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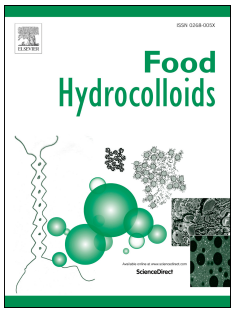

The Effect of Ultrasound Treatment on the Structural, Physical and Emulsifying Properties of Animal and Vegetable Proteins

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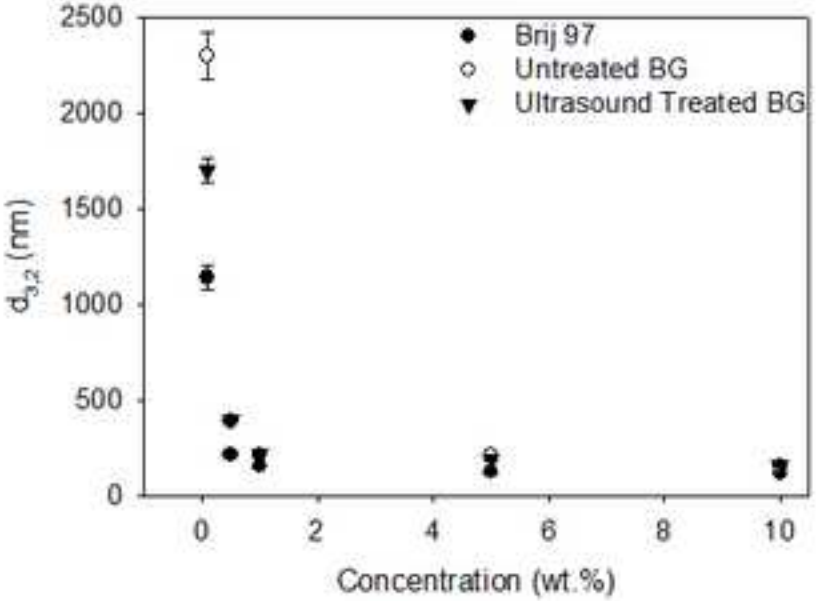
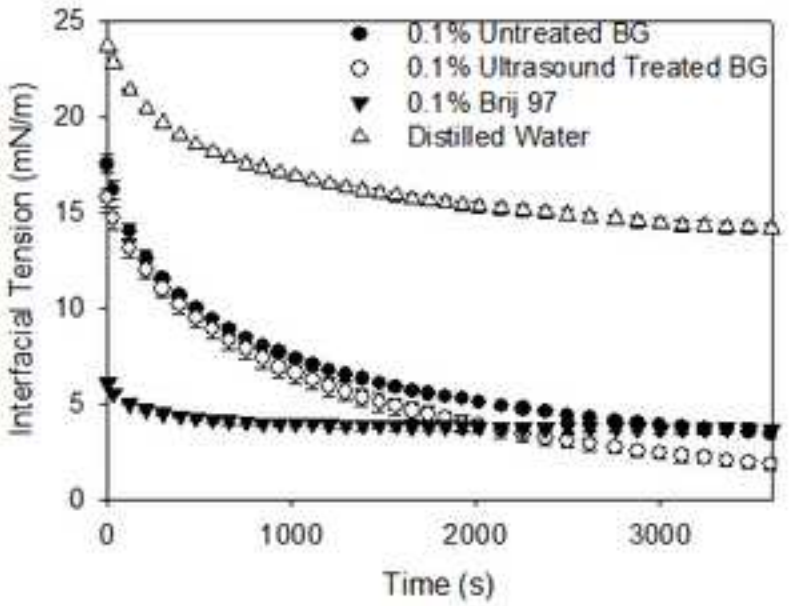

2500
2000
1500
1000
500
0
$d_{3,2}$ (nm)
0
2
4
6
8
10
Concentration (wt.%)
Brij 97
Untreated BG
Ultrasound Treated BG


25
20
15
10
5
0
Interfacial Tension (mN/m)
0
1000
2000
3000
Time (s)
0.1% Untreated BG
0.1% Ultrasound Treated BG
0.1% Brij 97
Distilled Water

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The Effect of Ultrasound Treatment on the Structural, Physical and Emulsifying Properties of Animal and Vegetable Proteins

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Abstract:

The ultrasonic effect on the physicochemical and emulsifying properties of three animal proteins, bovine gelatin (BG), fish gelatin (FG) and egg white protein (EWP), and three vegetable proteins, pea protein isolate (PPI), soy protein isolate (SPI) and rice protein isolate (RPI), was investigated. Protein solutions (0.1 – 10 wt. %) were sonicated at an acoustic intensity of ~34 W cm^{-2} for 2 minutes. The structural and physical properties of the proteins were probed in terms of changes in size, hydrodynamic volume and molecular structure using DLS and SLS, intrinsic viscosity and SDS-PAGE, respectively. The emulsifying performance of ultrasound treated animal and vegetable proteins were compared to their untreated counterparts and Brij 97.

Ultrasound treatment reduced the size of all proteins, with the exception of RPI, and no reduction in the primary structure molecular weight profile of proteins was observed in all cases. Emulsions prepared with all untreated proteins yielded submicron droplets at concentrations ≤ 1 wt. %, whilst at concentrations > 5 wt. % emulsions prepared with EWP, SPI and RPI yielded micron sized droplets (> 10 μm) due to pressure denaturation of protein from homogenisation. Emulsions produced with sonicated FG, SPI and RPI had the similar droplet sizes as untreated proteins at the same concentrations, whilst sonicated BG, EWP and PPI emulsions at concentrations ≤ 1 wt. % had a smaller droplet size compared to emulsions prepared with their untreated counterparts. This effect was consistent with the observed reduction in the interfacial tension between these untreated and ultrasound treated proteins.

Keywords: Gelatin, Egg white protein, Pea protein isolate, Soy protein isolate, Rice protein isolate, Ultrasound

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1. Introduction

Proteins perform a vast array of functions in both the food and pharmaceutical industries, such as emulsification, foaming, encapsulation, viscosity enhancement and gelation. This functionality arises from the complex chemical make-up of these molecules (O'Connell & Flynn, 2007; Walstra & van Vliet, 2003). Proteins are of particular interest in food systems as emulsifiers, due to their ability to adsorb to oil-water interfaces and form interfacial films (Foegeding & Davis, 2011; Lam & Nickerson, 2013). The surface activity of proteins owes to the amphiphilic nature these molecules possess, because of the presence of both hydrophobic and hydrophilic regions in their peptide chains (Beverung, Radke, & Blanch, 1999; O'Connell & Flynn, 2007). Due to proteins larger molecular weight lending to their bulkier structure by comparison to low molecular weight emulsifiers (e.g. Brij 97) proteins diffuse more slowly to the oil-water interface through the continuous phase (Dickinson, 1999; McClements, 2005). Once at the interface proteins undergo surface denaturation and rearrange themselves in order to position their hydrophobic and hydrophilic amino groups in the oil and aqueous phase respectively, reducing the interfacial tension and overall free energy of the system (Caetano da Silva Lannes & Natali Miquelim, 2013; McClements, 2004). Proteins provide several advantages for emulsion droplet stabilisation, such as protein-protein interactions at interfaces, and electrostatic and steric stabilisation due to the charged and bulky nature of these biopolymers (Lam & Nickerson, 2013; McClements, 2004; O'Connell & Flynn, 2007).

Ultrasound is an acoustic wave with a frequency greater than 20 kHz, the threshold for human auditory detection (Knorr, Zenker, Heinz, & Lee, 2004). Ultrasound can be classified in two distinct categories based on the frequency range, high frequency (100 kHz –

1 MHz) low power (< 1 W cm^{-2}) ultrasound, utilised most commonly for the analytical evaluation of the physicochemical properties of food (Chemat, Zill-e-Huma, & Khan, 2011), and low frequency (20 – 100 kHz) high power (10 – 1000 W cm^{-2}) ultrasound recently employed for the alteration of foods, either physically or chemically (McClements, 1995). The effects of high power ultrasound on food structures is attributed to the ultrasonic cavitations, the rapid formation and collapse of gas bubbles, which is generated by localised pressure differentials occurring over short periods of times (a few microseconds). These ultrasonic cavitations cause hydrodynamic shear forces and a rise in temperature at the site of bubble collapse (up to 5000 ^{o}C) contribute to the observed effects of high power ultrasound (Güzey, Gülseren, Bruce, & Weiss, 2006; O'Brien, 2007; O'Donnell, Tiwari, Bourke, & Cullen, 2010).

Ultrasound treatment of food proteins has been related to affect the physicochemical properties of a number of protein sources including soy protein isolate/concentrate (including soy flakes; Arzeni et al., 2012; Hu et al., 2013; Jambrak, Lelas, Mason, Krešić, & Badanjak, 2009; Karki et al., 2009, 2010) and egg white protein (Arzeni et al., 2012; Arzeni, Pérez, & Pilosof, 2012; Krise, 2011). Arzeni et al., (2012(a), 2012(b)) studied the effect of ultrasound upon the structural and emulsifying properties of egg white protein (EWP) and observed an increase in the hydrophobicity and emulsion stability of ultrasound treated EWP by comparison to untreated EWP. In addition, Krise, (2011) reported no significant reduction in the primary protein structure molecular weight profile of EWP after sonication at 55 kHz for 12 minutes. Similarly, Karki et al., (2010) and Hu et al., (2013) observed no significant changes in the primary protein structure molecular weight profile of ultrasound treated soy protein. Furthermore, Arzeni et al., (2012) described a significant reduction in protein aggregate size for soy protein isolate (SPI). However, the effect of ultrasound treatment upon

gelatin, either mammalian or piscine derived, pea protein isolate or rice protein isolate has yet to be investigated.

Gelatin is a highly versatile biopolymer widely used in a myriad of industries, from the food industry for gelation and viscosity enhancement, and the pharmaceutical industry for the manufacture of soft and hard capsules (Duconseille, Astruc, Quintana, Meersman, & Sante-Lhoutellier, 2014; Haug, Draget, & Smidsrød, 2004; Schrieber & Gareis, 2007). Gelatin is prepared from the irreversible hydrolysis of collagen (a water insoluble structural protein of connective tissues in animals) under either acidic or alkaline conditions in the presence of heat, yielding a variety of peptide-chain species (Schrieber & Gareis, 2007; Veis, 1964). Gelatin is a composite mixture of three main protein fractions: free α-chains, β-chains, the covalent linkage between two α-chains, and γ-chains, the covalent linkage between three α-chains (Haug & Draget, 2009). Gelatin is unique among proteins owing to the lack of appreciable internal structuring, so that in aqueous solutions at sufficiently high temperatures the peptide chains take up random configurations, analogous to the behaviour of synthetic linear-chain polymers (Veis, 1964).

Egg white protein (EWP) is a functional ingredient widely used in the food industry, due to its emulsifying, foaming and gelation capabilities, and utilised within a wide range of food applications, including noodles, mayonnaise, cakes and confectionary (McClements, 2009; Mine, 2002). EWP is globular in nature with highly defined tertiary and quaternary structures. The main protein fractions of egg white protein include ovalbumin (~55%), ovotransferrin (~12%) and ovomucin (~11%), as well as over 30 other protein fractions (Anton, Nau, & Lechevalier, 2009).

Pea protein isolate (PPI) is a nutritional ingredient used in the food industry owing to its emulsifying (Gharsallaoui, Saurel, Chambin, & Voilley, 2011; Liang & Tang, 2014) and

gelation properties (Sun & Arntfield, 2012), and additionally its hypoallergenic attributes (Boye, Zare, & Pletch, 2010). PPI, a pulse legume, is extracted from *Pisum sativum*, and is the main cultivated protein crop in Europe (Gonzalez-Perez & Arellano, 2009). The major protein fractions found in PPI are albumins (2S; 5 – 80 kDa) and globulins, the major fractions in pulse legumes are legumin (11S; ~40 kDa), vicilin (7S; ~175 kDa) and convicilin (7-8S; ~290 kDa) (Boye et al., 2010; Gonzalez-Perez & Arellano, 2009). Other minor proteins found in pulses include prolamins and glutelins (Saharan & Khetarpaul, 1994).

Soy protein isolate (SPI) is of particular interest to the food industry, as it is the largest commercially available vegetable protein source owing to its high nutritional value and current low cost, and a highly functional ingredient due to its emulsifying and gelling capabilities, however, this functionality is dependent upon the extraction method utilised for the preparation of the isolate (Achouri, Zamani, & Boye, 2012; Molina, Defaye, & Ledward, 2002; Sorgentini, Wagner, & Aiidn, 1995). SPI, extracted from *Glycine max*, is an oilseed legume grown primarily in the United Sates, Brazil, Paraguay and Uruguay (Gonzalez-Perez & Arellano, 2009). Similar to pulse legumes, like PPI, the major protein factions in oilseed legumes are albumins (2S; < 80 kDa) and globulins, the dominant fractions in SPI are glycinin (11S; 300-360 kDa) and β-conglycinin (7S; 150-190 kDa) a trimeric glycoprotein (Gonzalez-Perez & Arellano, 2009; Shewry, Napier, & Tatham, 1995).

Rice protein isolate (RPI) is a food ingredient of great importance, reflected by the large annual consumption of rice, 440 million metric tonnes in 2009 (Romero et al., 2012). Up until recently the protein component of rice (~8%) was usually discarded, as the starch component (~80%) yielded greater commercial value (Cao, Wen, Li, & Gu, 2009; Gonzalez-Perez & Arellano, 2009). Despite rice proteins being common ingredients in gels, ice creams and infant formulae (Chrastil, 1992), few studies have been conducted on these proteins to ascertain emulsifying, foaming and gelling capabilities (Agboola, Ng, & Mills, 2005; Romero

et al., 2012). RPI is extracted from *Oryza sativa*, a cereal grain, and is cultivated primarily in Asia (Gonzalez-Perez & Arellano, 2009). Similar to PPI and SPI, RPI has four main protein fractions albumin (~5%), globulin (~12%), glutelin (~80%) and prolamin (~3%), which are water-, salt-, alkali- and alcohol-soluble, respectively (Juliano, 1985).

In this work, three animal proteins, bovine gelatin (BG), fish gelatin (FG) and egg white protein (EWP), and three vegetable proteins, pea protein isolate (PPI), soy protein isolate (SPI) and rice protein isolate (RPI), all of which are composite mixtures of a number of protein fractions, were investigated in order to assess the significance of high power ultrasound treatment on industrially relevant food proteins. The objectives of this research were to discern the effects of ultrasound treatment upon animal and vegetable proteins, in particular changes in physicochemical properties, measured in terms of size, molecular structure and intrinsic viscosity. Furthermore, differences in the performance of proteins as emulsifiers after ultrasound treatment was assessed in terms emulsion droplet size, emulsion stability and interfacial tension. Oil-in-water emulsions were prepared with either untreated or ultrasound treated BG, FG, EWP, PPI, SPI and RPI at different concentrations and compared between them and to a low molecular weight emulsifier, Brij 97.

2. Materials and Methodology

2.1. Materials

Bovine gelatin (BG; 175 Bloom), cold water fish gelatin (FG; 200 Bloom), egg white protein from chickens (EWP), Brij® 97 and sodium azide were purchased from Sigma Aldrich (UK). Pea protein isolate (PPI), soy protein isolate (SPI) and rice protein isolate (RPI) were all kindly provided by Kerry Ingredients (Listowel, Ireland). The composition of the animal and vegetable proteins used in this study is presented in Table 1, acquired from the material specification forms from suppliers. The oil used was commercially available

rapeseed oil. The water used in all experiments was passed through a double distillation unit (A4000D, Aquatron, UK).

2.2. Methods

2.2.1. Preparation of untreated protein solutions

Bovine gelatin (BG), fish gelatin (FG) and rice protein isolate (RPI) solutions were prepared by dispersion in water and adjusting the pH of the solution to 7.08 ± 0.04 with 1 M NaOH, as the initial pH of the solution is close to the isoelectric point, 5.32, 5.02 and 4.85, for BG, FG and RPI, respectively. BG, FG, EWP, PPI, SPI and RPI were dispersed in water to obtain solutions within a protein concentration range of 0.1 – 10 wt. %, where all the animal proteins were soluble at the range of concentrations, whilst the vegetable proteins possessed an insoluble component regardless of hydration time. Sodium azide (0.02 wt. %) was added to the solution to mitigate against microbial activity.

2.2.2. Ultrasound treatment of protein solutions

An ultrasonic processor (Viber Cell 750, Sonics, USA) with a 12 mm diameter stainless steel probe was used to ultrasound treat 50 ml aliquots of BG, FG, EWP, PPI, SPI and RPI solutions in 100 ml plastic beakers, which were placed in an ice bath to reduce heat gain. The protein solutions were sonicated with a frequency of 20 kHz and amplitude of 95% (wave amplitude of 108 μm at 100% amplitude) for up to 2 minutes. This yielded an ultrasonic power intensity of ~34 W cm^{-2}, which was determined calorimetrically by measuring the temperature rise of the sample as a function of treatment time, under adiabatic conditions. The acoustic power intensity, I_a (W cm^{-2}), was calculated as follows (Margulis & Margulis, 2003):

$$I_a = \frac{P_a}{S_A}\ , where \quad P = m.c_p\left(\frac{dT}{dt}\right) \tag{1}$$

where P_a (W) is the acoustic power, S_A is the surface area of the ultrasound emitting surface (1.13 cm^2), m is the mass of ultrasound treated solution (g), c_p is the specific heat of the medium (4.18 kJ/gK) and dT/dt is the rate of temperature change with respect to time, starting at t = 0 ($^oC/s$).

The temperature of the protein solutions was measured before and after sonication by means of a digital thermometer (TGST3, Sensor-Tech Ltd., Ireland), with an accuracy of ± 0.1 °C. Prior to ultrasound treatment, the temperature of protein solutions was within the range of 5 – 10 ^oC, whilst the temperature BG and FG solutions was within a temperature range of 45 – 50 ^oC, above the helix coil transition temperature. After ultrasonic irradiation, the temperature of all protein solutions raised to approximately ~45 °C.

2.2.3. Characterisation of untreated and ultrasound treated proteins

2.2.3.1. pH measurements

The pH of animal and vegetable protein solutions was measured before and after sonication at a temperature of 20 ^oC. pH measurements were made by using a SevenEasy pH meter (Mettler Toledo, UK). This instrument was calibrated with buffer standard solutions of known pH. The pH values are reported as the average and the standard deviation of three repeat measurements.

2.2.3.2. Microstructure characterisation

The size of untreated and ultrasound treated animal proteins was measured by dynamic light scattering (DLS) using a Zetasizer Nano Series (Malvern Instruments, UK), and the size of untreated and ultrasound treated vegetable proteins was measured by static light scattering (SLS) using the Mastersizer 2000 (Malvern Instruments, UK). Protein size values are reported as Z-average (D_z). The width of the protein size distribution was

194 expressed in terms of span ($Span = D_{v0.9} - D_{v0.1}/D_{v0.5}$), where $D_{v0.9}$, $D_{v0.1}$, and $D_{v0.5}$ are the
195 equivalent volume diameters at 90, 10 and 50% cumulative volume, respectively. Low span
196 values indicate a narrow size distribution. The protein size and span values are reported as the
197 average and the standard deviation of three repeat measurements.

198 **2.2.3.3. Microstructure Visualisation**

199 Cryogenic scanning electron microscopy (Cryo-SEM; Philips XL30 FEG ESSEM) was
200 used to visualise the microstructure of untreated and ultrasound treated proteins. One drop of
201 protein solution was frozen to approximately -180 °C in liquid nitrogen slush. Samples were
202 then fractured and etched for 3 min at a temperature of -90 °C inside a preparation chamber.
203 Afterwards, samples were sputter coated with gold and scanned, during which the temperature
204 was kept below -160 °C by addition of liquid nitrogen to the system.

205 **2.2.3.4. Molecular structure characterisation**

206 The molecular structure of untreated and ultrasound treated animal and vegetable
207 proteins was determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis
208 (SDS-PAGE), using a Mini-Protean 3 Electrophoresis System (Bio-Rad, UK), where proteins
209 were tested using the reducing method. 100 µL of protein solution at a concentration of 1 wt.
210 % was added to 900 µL of Laemmli buffer (Bio-Rad, UK; 65.8 mM Tris-HCl, 2.1% SDS,
211 26.3% (w/v) glycerol, 0.01% bromophenol blue) and 100 µL of β-mercaptoethanol (Bio-Rad,
212 UK) in 2 mL micro tubes and sealed. These 2 mL micro tubes were placed in a float in a
213 water bath at a temperature of 90 ^{o}C for 30 minutes, to allow the reduction reaction to take
214 place. A 10 µL aliquot was taken from each sample and loaded onto a Tris-acrylamide gel
215 (Bio-Rad, UK; 4-20% Mini Protean TGX Gel, 10 wells). A molecular weight standard (Bio-
216 Rad, UK; Precision Plus Protein^{TM} All Blue Standards) was used to determine the primary
217 protein structure molecular weight profile of the samples. Gel electrophoresis was carried out

initially at 55 V (I > 20 mA) for 10 min, then at 155 V (I > 55 mA) for 45 min in a running buffer (10x Tris/Glycine/SDS Buffer, Bio-Rad, UK; 4% Tris, 15% glycine, 0.5% SDS). The gels were removed from the gel cassette and stained with Coomassie Bio-safe stain (Bio-Rad, UK; 4% phosphoric acid, 0.5% methanol, 0.05% ethanol) for 1 hr and de-stained with distilled water overnight.

2.2.3.5. Intrinsic viscosity measurements

The intrinsic viscosity of untreated and ultrasound treated animal and vegetable proteins was determined by a double extrapolation to a zero concentration method, as described by Morris, Cutler, Ross-Murphy, Rees, & Price, (1981), using the models of Huggins' and Kraemer, as follows:

Huggins (Huggins, 1942): $$\frac{\eta_{sp}}{c} = [\eta] + k_H[\eta]^2 c \quad (2)$$

Kraemer (Kraemer, 1938): $$\frac{\ln \eta_{rel}}{c} = [\eta] + k_K[\eta]^2 c \quad (3)$$

where η_{sp} is the specific viscosity (viscosity of the solvent, η_0 / viscosity of the solution, η), c the protein concentration (w/v%), $[\eta]$ the intrinsic viscosity (dL/g), k_H the Huggins constant. η_{rel} is the relative viscosity (viscosity of the solution, η / viscosity of the solvent, η_0) and k_K is the Kraemer constant.

The concentration ranges used for the determination of the intrinsic viscosity of BG, FG, EWP, PPI, SPI and RPI were 0.1 – 0.5 wt. %, 0.25 – 1.5 wt. %, 1.5 – 3 wt. %, 0.5 – 0.8 wt. %, 1.5 – 3 wt. % and 0.5 – 2 wt. %, respectively. The validity of the regression procedure is confined within a discrete range of η_{rel}, $1.2 < \eta_{rel} < 2$. The upper limit is due to the hydrodynamic interaction between associates of protein molecules, and the lower limit is due to inaccuracy in the determination of very low viscosity fluids. A value of η_{rel} approaching 1 indicates the lower limit (Morris et al., 1981).

241 The viscosity of the protein solutions was measured at 20 °C using a Kinexus
242 rheometer (Malvern Instruments, UK) equipped with a double gap geometry (25 mm
243 diameter, 40 mm height). For the determination of intrinsic viscosity by extrapolation to
244 infinite dilution, there must be linearity between shear stress and shear rate, which indicates a
245 Newtonian behaviour region on the range of shear rate used in the measurements. The
246 Newtonian plateau region of the BG, FG, EWP, PPI, SPI and RPI solutions at the range of
247 concentrations used, was found within a shear rate range of 25 - 1000 s^{-1} (data not shown).
248 Thus, the values of viscosity of the protein solutions and that of the solvent (distilled water)
249 were selected from the flow curves data at a constant shear rate of 250 s^{-1} (within the
250 Newtonian region), which were subsequently used to determine the specific viscosity, η_{sp}, the
251 relative viscosity, η_{rel}, and the intrinsic viscosity, $[\eta]$. At least three replicates of each
252 measurement were made.

253 **2.2.4. Preparation of oil-in-water emulsions**

254 10 wt. % dispersed phase (rapeseed oil) was added to the continuous aqueous phase
255 containing either untreated or sonicated animal or vegetable proteins or Brij 97 at different
256 concentrations, ranging from 0.1 to 10 wt. %. An oil-in-water pre-emulsion was prepared by
257 emulsifying this mixture at 8000 rpm for 2 min using a high shear mixer (SL2T, Silverson,
258 UK). Submicron oil-in-water emulsions were then prepared by further emulsifying the pre-
259 emulsion using a high-pressure valve homogeniser (Panda NS 1001L-2K, GEA Niro Soavi,
260 UK) at 125 MPa for 2 passes. The initial temperature of EWP, PPI, SPI and RPI emulsions
261 was a temperature of 5 ^{o}C to prevent thermal denaturation of proteins from high pressure
262 homogenisation, whilst denaturation may still occur due the high shear during high pressure
263 processing. The initial temperature of BG and FG emulsions was at a temperature of 50 ^{o}C to
264 prevent gelation of gelatin (bovine or fish) during the homogenisation process. High pressure

265 processing increases the temperature of the processed material, and consequently, the final
266 temperatures of emulsions prepared with EWP, PPI, SPI and RPI, and gelatin (BG and FG),
267 after homogenisation were ~45 ^{o}C and ~90 ^{o}C, respectively.

268 **2.2.5. Characterisation of oil-in-water emulsions.**

269 **2.2.5.1. Droplet size measurements**

270 The droplet size of the emulsions was measured by SLS using a Mastersizer 2000
271 (Malvern Instruments, UK) immediately after emulsification. Emulsion droplet size values
272 are reported as the volume-surface mean diameter (Sauter diameter; $d_{3,2}$). The stability of the
273 emulsions was assessed by droplet size measurements over 28 days, where emulsions were
274 stored under refrigeration conditions (4 ^{o}C) throughout the duration of the stability study.
275 The droplet sizes and error bars are reported as the mean and standard deviation, respectively,
276 of measured emulsions prepared in triplicate.

277 **2.2.5.2. Interfacial tension measurements**

278 The interfacial tension between the aqueous phase (pure water, animal or vegetable
279 protein solutions, or surfactant solution) and oil phase (rapeseed oil) was measured using a
280 tensiometer K100 (Krűss, Germany) with the Wilhelmy plate method. The Wilhelmy plate
281 has a length, width and thickness of 19.9 mm, 10 mm and 0.2 mm, respectively and is made
282 of platinum. The Wilhelmy plate was immersed in 20 g of aqueous phase to a depth of 3 mm.
283 Subsequently, an interface between the aqueous phase and oil phase was created by carefully
284 pipetting 50 g of the oil phase over the aqueous phase. The test was conducted over 3,600 s
285 and the temperature was maintained at 20 °C throughout the duration of the test. The
286 interfacial tension values and the error bars are reported as the mean and standard deviation,
287 respectively, of three repeat measurements.

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2.2.5.3. Emulsion Visualisation

Cryogenic scanning electron microscopy (Cryo-SEM; Philips XL30 FEG ESSEM) was used to visualise the microstructure of pre-emulsions using untreated and sonicated proteins. One drop of pre-emulsion was frozen to approximately -180 °C in liquid nitrogen slush. Samples were then fractured and etched for 3 min at a temperature of -90 °C inside a preparation chamber. Afterwards, samples were sputter coated with gold and scanned, during which the temperature was kept below -160 °C by addition of liquid nitrogen to the system.

2.3. Statistical analysis

Student's t-test with a 95% confidence interval was used to assess the significance of the results obtained. t-test data with $P < 0.05$ were considered statistically significant.

3. Results and Discussion

3.1. Effect of ultrasound treatment on the structural and physical properties of BG, FG, EWP, PPI, SPI and RPI

The effect of duration of ultrasonic irradiation on the size and pH of BG, FG, EWP, PPI, SPI and RPI was initially investigated. 0.1 wt. % solutions of BG, FG, EWP, PPI, SPI and RPI were sonicated for 15, 30, 60 and 120 s, with an ultrasonic frequency of 20 kHz and an amplitude of 95%. Protein size and pH measurements for untreated, and ultrasound treated BG, FG, EWP, PPI, SPI and RPI as a function of time are shown in Fig. 1 and Table 2. The size of the vegetable proteins isolates presented in Fig. 1 prior to sonication (i.e. $t = 0$) are in a highly aggregated state due to protein denaturation from the processing to obtain these isolates. Fig. 1 shows that there is a significant reduction ($P < 0.05$) in protein size with an increase in the sonication time, and the results also highlight that after a sonication of 1 minute there is minimal further reduction in protein size of BG, FG, EWP, PPI and SPI. This

311 decrease in protein size is attributed to disruption of the hydrophobic and electrostatic
312 interactions which maintain untreated protein aggregates from the high hydrodynamic shear
313 forces associated with ultrasonic cavitations. However, there is no significant reduction ($P > 0.05$) in the size of RPI agglomerates, irrespective of treatment time, due to the highly
314 aggregated structure of the insoluble component of RPI, ascribed to both the presence of
315 carbohydrate within the aggregate structure and the denaturation of protein during the
316 preparation of the protein isolate, restricting size reduction by way of ultrasound treatment
317 (Guraya & James, 2002; Marshall & Wadsworth, 1994; Mujoo, Chandrashekar, & Zakiuddin
318 Ali, 1998). The pH of all animal and vegetable protein solutions, with the exception of RPI,
319 decreased significantly ($P < 0.05$) with increasing sonication time. Equivalent to the protein
320 size measurements, after a treatment time of 1 min the pH of protein solutions decreased no
321 further. The decrease in pH of animal and vegetable protein solutions is thought to be
322 associated with the transitional changes resulting in deprotonation of acidic amino acid
323 residues (Sakurai, Konuma, Yagi, & Goto, 2009) which were contained within the interior of
324 associated structures of untreated proteins prior to ultrasound treatment. Our results are in
325 agreement with those of O'Sullivan et al., (2014), who showed that an increased sonication
326 led to a significant reduction of protein size and pH for dairy proteins up to a sonication time
327 of 1 min, as with animal and vegetable proteins, with an ultrasound treatment of 20 kHz and
328 an amplitude of 95%.

329 The stability of sonicated animal and vegetable proteins solutions as a function of
330 time was investigated by protein size and protein size distribution (span) of sonicated BG,
331 FG, EWP, PPI, SPI and RPI. Animal and vegetable protein solutions with a concentration of
332 0.1 wt. % were ultrasound treated at 20 kHz and ~34 W cm^{-2} for a sonication time of 2 min,
333 as no further decrease in protein size after a sonication time of 1 min was observed (cf. Table
334 2). The protein size and span values of sonicated animal and vegetable proteins were
335

336 measured immediately after treatment and after 1 and 7 days, in order to assess the stability
337 of protein size and protein size distribution. Protein size measurements and span values
338 obtained from DLS and SLS for untreated and ultrasound treated BG, FG, EWP, PPI, SPI and
339 RPI are shown in Table 3.

340 As can be seen from Table 3, ultrasound treatment produced a significant reduction ($P < 0.05$)
341 in the size and span of BG, FG and EWP. However, 7 days after sonication an
342 increase in the size and the broadening of the distribution was observed for BG, FG and
343 EWP. The effective size reduction of the ultrasound treatment to BG, FG and EWP on day 7
344 was 85.6%, 80% and 74.25% respectively. In the case of PPI and SPI, the results in Table 3
345 show that ultrasound treatment significantly ($P < 0.05$) reduced the aggregate size and a
346 broadening of the protein size distribution. The size distribution of PPI and SPI after
347 ultrasound treatment is bimodal, one population having a similar size as the parent untreated
348 protein, and the other population is nano-sized (~120 nm). The span of the distribution and
349 protein size on day 7 for PPI and SPI was quite similar to that after immediate sonication,
350 representing an effective protein size reduction of 95.7% and 82.3% for PPI and SPI
351 respectively. This significant reduction in aggregate size of both PPI and SPI from ultrasound
352 treatment allows for improved solubilisation and prolonged stability of these vegetable
353 protein isolates to sedimentation. Our results are in agreement with those of Jambrak et al.,
354 (2009), who observed a significant reduction in the size of SPI aggregates. Arzeni et al.,
355 (2012) also observed a decrease in the protein size for sonicated SPI but an increase in size
356 for EWP treated by ultrasound, whereby this increase in size of EWP aggregates is associated
357 with thermal aggregation during the ultrasound treatment. The reason for the observed
358 decrease in the protein size of BG, FG, EWP, PPI and SPI is due to disruption of non-
359 covalent associative forces, such as hydrophobic and electrostatic interactions, and hydrogen
360 bonding, which maintain protein aggregates in solution induced by high levels hydrodynamic

shear and turbulence due to ultrasonic cavitations. The observed increase in size for BG, FG and EWP after 7 days is thought to be due to reorganisation of proteins into sub-aggregates due to non-covalent interactions (electrostatic and hydrophobic). In the case of PPI and SPI, the static size observed is due to the more defined structure of the PPI and SPI aggregates in comparison to the fully hydrated animal proteins, which allows for greater molecular interactions and mobility (Veis, 1964). In order to validate these hypotheses, cryo-SEM micrographs were captured of untreated and 7 days after sonication of BG, EWP, SPI and PPI solution at 1 wt. % for all proteins tested (Fig. 2).

Untreated BG in solution (cf. Fig. 2a) appears to be distributed into discrete fibres, which is consistent with the literature, describing gelatin as a fibrous protein (Schrieber & Gareis, 2007; Veis, 1964), whilst BG treated by ultrasound (cf. Fig. 2b) appears to be in the form of fibrils of the parent untreated BG fibre, where the width of the fibres and the fibrils is equivalent, yet the length of the fibrils is shorter than the untreated BG fibres. In the case of untreated SPI (cf. Fig. 2c) large aggregates of protein can be seen, composed of discrete entities, whereas sonicated SPI (cf. Fig. 2d) has a notably reduced protein size, with a monodisperse size distribution. Similar results were observed for FG, EWP and PPI (data not shown). These results are in agreement with previously discussed observations (cf. Table 3), and adds evidence to the hypothesis that ultrasound treatment causes disruption of protein aggregates, that subsequently reorganise themselves into smaller sub-associates.

The molecular structure of untreated and ultrasound treated animal and vegetable proteins was investigated next. Protein solutions at a concentration of 1 wt. % were ultrasound treated for 2 min at 20 kHz, with a power intensity of ~34 W cm^{-2}. Electrophoretic profiles obtained by SDS-PAGE for untreated and ultrasound treated BG, FG, EWP, SPI, PPI and RPI, and the molecular weight standard, are shown in Fig. 3. No difference in the protein fractions was observed between untreated and sonicated BG, FG, EWP, SPI, PPI and RPI (cf.

Fig. 3). These results are in concurrence with those reported by Krise, (2011) who showed no difference in the primary structure molecular weight profile between untreated and ultrasound treated egg white, with a treatment conducted at 55 kHz, 45.33 W cm^{-2} for 12 min. Moreover, the obtained protein fractions are in agreement with the literature for gelatin (Gouinlock, Flory, & Scheraga, 1955; Veis, 1964), EWP (Anton et al., 2009), SPI (Gonzalez-Perez & Arellano, 2009), PPI (Sun & Arntfield, 2012) and RPI (Hamaker, 1994; Juliano, 1985).

The intrinsic viscosity, $[\eta]$, was obtained by the fitting of experimental viscosity data to the Huggins' and Kraemer equations, for untreated and ultrasound irradiated animal and vegetable protein solutions, as shown in Fig. 4 for EWP and PPI. The other proteins investigated as part of this study (BG, FG, SPI and RPI) display similar behaviour to EWP (i.e. negative k_H and k_K values). The values of $[\eta]$ and the Huggins', k_H, and Kraemer, k_K, constants for each of the proteins investigated in this study are listed in Table 4.

Intrinsic viscosity, $[\eta]$, demonstrates the degree of hydration of proteins and provides information about the associate hydrodynamic volume, which is related to molecular conformation of proteins in solution (Behrouzian, Razavi, & Karazhiyan, 2014; Harding, 1997; Sousa, Mitchell, Hill, & Harding, 1995). A comparison of the $[\eta]$ between untreated and ultrasound treated animal and vegetable proteins (cf. Table 4) demonstrates that ultrasound treatment induced a significant reduction ($P < 0.05$) in the intrinsic viscosity of BG, FG, EWP, PPI and SPI in solution, and consequently a significant reduction in the hydrodynamic volume occupied by the proteins and the solvents entrained within them. These results are in agreement with the reduction in associate size (cf. Table 3) and cryo-SEM micrographs (cf. Fig. 2), however, for the case of RPI, there is no reduction in the intrinsic viscosity, which is consistent with the previous size measurements (cf. Table 3). Gouinlock, Flory, & Scheraga, (1955), Lefebvre, (1982) and Prakash, (1994) reported intrinsic viscosity values of 6.9 dL/g for gelatin, 0.326 dL/g for ovalbumin and 0.46 dL/g for

glycinin (11S; soy globulin), respectively. These values differ to those obtained in this work untreated BG, EWP and SPI (cf. Table 4). These differences may be a consequence of the complexity of EWP and SPI solutions, which are composed of a mixture of protein fractions rather than single component ovalbumin and glycinin (Lefebvre, 1982; Prakash, 1994), and in case of gelatin, differences may arise due to variability in preparation of the gelatin from collagen, which determines the molecular weight profile of the resulting gelatin (Veis, 1964). Extrinsic variations in solvent quality greatly affect the determination of intrinsic viscosity and further accounts for the differences between the single fraction proteins and the multi-component proteins investigated in this study. Extrinsic factors affecting intrinsic viscosity include temperature, pH, initial mineral content and composition, co-solvents, additional salts and their concentration (Harding, 1997). Furthermore, the large $[\eta]$ of both BG and FG by comparison to the other proteins investigated as part of this study is due to the random coil conformation of these molecules in solutions, which consequently entrain more water giving a larger overall hydrodynamic volume.

Intrinsic viscosity of a protein solution can be used to indicate the degree of hydrophobicity of the protein (Tanner & Rha, 1980). The intrinsic viscosity of protein associates in solution is dependent on its conformation and degree of hydration, which dictate the amount of hydrophobic residues that are within the interior of protein associates. A decrease in the intrinsic viscosity also leads to dehydration of amphiphilic biopolymers, increasing the hydrophobicity of the biopolymer and thus reducing the energy required for adsorption of amphiphilic biopolymers to the oil-water interface (Khan, Bibi, Pervaiz, Mahmood, & Siddiq, 2012). Thus, the significant reduction ($P < 0.05$) of intrinsic viscosity induced by ultrasound treatment (cf. Table 4), expresses an increase in the degree of hydrophobicity of BG, FG, EWP, PPI and SPI.

The Huggins' and Kraemer coefficients are adequate for the assessment of solvent quality. Positive values of the Huggins' coefficient, k_H, within a range of 0.25 – 0.5 indicate good solvation, whilst k_H values within a range of 0.5 – 1.0 are related to poor solvents (Delpech & Oliveira, 2005; Pamies, Hernández Cifre, del Carmen López Martínez, & García de la Torre, 2008). Conversely negative values for the Kraemer coefficient, k_K, indicate good solvent, yet positive values express poor solvation (Delpech & Oliveira, 2005; Harding, 1997; Pamies et al., 2008). The values for the k_H and k_K (cf. Table 4) are both negative, with the exception of untreated PPI exhibiting a positive k_H value, indicating good solvation when considering k_K, yet unusual behaviour in the case of k_H. Nonetheless, negative values of k_H have been reported in the literature for biopolymers with amphiphilic properties, such as bovine serum albumin (Curvale, Masuelli, & Padilla, 2008), sodium caseinate, whey protein isolate and milk protein isolate (O'Sullivan et al., 2014; O'Sullivan, Pichot, & Norton, 2014), all dispersed within serum. Positive k_H values are associated with uniform surface charges of polymers (Sousa et al., 1995), indicating that untreated PPI aggregates have a uniform surface charge, and after ultrasound treatment conformational changes occur yielding an amphiphatic character on the surface of the ultrasound treated PPI, observed by the negative k_H value. It is also important to observe that the relation $k_H + k_K = 0.5$, generally accepted to indicate adequacy of experimental results for hydrocolloids, was not found for any of the proteins investigated in this study (cf. Table 4). This effect is thought to be associated with the amphiphatic nature of the proteins used in this study (by comparison to non-amphiphilic polysaccharides) yielding negative values of k_H and k_K. Similar results have been reported in the literature for other amphiphilic polymers (Curvale et al., 2008; O'Sullivan, Arellano, et al., 2014; Yilgor, Ward, Yilgor, & Atilla, 2006). In addition, the values of k_H and k_K tend to decrease after ultrasound treatment indicating improved solvation of proteins (Delpech & Oliveira, 2005).

460 *3.2. Comparison of the emulsifying properties of untreated and ultrasound treated BG, FG,*
461 *EWP, PPI, SPI and RPI*

462 Oil-in-water emulsions were prepared with 10 wt. % rapeseed oil and an aqueous
463 continuous phase containing either untreated or ultrasound irradiated (2 min at 20 kHz, ~34
464 W cm^{-2}) BG, FG, EWP, PPI, SPI and RPI, or a low molecular weight surfactant, Brij 97, at a
465 range of emulsifier concentrations (0.1 – 10 wt. %). Emulsions were prepared using high-
466 pressure valve homogenisation (125 MPa for 2 passes) and droplet sizes as a function of
467 emulsifier type and concentration are shown in Fig. 5. The emulsion droplet sizes were
468 measured immediately after emulsification, and all exhibited unimodal droplet size
469 distributions.

470 Emulsions prepared with sonicated BG (cf. Fig 5a), EWP (cf. Fig. 5c) and PPI (cf.
471 Fig. 5d) at concentrations < 1 wt. % yielded a significant ($P < 0.05$) reduction in emulsion
472 droplet size by comparison to their untreated counterparts. At concentrations ≥ 1 wt. % the
473 emulsions prepared with untreated and ultrasound treated BG, EWP and PPI exhibited similar
474 droplet sizes. The decrease in emulsion droplet size after ultrasound treatment at
475 concentrations < 1 wt. % is consistent with the significant reduction ($P < 0.05$) in protein size
476 (increase in surface area-to-volume ratio) upon ultrasound treatment of BG, EWP and PPI
477 solutions (cf. Table 3) which allows for more rapid adsorption of protein to the oil-water
478 interface, as reported by Damodaran & Razumovsky (2008). In addition, the significant
479 increase of hydrophobicity of ultrasound treated BG, EWP and PPI and the decrease in
480 intrinsic viscosity (cf. Table 4; Khan et al., 2012) would lead to an increased rate of protein
481 adsorption to the oil-water interface, reducing interfacial tension allowing for improved
482 facilitation of droplet break-up. The submicron droplets obtained for untreated PPI are in
483 agreement with droplet sizes obtained by those measured by Donsì, Senatore, Huang, &
484 Ferrari (2010), in the order of ~200 nm for emulsions containing pea protein (4 wt. %).

Emulsions prepared with the tested concentrations of untreated and ultrasound treated FG (cf. Fig. 5b), SPI (data not shown) and RPI (data not shown) yielded similar droplet sizes, where emulsions prepared with 0.1 wt. % FG yielded emulsion droplets ~5 μm, and both SPI and RPI yielded ~2 μm droplets at the same concentration. Furthermore, at similar concentrations PPI yielded smaller emulsion droplets than those prepared with SPI, making SPI a poorer emulsifier, in agreement with the results of Vose, (1980). This behaviour was anticipated for RPI, where no significant reduction ($P > 0.05$) in protein size was observed (cf. Table 3), yet unexpected when considering the significant reduction ($P < 0.05$; increase in surface area-to-volume ratio) of protein size observed for both sonicated FG and SPI (cf. Table 3). Moreover, the significant increase in hydrophobicity of ultrasound treated FG and SPI expressed by the decrease in intrinsic viscosity (cf. Table 4; Khan et al., 2012; Tanner & Rha, 1980) would also be expected to result in faster adsorption of protein to the oil-water interface, however it appears that the rate of protein adsorption of ultrasound treated FG and SPI to the oil-water interface remains unchanged regardless of the smaller protein associate sizes and increase in hydrophobicity, when compared with untreated FG and SPI. Even though ultrasound treatment reduces the aggregate size of SPI, proteins possessing an overall low molecular weight, such as EWP (ovalbumin is ~44 kDa), are capable of forming smaller emulsion droplets than larger molecular weight proteins (glycinin is 360 kDa) as lower molecular weight species have greater molecular mobility through the bulk for adsorbing to oil-water interfaces (Beverung et al., 1999; Caetano da Silva Lannes & Natali Miquelim, 2013). The submicron droplets achieved for untreated FG are consistent with droplet sizes obtained by Surh, Decker, & McClements (2006), in the order of ~300 nm for emulsions containing either low molecular weight (~55 kDa) or high molecular weight (~120 kDa) fish gelatin (4 wt. %).

509 At protein concentrations > 1 wt. % for emulsions prepared with either untreated or
510 ultrasound treated EWP (cf. Fig. 5c), SPI and RPI micron sized entities (> 10 μm) were
511 formed. Unexpectedly, emulsions prepared with PPI did not exhibit the formation of these
512 entities, even though the structure of PPI is similar to that of SPI. The degree and structure of
513 the denatured component of PPI likely varies to that of SPI and accounts for the non-
514 aggregating behaviour of PPI. Emulsions being processed using high pressure
515 homogenisation experience both increases in temperature and regions of high hydrodynamic
516 shear, both of these mechanisms result in denaturation of proteins. These micron sized
517 entities are attributed to denaturation and aggregation of protein due to the high levels of
518 hydrodynamic shear present during the homogenisation process, as thermal effects were
519 minimised by ensuring that the emulsions were processed at a temperature of 5 ^{o}C, and the
520 outlet temperature was less than 45 ^{o}C in all cases, lower than the thermal denaturation
521 temperatures of EWP, SPI and RPI (Ju, Hettiarachchy, & Rath, 2001; Sorgentini et al., 1995;
522 Van der Plancken, Van Loey, & Hendrickx, 2006). Hydrostatic pressure induced gelation of
523 EWP, SPI and RPI has been reported in the literature (Messens, Van Camp, & Huyghebaert,
524 1997; Molina et al., 2002; Tang & Ma, 2009; Zhang-Cun et al., 2013) and the formation of
525 these entities is attributed to the high shear forces exerted upon the proteins while under high
526 shear conditions, whereby the excess of bulk protein allows for greater interpenetration of
527 protein chains under high shear yielding the formation of discrete entities composed of oil
528 droplets within denatured aggregated protein. Unexpectedly, emulsions prepared with a
529 higher concentration of protein (10 wt. %) yielded a significant ($P < 0.05$) reduction in entity
530 size in comparison to those prepared with the lower concentration (5 wt. %). This behaviour
531 is ascribed to an increased rate of formation and number of aggregates formed at higher
532 concentrations during the short time within the shear field.

Emulsion droplets sizes for all animal and vegetable proteins investigated (cf. Fig. 5) are smaller than that of the size of the untreated proteins (cf. Table 3). Be that as it may, the reported proteins sizes (cf. Table 3) represent aggregates of protein molecules and not discrete protein fractions. Native ovalbumin and glycinin have hydrodynamic radii (R_h) of approximately 3 nm and 12.5 nm respectively (García De La Torre, Huertas, & Carrasco, 2000; Peng, Quass, Dayto, & Allen, 1984), in comparison to size data presented in Table 3, whereby the EWP and SPI have D_z values of EWP and SPI of approximately 1.6 and 1.7 μm, respectively. This disparity in size is due to the preparation of these protein isolates whereby shear and temperature result in the formation of insoluble aggregated material, in comparison to the soluble native protein fractions. Proteins in aqueous solutions associate together to form aggregates due to hydrophobic and electrostatic interactions (O'Connell, Grinberg, & de Kruif, 2003), however in the presence of a hydrophobic dispersed phase (i.e. rapeseed oil) the protein fractions which comprise the aggregate disassociates and adsorb to the oil-water interface (Beverung et al., 1999; O'Connell & Flynn, 2007), which accounts for the fabrication of submicron droplets presented in this study.

The emulsion droplet sizes presented in Fig. 5, which were shown to be dependent on the emulsifier type, can be interpreted by comparing the interfacial tension of the studied systems. Fig. 5 presents the interfacial tension between water and rapeseed oil, for untreated and ultrasound treated BG, FG, PPI and SPI, and Brij 97, all at an emulsifier concentration of 0.1 wt. %. In order to assess the presence of surface active impurities within the dispersed phase, the interfacial tension between distilled water and rapeseed oil was measured. Fig. 6 shows that the interfacial tension of all systems decreases continually as a function of time. In light of these results, the decrease of interfacial tension with time is attributed primarily to the nature of the dispersed phase used, and to a lesser degree the type of emulsifier. Gaonkar (1989, 1991) explained that the time dependent nature of interfacial tension of commercially

available vegetable oils against water was due to the adsorption of surface active impurities present within the oils at the oil-water interface. Gaonkar, (1989, 1991) also reported that after purification of the vegetable oils (percolation through a synthetic magnesium silicate bed), the time dependency of interfacial tension was no longer observed.

No significant differences ($P > 0.05$) were observed in the obtained values of interfacial tension between untreated and ultrasound treated FG (cf. Fig. 6b) and RPI (data not shown). These results are consistent with droplet size data, where no significant difference in the droplet size was observed. Significant differences were shown for the initial rate of decrease of interfacial tension when comparing untreated and ultrasound treated PPI (cf. Fig. 6c). Ultrasound treated PPI aggregates are smaller than untreated PPI (cf. Table 3) and have greater hydrophobicity (i.e. reduction in $[\eta]$; cf. Table 4) accounting for the significant reduction of initial interfacial tension, enhancing droplet break-up during emulsification. Significant differences ($P < 0.05$) in the equilibrium interfacial tension values were observed when comparing untreated and sonicated BG (cf. Fig. 6a), EWP (data not shown) and SPI (cf. Fig. 6d). These results are consistent with the observed significant reduction ($P < 0.05$) in emulsion droplet size for BG (cf. Fig. 5a) and EWP (cf. Fig. 5c) and adds evidence to the hypotheses that aggregates of sonicated BG and EWP adsorb faster to the interface due to higher surface area-to-volume ratio (cf. Table 3; smaller protein size) and increased hydrophobicity (i.e. reduction in $[\eta]$; cf. Table 4), significantly reducing the equilibrium interfacial tension, yielding smaller emulsion droplets. No significant reduction ($P > 0.05$) in emulsion droplet size was noted for SPI, despite the observed reduction in equilibrium interfacial tension of SPI (cf. Fig. 6d) which may be a consequence of alternative protein conformations at the oil-water interface. These hypotheses were explored by cryo-SEM of pre-emulsions, to allow for visualisation emulsion droplet interface, prepared with

582 untreated and ultrasound treated BG and SPI at an emulsifier concentration of 1 wt. % for all
583 pre-emulsions tested (cf. Fig. 7).

584 Emulsion droplets of pre-emulsions prepared with untreated BG (cf. Fig. 7a) show
585 fibres of gelatin tracking around the surface of the droplets whereas emulsion droplets of pre-
586 emulsions prepared with ultrasound treated BG (cf. Fig. 7b) show the smaller fibrils of
587 gelatin at the interface of the droplets, yielding improved interfacial packing of protein,
588 accounting for the lower equilibrium interfacial tension (cf. Fig. 6a) and the decrease in
589 droplet size (cf. Fig. 5a). The droplet surfaces of pre-emulsions prepared with ultrasound SPI
590 (cf. Fig. 7d) appear to be are smoother by comparison to the seeming more textured droplet
591 interfaces observed for pre-emulsions prepared with untreated SPI (cf. Fig. 7c). These
592 findings are consistent with the interfacial tension data (cf. Fig. 6), where a significant
593 reduction ($P < 0.05$) of the equilibrium interfacial tension upon sonication of BG and SPI
594 was observed, and accounted for by visualisation of the improved interfacial packing of
595 protein.

596 The stability of oil-in-water emulsions prepared with untreated and sonicated BG, FG,
597 EWP, PPI, SPI and RPI, and Brij 97 for comparative purposes, was assessed over a 28 day
598 period. Fig. 8 shows the development of droplet size ($d_{3,2}$) as a function of time for emulsions
599 prepared with untreated and ultrasound irradiated BG, FG, PPI and SPI, as well as Brij 97, at
600 an emulsifier concentration of 0.1 wt. %.

601 Emulsions prepared with untreated BG (cf. Fig. 8a) exhibited a growth in droplet size,
602 and this coalescence was also observed for emulsions prepared with 0.5 wt. % untreated BG,
603 while emulsions prepared with higher concentrations ($\geq$ 1 wt. %) of untreated BG were stable
604 for the 28 days of the study (data not shown). However, it can also be seen (cf. Fig. 8a) that
605 emulsions prepared with ultrasound treated BG were resistant to coalescence over the 28 days

of the study, and had the same stability of Brij 97. The behaviour exhibited by 0.1 wt. % ultrasound treated BG was observed at all concentrations investigated in this study (data not shown). This improved stability of ultrasound treated BG by comparison to untreated BG is thought to be associated with an increase in the hydrophobicity (i.e. decrease in the intrinsic viscosity; cf. Table 4) and improved interfacial packing of ultrasound treated BG by comparison to untreated BG as observed by a decrease in the equilibrium interfacial tension (cf. Fig. 6a) and cryo-SEM visualisation (cf. Fig. 7a, b). In contrast, results in Fig 8b show that emulsions prepared with both untreated and ultrasound treated FG display coalescence, yet ultrasound treated FG displayed a notable decrease in emulsion stability by comparison to untreated FG. The emulsion stability of untreated and ultrasound treated FG is analogous to untreated BG, where coalescence was observed at concentration of 0.5 wt. %, and stable emulsions were achieved with higher emulsifier concentrations (≥ 1 wt. %; data not shown). This decrease in emulsion stability after ultrasound treatment of FG is thought to be associated with a weaker interfacial layer of ultrasound treated FG by comparison to untreated FG allowing for a greater degree of coalescence, accounting for the decrease in emulsion stability. Emulsions prepared with either untreated or sonicated EWP (data not shown), PPI (cf. Fig. 8c), SPI (cf. Fig. 8d) and RPI (data not shown), and Brij 97 (cf. Fig 8) were all stable against coalescence and bridging flocculation over the 28 days of this study. This stability was observed for all concentrations probed in this study (≥ 0.5 wt. %) of untreated and ultrasound treated EWP, PPI, SPI and RPI investigated, as well as for Brij 97 (data not shown). In all cases no phase separation was observed in the emulsions, whilst emulsions with droplet sizes > 1 μm exhibited gravitational separation with a cream layer present one day after preparation. Furthermore, the $d_{3,2}$ is lower in all cases at an emulsifier concentration of 0.1wt. % for ultrasound treated proteins by comparison to that of their untreated counterparts, as previously discussed.

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4. Conclusions

This study showed that ultrasound treatment (20 kHz, ~34 W cm^{-2} for 2 min) of animal and vegetable proteins significantly ($P < 0.05$) reduced aggregate size and hydrodynamic volume, with the exception of RPI. The reduction in protein size was attributed to the hydrodynamic shear forces associated with ultrasonic cavitations. In spite of the aggregate size reduction, no differences in primary structure molecular weight profile were observed between untreated and ultrasound irradiated BG, FG, EWP, PPI, SPI and RPI.

Unanticipatedly, emulsions prepared with the ultrasound treated FG, SPI and RPI proteins had the same droplet sizes as those obtained with their untreated counterparts, and were stable at the same concentrations, with the exception of emulsions prepared with ultrasound treated FG where reduced emulsion stability at lower concentrations (< 1 wt. %) was exhibited. These results suggest that sonication did not significantly affect the rate of FG or RPI surface denaturation at the interface, as no significant ($P > 0.05$) reduction in the equilibrium interfacial tension between untreated and ultrasound irradiated FG or RPI was observed. By comparison, emulsions fabricated with ultrasound treated BG, EWP and PPI at concentrations < 1 wt. % had smaller emulsion sizes than their untreated counterparts at the same concentrations. This behaviour was attributed to a reduction in protein size (i.e. increased mobility through the bulk) and an increase in the hydrophobicity (reflected by a decrease in the intrinsic viscosity) of sonicated BG, EWP and PPI. Furthermore, emulsions prepared with ultrasound treated BG had improved stability against coalescence for 28 days at all concentrations investigated. This enhancement in emulsion stability attributed to improved interfacial packing, observed by a lower equilibrium interfacial tension and cryo-SEM micrographs.

Ultrasound treatment can thus improve the solubility of previously poorly soluble vegetable proteins (PPI and SPI) and moreover, is capable of improving the emulsifying performance of other proteins (BG, EWP and PPI).

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Table 1. Composition and pH (measured at a concentration of 1 wt. % and a temperature of 25 ^{o}C)of bovine gelatin (BG), fish gelatin (FG), egg white protein (EWP), pea protein isolate (PPI), soy protein isolate (SPI) and rice protein isolate (RPI).

Table 2. Effect of sonication time on pH of BG, FG, EWP, PPI, SPI and RPI solutions at a concentration of 0.1 wt. %. The standard deviation for all pH measurements was < 0.04.

Table 3. Average protein size (D_z) and span of untreated and ultrasound treated BG, FG, EWP, PPI, SPI and RPI at a concentration of 0.1 wt. %.

Table 4. Intrinsic viscosity ([η]), Huggins (k_H) and Kraemer (k_K) constants obtained for untreated and ultrasound treated BG, FG, EWP, PPI, SPI and RPI solutions.

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	BG	FG	EWP	PPI	SPI	RPI
Protein (wt. %)	86	86	85	86	86	84.5
Moisture (wt. %)	10	12	8.4	7.2	6.2	7.7
Fat (wt. %)	0	0	< 0.1	0	3.5	3
Carbohydrate (-)	neg.	neg.	neg.	pos.	pos.	pos.
Ash (wt. %)	0.76	0.09	4.11	4.85	4.96	0.72
pH (-)	5.32	5.02	6.26	7.45	6.95	4.85

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	pH (-)				
Time (s)	0	15	30	60	120
BG	7.09	6.97	6.84	6.71	6.63
FG	7.11	7.02	6.82	6.68	6.77
EWP	6.28	6.19	6.11	6.07	6.04
PPI	7.45	7.36	7.26	7.14	7.12
SPI	6.94	6.8	6.69	6.61	6.59
RPI	7.05	7.04	7.04	7.03	7.02

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		Ultrasound Treated		
D_z (nm)	Untreated	Day 0	Day 1	Day 7
BG	812 ± 19	61 ± 7	112 ± 11	117 ± 8
FG	554 ± 23	52 ± 9	104 ± 13	111 ± 17
EWP	1,600 ± 120	244 ± 5	398 ± 7	412 ± 22
PPI	5,250 ± 230	187 ± 7	198 ± 6	222 ± 4
SPI	1,700 ± 320	265 ± 10	293 ± 9	298 ± 15
RPI	51,600 ± 920	52,800 ± 840	52,400 ± 680	52,500 ± 730
Span (-)	Untreated	Day 0	Day 1	Day 7
BG	1.93 ± 0.54	0.44 ± 0.03	0.67 ± 0.07	0.73 ± 0.06
FG	1.72 ± 0.43	0.35 ± 0.04	0.59 ± 0.06	0.66 ± 0.05
EWP	8.2 ± 0.44	5.8 ± 0.11	6 ± 0.11	5.8 ± 0.11
PPI	2.8 ± 0.13	48.1 ± 1.5	47.9 ± 1.7	46.6 ± 2.3
SPI	3.4 ± 0.43	23.5 ± 0.9	24.1 ± 1.2	24.4 ± 1.5
RPI	3.61 ± 0.23	3.57 ± 0.32	3.58 ± 0.43	3.6 ± 0.52

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Protein in solution	$[\eta]_{Untreated}$ (dL/g)	$k_{H\ Untreated}$	$k_{K\ Untreated}$	$[\eta]_{Ultrasound}$ (dL/g)	$k_{H\ Ultrasound}$	$k_{K\ Ultrasound}$
BG	2.75 ± 0.08	-2.88	-3.09	2.06 ± 0.09	-2.31	-2.39
FG	1.06 ± 0.07	-0.38	-0.41	0.76 ± 0.05	-0.18	-0.24
EWP	0.25 ± 0.001	-0.03	-0.033	0.21 ± 0.001	-0.023	-0.026
PPI	0.8 ± 0.005	0.59	0.034	0.76 ± 0.007	-0.24	-0.29
SPI	0.31 ± 0.002	-0.02	-0.032	0.27 ± 0.001	-0.023	-0.031
RPI	0.55 ± 0.009	-0.15	-0.16	0.56 ± 0.007	-0.13	-0.14

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Fig. 1. Effect of sonication time on the D_z (nm) of (a) BG, (b) EWP, (c) PPI and (d) RPI.

Fig. 2. Cryo-SEM micrographs of protein solutions: (a) 1% Untreated BG, (b) 1% Ultrasound treated BG, (c) 1% Untreated SPI and (d) 1% Ultrasound treated SPI. Scale bar is 2 μm in all cases.

Fig. 3. SDS-PAGE electrophoretic profiles of protein solutions: (a) Molecular weight standard (10 kDa – 250 kDa), (b) Untreated BG, (c) Ultrasound treated BG, (d) Untreated FG, (e) Ultrasound treated FG, (f) Untreated EWP, (g) Ultrasound treated EWP, (h) Untreated PPI, (i) Ultrasound treated PPI, (j) Untreated SPI, (k) Ultrasound treated SPI, (l) Untreated RPI and (m) Ultrasound treated RPI.

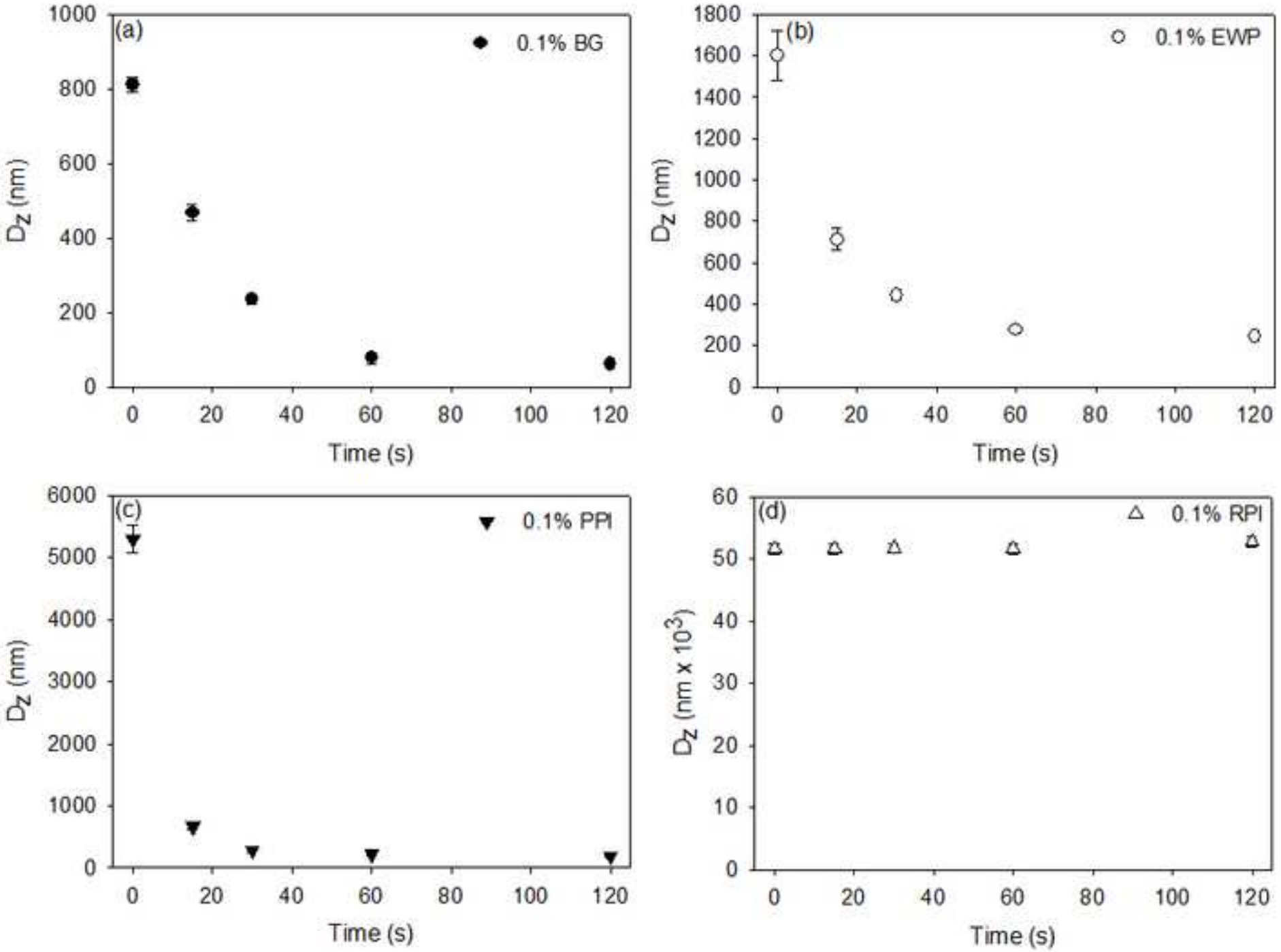
Fig 4. Fitting of the Huggins (●) and Kraemer (○) equations to the viscosity data of the studied protein solutions: (a) Untreated EWP, (b) Ultrasound treated EWP, (c) Untreated PPI and (d) Ultrasound treated PPI.

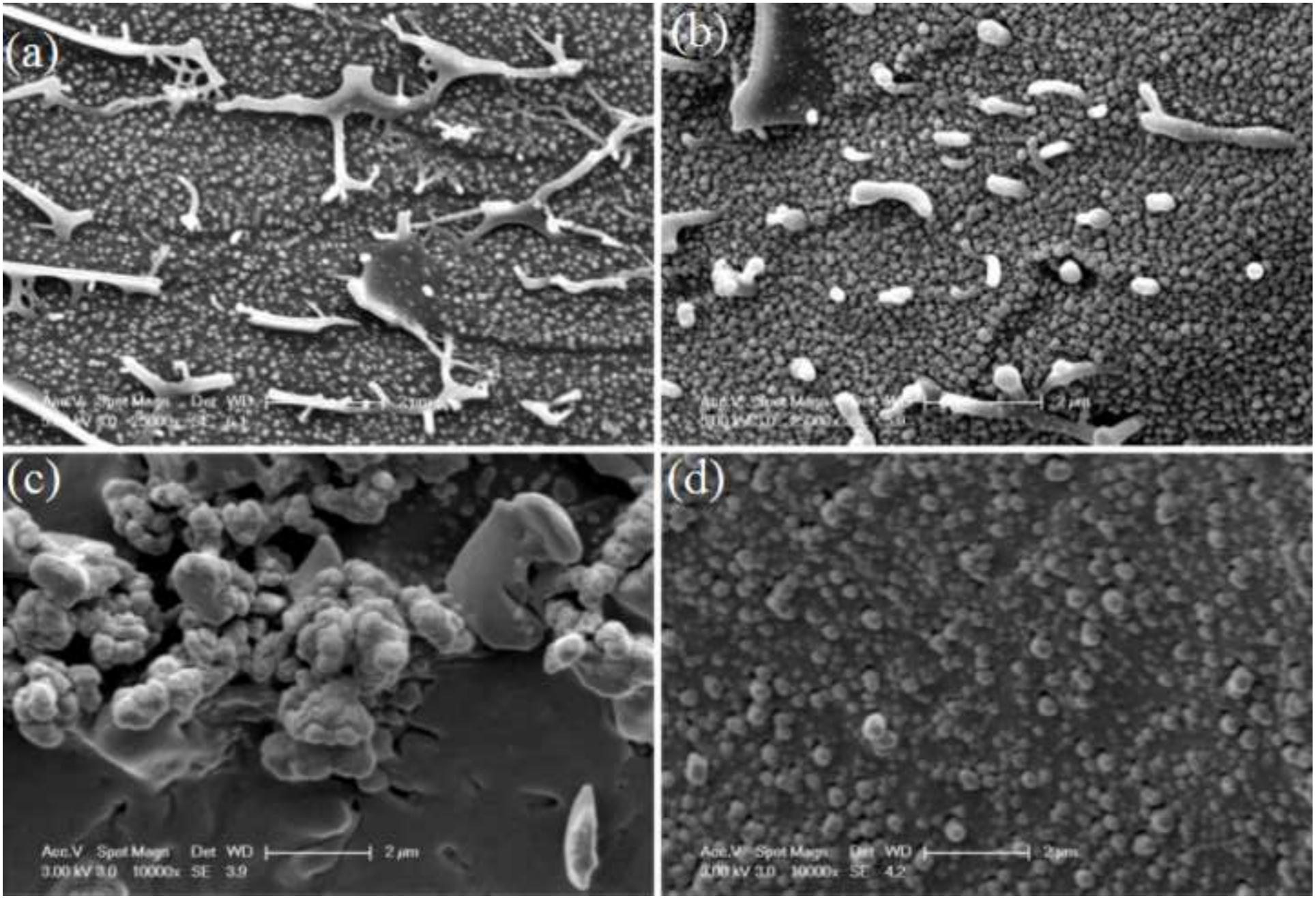
Fig. 5. Average droplet size as a function of concentrations of: (a) Untreated BG, sonicated BG and Brij 97, (b) Untreated FG, sonicated FG and Brij 97, (c) Untreated EWP, sonicated EWP and Brij 97 and (d) Untreated PPI, sonicated PPI and Brij 97.

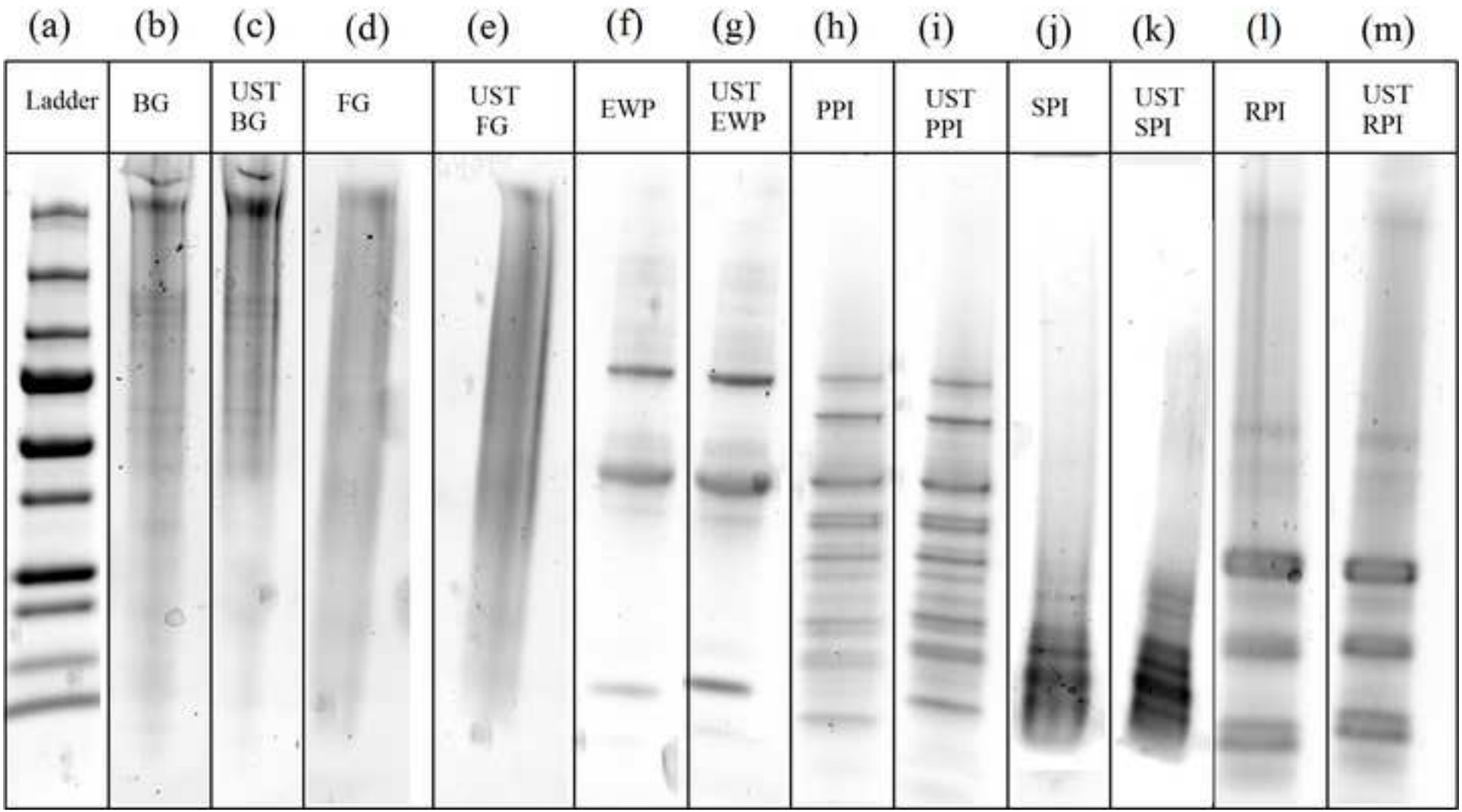
Fig. 6. Interfacial tension between water and pure vegetable oil as a function of emulsifier type: (a) Untreated BG, ultrasound treated BG and Brij 97, (b) Untreated FG, ultrasound treated FG and Brij 97, (c) Untreated PPI, ultrasound treated PPI and Brij 97 and (d) Untreated SPI, ultrasound treated SPI and Brij 97.

Fig. 7. Cryo-SEM micrographs of protein stabilised O/W pre-emulsions: (a) 1% Untreated BG stabilised emulsion, (b) 1% Ultrasound treated BG stabilised emulsion and (c) 1% Untreated SPI stabilised emulsion, (d) 1% Ultrasound treated SPI stabilised emulsion. Scale bar is 10 μm in all cases.

Fig. 8. Effect of emulsifier type on droplet size as a function of time for O/W emulsions stabilised by: (a) Untreated BG, ultrasound treated BG and Brij 97, (b) Untreated FG, ultrasound treated FG and Brij 97, (c) Untreated PPI, ultrasound treated PPI and Brij 97, (d) Untreated SPI, ultrasound treated SPI and Brij 97.


(a)
0.1% BG
D_Z (nm)
Time (s)
(b)
0.1% EWP
D_Z (nm)
Time (s)
(c)
0.1% PPI
D_Z (nm)
Time (s)
(d)
0.1% RPI
D_Z (nm x 10^3)
Time (s)


(a)
(b)
(c)
Acc.V Spot Magn Det WD 2 μm
3.00 kV 3.0 10000x SE 3.9
(d)


(a)
(b)
(c)
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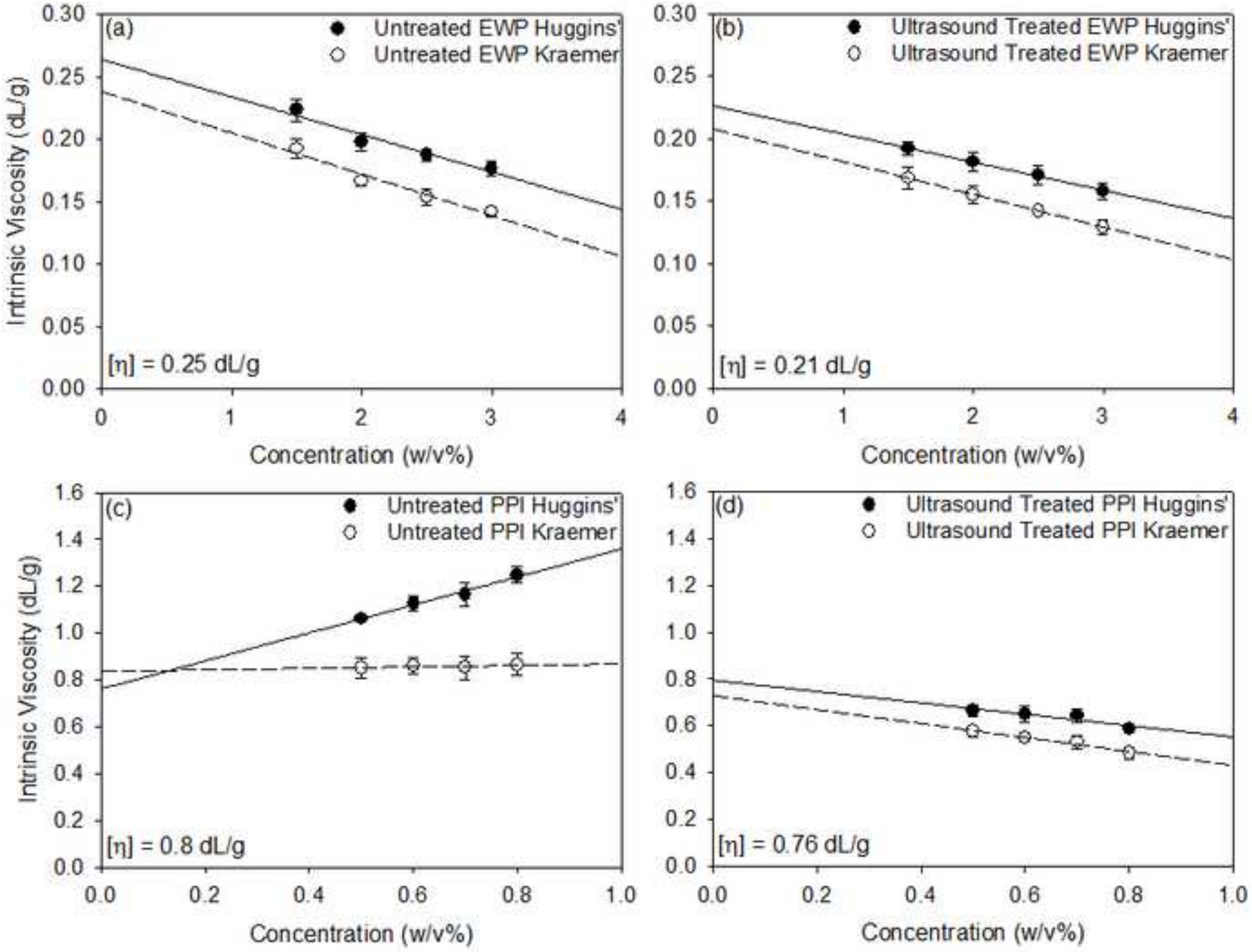
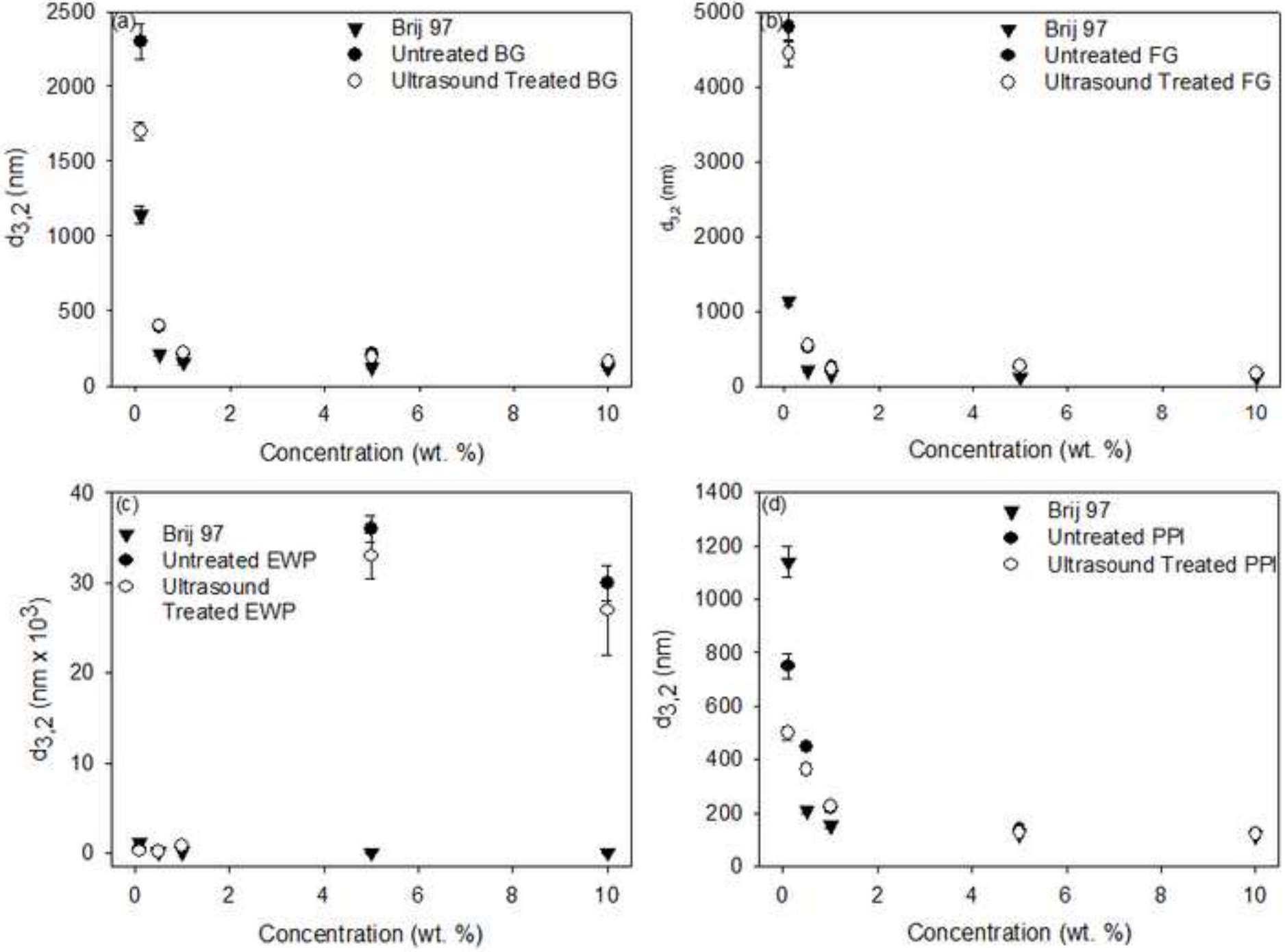
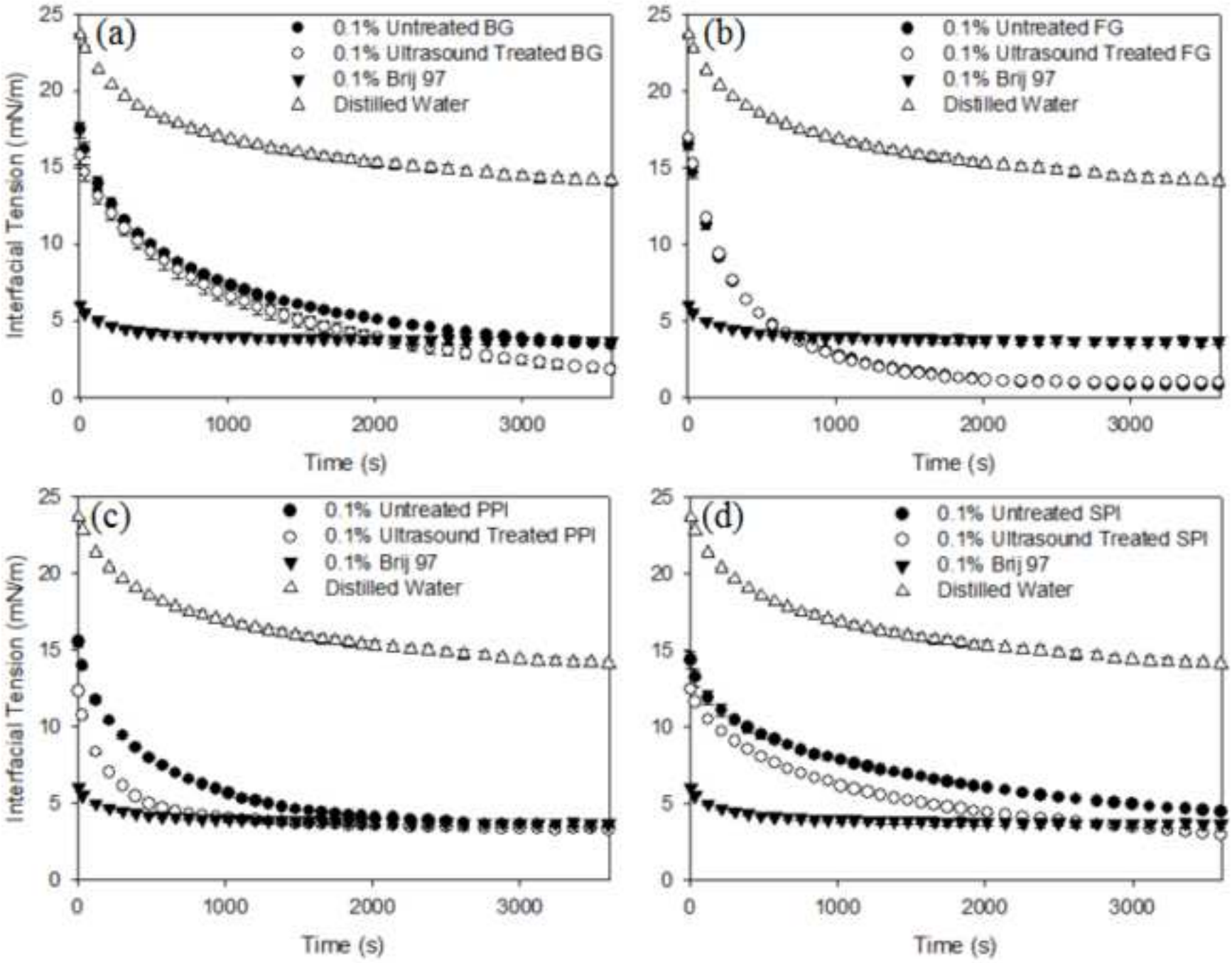
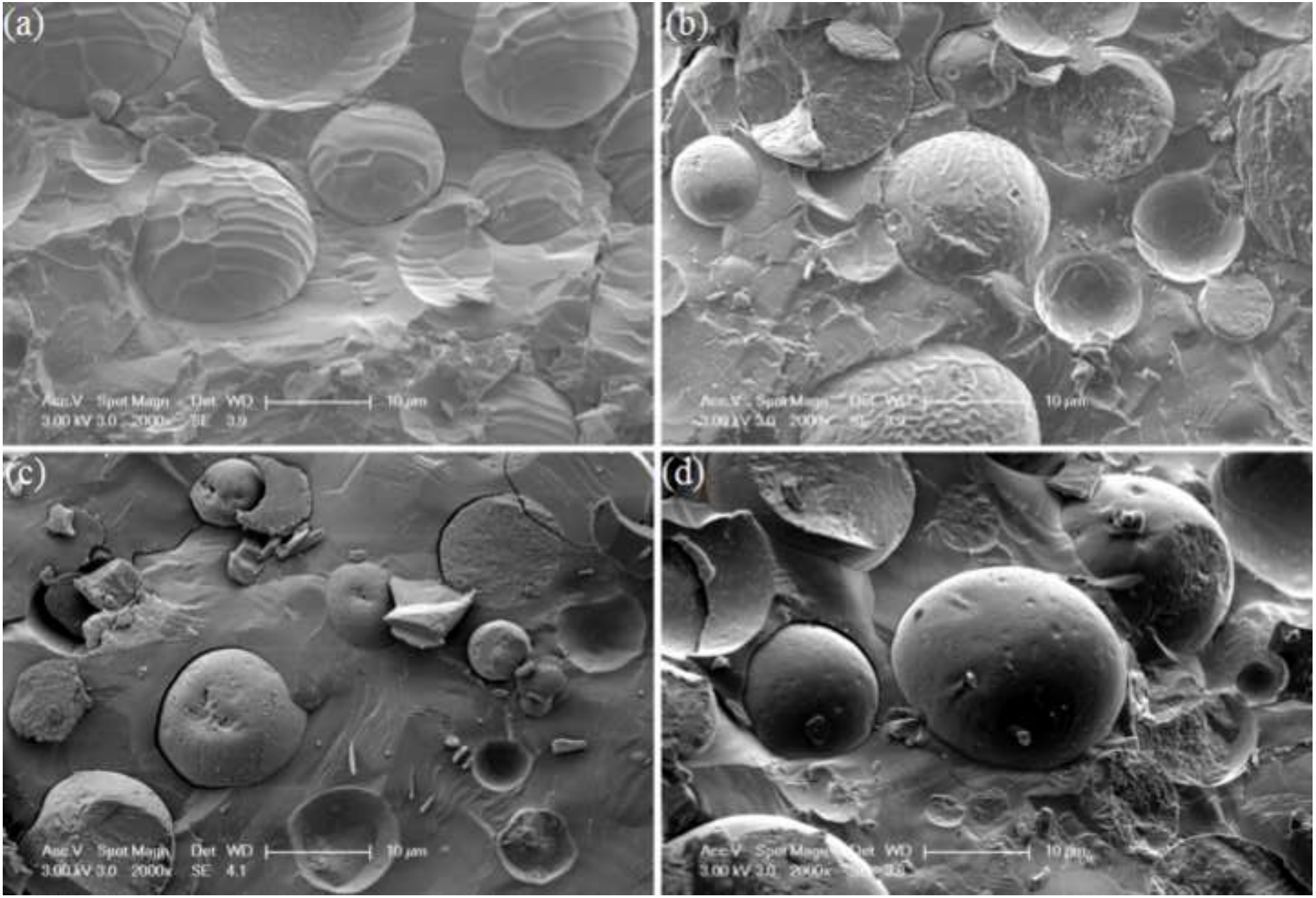
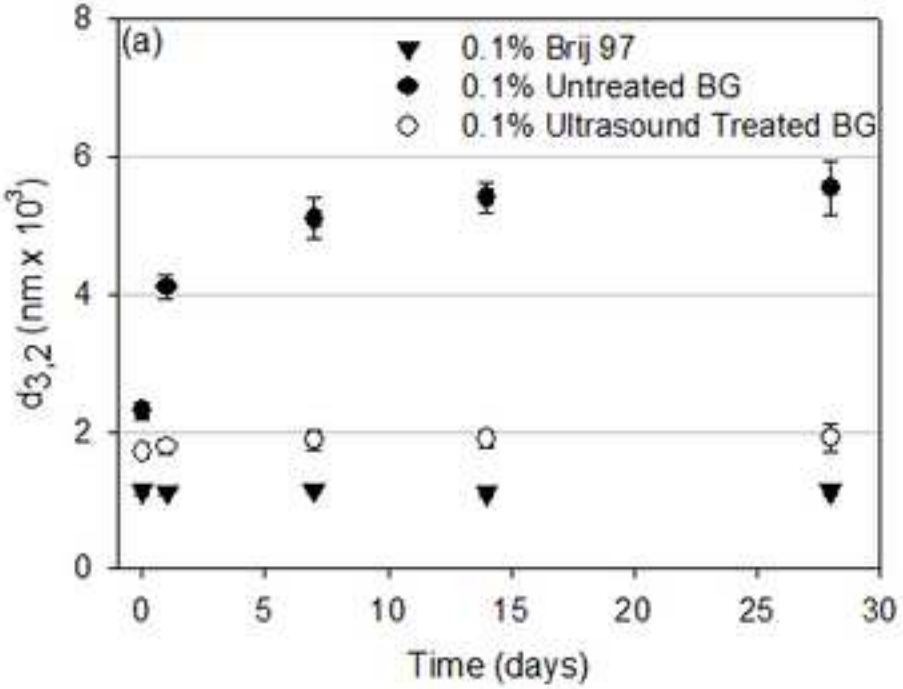
(e)
(f)
(g)
(h)
(i)
(j)
(k)
(l)
(m)
Ladder
BG
UST BG
FG
UST FG
EWP
UST EWP
PPI
UST PPI
SPI
UST SPI
RPI
UST RPI

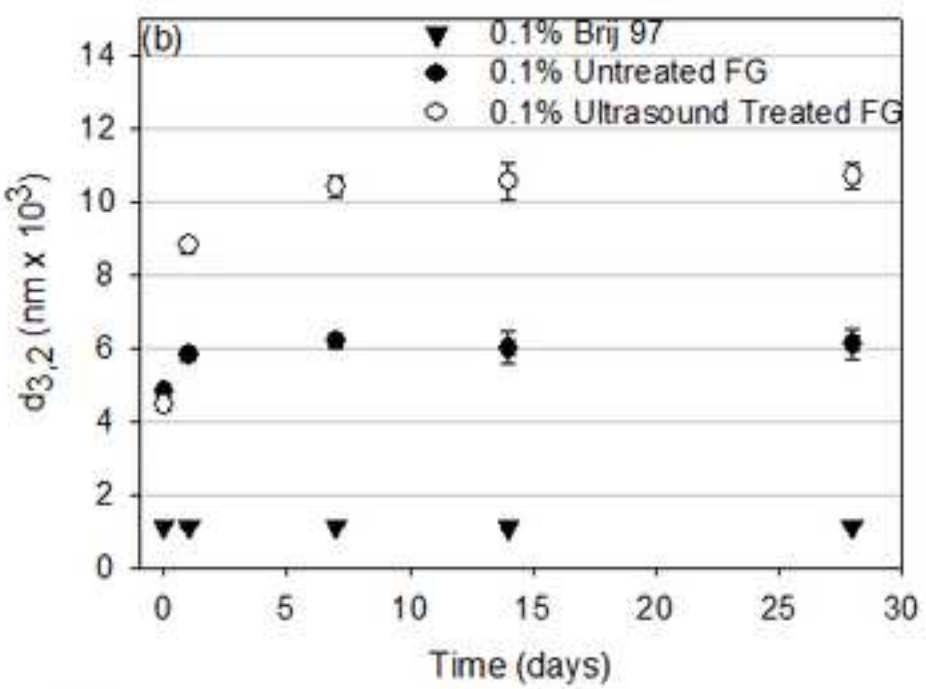

(a)
Untreated EWP Huggins'
Untreated EWP Kraemer
[η] = 0.25 dL/g
(b)
Ultrasound Treated EWP Huggins'
Ultrasound Treated EWP Kraemer
[η] = 0.21 dL/g
(c)
Untreated PPI Huggins'
Untreated PPI Kraemer
[η] = 0.8 dL/g
(d)
Ultrasound Treated PPI Huggins'
Ultrasound Treated PPI Kraemer
[η] = 0.76 dL/g
Intrinsic Viscosity (dL/g)
Concentration (w/v%)

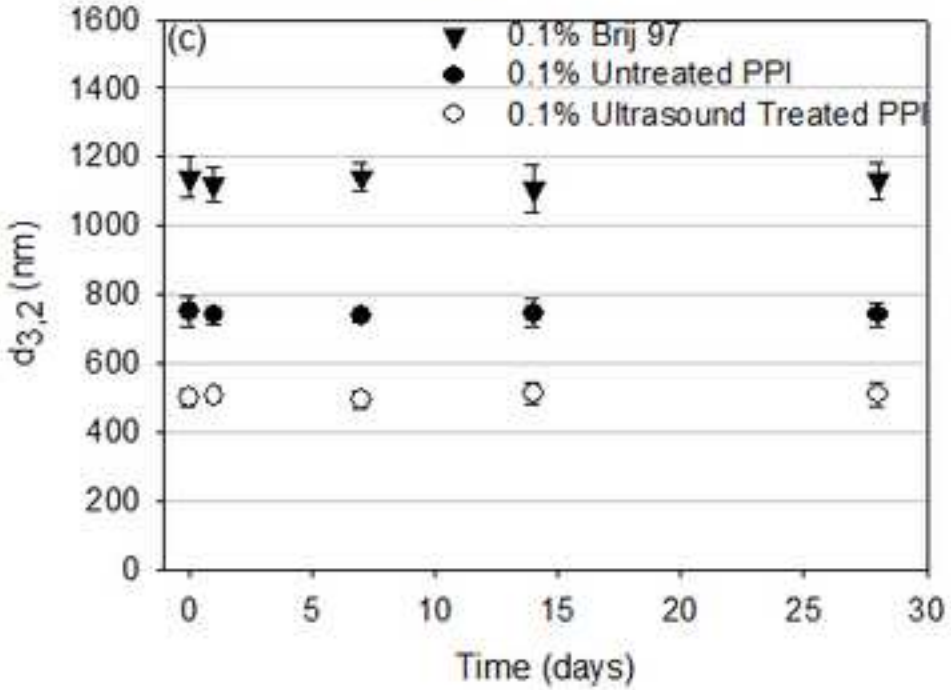

(a)
Brij 97
Untreated BG
Ultrasound Treated BG
$d_{3,2}$ (nm)
Concentration (wt. %)
(b)
Brij 97
Untreated FG
Ultrasound Treated FG
$d_{3,2}$ (nm)
Concentration (wt. %)
(c)
Brij 97
Untreated EWP
Ultrasound Treated EWP
$d_{3,2}$ (nm x 10^3)
Concentration (wt. %)
(d)
Brij 97
Untreated PPI
Ultrasound Treated PPI
$d_{3,2}$ (nm)
Concentration (wt. %)

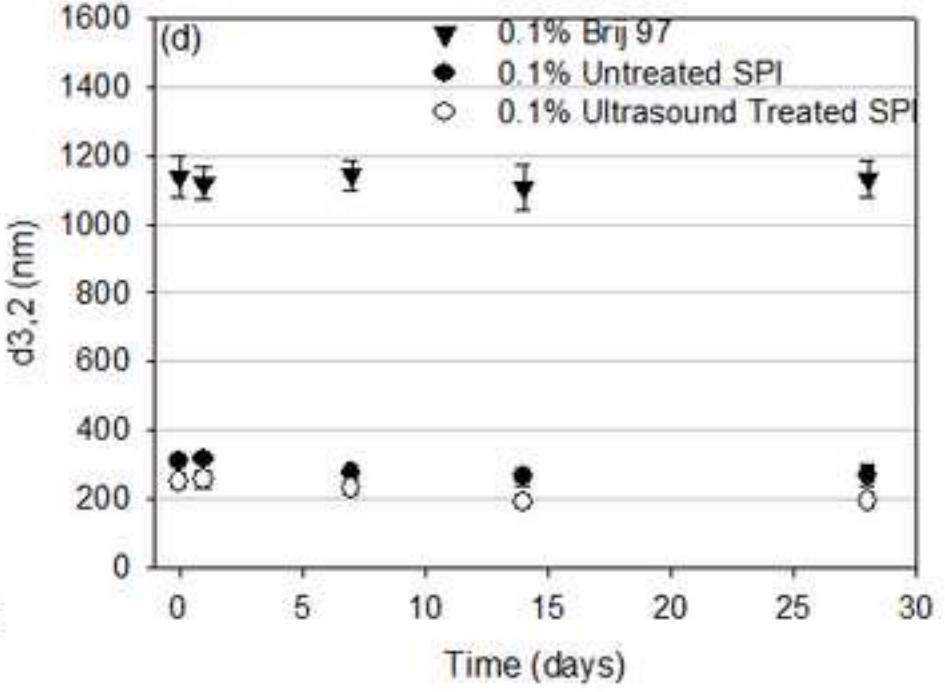

(a)
0.1% Untreated BG
0.1% Ultrasound Treated BG
0.1% Brij 97
Distilled Water
Interfacial Tension (mN/m)
Time (s)
(b)
0.1% Untreated FG
0.1% Ultrasound Treated FG
0.1% Brij 97
Distilled Water
Time (s)
(c)
0.1% Untreated PPI
0.1% Ultrasound Treated PPI
0.1% Brij 97
Distilled Water
Interfacial Tension (mN/m)
Time (s)
(d)
0.1% Untreated SPI
0.1% Ultrasound Treated SPI
0.1% Brij 97
Distilled Water
Time (s)


(a)
(b)
(c)
(d)


(a)
0.1% Brij 97
0.1% Untreated BG
0.1% Ultrasound Treated BG
$d_{3,2}$ (nm x 10^3)
Time (days)


(b)
0.1% Brij 97
0.1% Untreated FG
0.1% Ultrasound Treated FG
$d_{3,2}$ (nm x 10^3)
Time (days)


(c)
0.1% Brij 97
0.1% Untreated PPI
0.1% Ultrasound Treated PPI
$d_{3,2}$ (nm)
Time (days)


(d)
0.1% Brij 97
0.1% Untreated SPI
0.1% Ultrasound Treated SPI
d3,2 (nm)
Time (days)

ACCEPTED MANUSCRIPT

Highlights:

- Ultrasonic effect on properties of animal and vegetable proteins was assessed.
- High power ultrasound (~34 W cm^{-2}) reduced aggregate size of all animal proteins.
- SDS-PAGE confirmed UST had no effect on the molecular weight of animal proteins.
- UST BG and FG had similar droplet sizes as their untreated counterparts.
- UST BG, PPI and EWP produced smaller emulsion droplets than untreated counterparts.

ACCEPTED MANUSCRIPT