates at pH 3.0 was not associated with a higher interfacial

activity; in fact, the π_{int} values were slightly lower than those observed at pH 4.0 and 5.0 (Table 1). During ultrasound homogenization, TWC particles at pH 3.0 could feature a higher dual wettability by both the aqueous and oil phase, so a more efficient adsorption favors the increase of % P_A . The enhanced adsorption ability at the oil/water interface at pH 3.0 would decrease the re-coalescence during the homogenization, leading to a decrease of the particle size ($D_{3,2}$, $D_{4,3}$). Likewise, the efficient coverage of the interface would reduce the susceptibility to flocculation, being consistent with the lowest FD values observed at the mentioned pH (Table 2).

3.3. Effect of environmental stresses on the stability of emulsions

3.3.1. Freeze-thawing

As was stated in previous articles, freeze-thawing is a highly destabilizing treatment for o/w emulsions, especially in the absence of cryoprotectants (Ghosh et al., 2006; Ghosh and Coupland, 2008; Palazolo et al., 2011; Thanasukarn et al., 2004). The $D_{4,3}$ values of initial and freeze-thawed emulsions are shown in Fig. 3. For all emulsions, no oiling off was observed after freeze-thawing (data not shown), but an increase of $D_{4,3}$ was actually observed. Nevertheless, the destabilization degree was noticeably lower than that of model o/w emulsions prepared with aqueous dispersions of whey protein isolate or soy protein isolate and sunflower oil as dispersed phase in similar conditions of frozen storage (Cabezas et al., 2019; Palazolo et al., 2011). In this work, the emulsions were prepared with sunflower oil, which is rich in polyunsaturated fatty acids; the impact of the oil crystallization on the emulsion destabilization was negligible at relatively shorter periods of frozen storage (Palazolo et al., 2011). Thus the destabilization is mainly attributed to the stress imposed by the expanding ice. Indeed, the frozen state imposes two main effects leading to emulsion destabilization. First, the increase of ionic strength by the cryoconcentration can screen out any

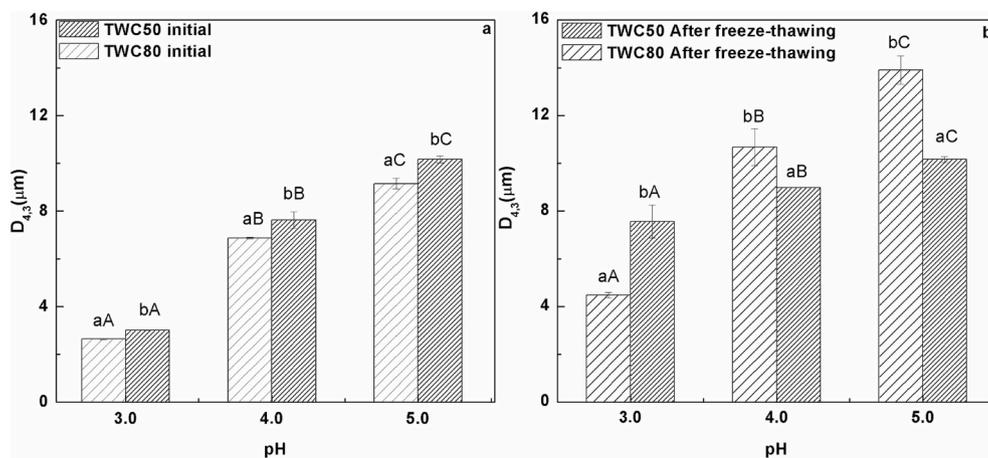


Fig. 3. De Brouckere, volume-weighted, mean diameter ($D_{4,3}$) of initial, unfrozen (a) and freeze-thawed (b) o/w emulsions prepared with aqueous dispersions of tofu whey concentrates (TWC50 and 80, pH 3.0, 4.0 and 5.0) and refined sunflower oil as dispersed phase (oil mass fraction, $\phi_m = 0.25$). Values are the means of three replicates ($n=3$) and error bars indicate standard deviation. At the top of the bars, different capital letters indicate that emulsions from the same sample made at different pHs are significantly different ($p < 0.05$), as determined by Fisher's test. Lowercase letters indicate that emulsions prepared at the same pH, but with different concentrates are significantly different ($p < 0.05$), as determined by Fisher's test.

electrostatic repulsion between droplets. Second, the oil dispersed oil phase increases, favoring the confinement of the droplets in a reduced volume of unfrozen aqueous phase. The droplets are forced to close together, favoring the flocculation, the rupture of interfacial film, and the destabilization after thawing (Ghosh et al., 2006; Ghosh and Coupland, 2008; Thanasukarn et al., 2004).

For emulsions prepared with both concentrates, $D_{4,3}$ values after freeze-thawing increased with increasing pH (Fig. 3). The initial $D_{4,3}$ followed the same tendency with regard to pH variation, so the smaller droplets could accommodate more efficiently in the unfrozen aqueous phase during the frozen storage. Moreover, the formation of a thicker interfacial film associated with an increase of % P_A and Γ with decreasing pH (Table 2) would also promote the emulsion stability. Nevertheless, although TWC80 emulsions showed lower initial $D_{4,3}$ values, after freeze-thawing the mean diameters were higher than those of emulsions prepared with TWC50 at pH 4.0 and 5.0; only at pH 3.0, TWC80 emulsions exhibited a higher stability (Fig. 3). In this regard, the presence of species of higher particle size and lower structural flexibility in aqueous dispersions did not give an additional protection of the interfacial film due to the stress imposed by the expanding ice at pH 4.0 and 5.0. In contrast, at pH 3.0 the higher dual wettability of the aggregates, which promoted an increase of η^*_{30} in the interfacial rheology assays, would have a decisive influence on the increase of emulsion stability.

3.3.2. Mechanical stirring

Collisions between droplets occur as result of their movement; at rest, they are produced due to Brownian motion and gravitational separation. Nevertheless, the applied mechanical forces a noticeable increase of the collision frequency between the droplets. In these conditions, the gravitational separation is inhibited, and the emulsion stability is governed by the collision efficiency, which depends on the balance of the repulsive and colloidal interactions between the droplets (McClements, 2016). In this regard, the emulsions were subjected to a controlled mechanical stirring, and the stability was monitored as a function of time. The results are shown in Fig. 4. At pH 4.0 and 5.0, the emulsions prepared with both concentrates showed a sharp increase of $D_{4,3}$ values due to a coalescence destabilization (Fig. 4b and c). When the stirring stopped after 40 min, at the end of the assay, the noticeable increase of particle size promoted the immediate gravitational separation (data not shown). Interestingly, at pH 3.0, a noticeable difference

between the stability of emulsions prepared with both concentrates was evidenced. TWC50 emulsion was highly destabilized; instead, TWC80 exhibited only a marginal increase of $D_{4,3}$ as a function of stirring time (Fig. 4a), this result being consistent with a high stability. For emulsions prepared with both concentrates, at pH 4.0 and 5.0, the high collision efficiency during the stirring would be attributed to a combination of higher initial particle size and relatively low values of % P_A and Γ (Fig. 3a; Table 2). Although at pH 3.0, TWC50 evidenced better emulsifying ability and higher values of % P_A and Γ , the stability was also low, so the noticeable increase observed for TWC80 emulsions would be ascribed to the adsorption of rigid aggregates that impart high viscoelasticity to the interfacial film. This result would be consistent with the highest η^*_{30} value observed for TWC80 dispersions at pH 3.0 (Table 1). In this regard, the increase of stability of soy protein-stabilized emulsions to mechanical stress due to the formation of high viscoelastic films has been previously reported by Mitidieri and Wagner (2002).

4. Conclusions

This study evaluated the emulsifying properties of TWCs and the stability of the resultant emulsions to freeze-thawing and controlled mechanical stirring. Results showed that the increase of temperature during the preparation of TWCs promoted the formation of species of higher particle size and more rigid biopolymer structures associated with an increase of film viscoelasticity in interfacial rheology measurements. Broadly, TWC80 showed better emulsifying properties than TWC50 whatever the pH, especially at pH 3.0 where the highest values of adsorbed protein percentage and protein load were observed. For TWC80 emulsions, the interfacial adsorption of more rigid structures would also have a decisive role to promote the emulsion stability against freeze-thawing and stirring. Tofu-whey concentrates are rich in polysaccharides, and the increase of temperature during the concentration stage promotes their solubilization. In this work, in preparing the concentrates, no protein-polysaccharide complexation strategy was assessed; thus further experiments are needed to ascertain the role of polysaccharides in the stability of emulsions to environmental stresses and their interactions with the proteins in the continuous aqueous phase and the oil/water interface. Notwithstanding this, it is expected that the main findings of this paper will be important for the utilization of tofu whey concentrates as emulsifier for the preparation of acidic emulsion-based products such as beverages, sauces, and dressings with high

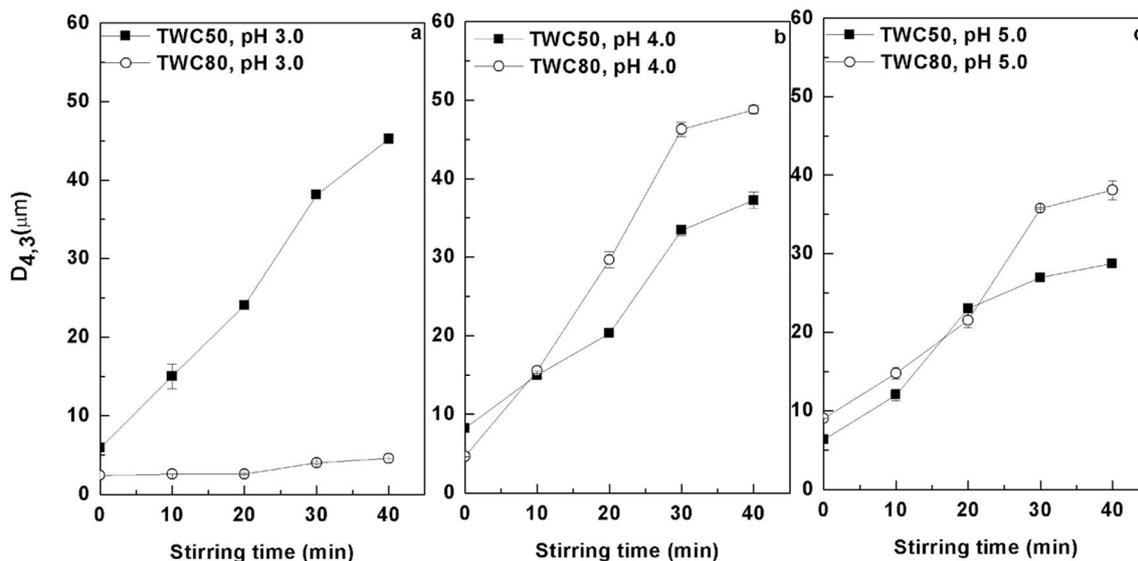


Fig. 4. Effect of stirring time on De Brouckere, volume-weighted, mean diameters ($D_{4,3}$) of o/w emulsions prepared with aqueous dispersions of tofu whey concentrates (TWC50 and TWC80) and refined sunflower oil as dispersed phase (oil mass fraction, $\varphi_m = 0.25$); a) pH 3.0, b) pH 4.0 and c) pH 5.0. Values are the means of three replicates ($n=3$) and error bars indicate standard deviation.

stability against environmental stresses.

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CRediT authorship contribution statement

J. Sebastian Henao Ossa: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Writing – original draft, preparation, Writing – review & editing. **Jorge R. Wagner:** Conceptualization, Methodology, Resources, Investigation, Formal analysis, Funding acquisition, Project administration, Supervision. **Gonzalo G. Palazolo:** Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Project administration, Supervision, Writing – review & editing.

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.

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