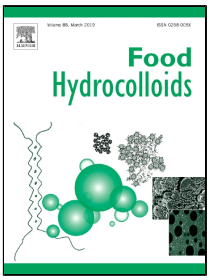
Accepted Manuscript



Emulsifying properties of hemp proteins: effect of isolation technique

Tamara Dapčević-Hadnađev, Manda Dizdar, Milica Pojić, Veljko Krstonošić, Lisa M. Zychowski, Miroslav Hadnađev

PII:	S0268-005X(18)31083-X
DOI:	10.1016/j.foodhyd.2018.12.002
Reference:	FOOHYD 4806
To appear in:	*Food Hydrocolloids*
Received Date:	13 June 2018
Accepted Date:	03 December 2018

Please cite this article as: Tamara Dapčević-Hadnađev, Manda Dizdar, Milica Pojić, Veljko Krstonošić, Lisa M. Zychowski, Miroslav Hadnađev, Emulsifying properties of hemp proteins: effect of isolation technique, *Food Hydrocolloids* (2018), doi: 10.1016/j.foodhyd.2018.12.002

Emulsifying properties of hemp proteins: effect of isolation technique

Tamara Dapčević-Hadnađev[1], Manda Dizdar[2], Milica Pojić[1], Veljko Krstonošić[3], Lisa M. Zychowski[4], Miroslav Hadnađev[1*]

[1]University of Novi Sad, Institute of Food Technology, Bulevar cara Lazara 1, 21000 Novi Sad, Serbia

[2]University Business Academy in Novi Sad, Faculty of Pharmacy, Trg mladenaca 5, 21000 Novi Sad, Serbia

[3]University of Novi Sad, Faculty of Medicine, Department of Pharmacy, Hajduk Veljkova 3, 21000 Novi Sad, Serbia

[4]Food Chemistry and Technology Department, Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland

Abstract

Hemp protein was isolated from hemp seed meal using two different isolation procedures: alkali extraction/isoelectric precipitation (HPI) and micellization (HMI). The ability of these proteins to form and stabilize 10% (w/w) sunflower oil-in-water emulsions (at pH = 3.0) was studied at three different concentrations, 0.25, 0.75 and 1.5% (w/w), by monitoring emulsion droplet size distribution, microstructural and morphological properties, rheological behaviour and stability against flocculation, coalescence and creaming. In addition, hemp proteins were analysed for water solubility, denaturation degree and surface/interfacial activity. HMI protein, which was found to be less denatured after isolation, exhibited higher solubility and slightly higher surface/interfacial activity than HPI protein. HMI emulsions possessed a smaller volume mean droplet diameter ($d_{4,3}$ = 1.92 - 3.42 μm in 2% SDS) than HPI emulsions ($d_{4,3}$ = 2.25 - 15.77 μm in 2% SDS). While HMI stabilized emulsions were characterized with individual droplets covered by protein film, both confocal laser scanning microscopy and flocculation indices indicated occurrence of bridging flocculation in HPI stabilized emulsions. Protein aggregation, which induced flocculation of the droplets, contributed to higher apparent viscosity of HPI stabilized emulsions compared to HMI stabilized emulsions. Interestingly, emulsions stabilized with 1.5% (w/w) HPI exhibited much better creaming and coalescence stability than other emulsions due to the formation of a weak transient network of floccules and higher continuous phase viscosity which both suppressed the movement of the droplets.

Key words: *hemp protein, isolation technique, emulsion stability, droplet size distribution, bridging flocculation*

*Corresponding author. Tel.: +381214853811; e-mail: miroslav.hadnadjev@fins.uns.ac.rs.

1 Introduction

Proteins commonly used for food and pharmaceutical applications are whey protein, casein, soy, egg white proteins, gelatin, bovine serum albumin, collagen, etc. (Lam & Nickerson, 2013). However, over the past few years, food and cosmetics industries have been testing the potential use of vegetable proteins from alternative sources due to increased interest.

The use of plant-based proteins as an alternative to animal derived proteins is desired due to the higher sustainability, abundancy, and wide variety of sources (Bučko et al., 2015; Hadnađev et al., 2017). Oilseeds are rich source of proteins, which can be extracted from the oil seed meal, a by-product of the oil processing.

Hemp (*Cannabis sativa* L.) is a widely cultivated plant of industrial importance. In the past, it was grown to produce hemp fibre for durable fabrics, while today it is becoming increasingly utilized for edible oil extraction (Pojić et al., 2014). Hemp seed contains over 30% oil and 25% protein, with a considerable amount of dietary fibre, vitamins and minerals. Hemp seed main proteins are albumin and edestin. Hempseed proteins are an excellent source of digestible amino acids when compared to other vegetable proteins like borage meal, canola meal (Callaway, 2004). A direct comparison of the amino acid profiles from egg white, hempseed and soy bean has shown that hemp seed is comparable to both egg white and soybean, which are both considered to be high quality proteins (Tang, Ten, Wang, & Yang, 2006). In order to isolate the proteins from hempseed meal (HMP), a by-product obtained after processing the seeds into edible oil, alkali extraction is usually employed, followed by isoelectric protein precipitation or micellization techniques (Hadnađev et al., 2018; Malomo & Aluko, 2015; Tang et al., 2006). Malomo, He, & Aluko (2014) have reported that after protein alkali extraction, the protein content in the obtained isolate (84.15%) was significantly higher than the determined value in the starting material - HMP (44.32%). Comparing two isolation techniques, Hadnađev et al. (2018) concluded that micellization (a “salting in-salting out” process) resulted in higher hemp protein purity (98.87%) than the alkaline extraction/isoelectric precipitation technique (91.44%).

Proteins generally exhibit a wide range of techno-functionalities such as foaming, emulsifying, gelling and film-forming ability, fat absorption capacity, etc. (Pojić, Mišan, & Tiwari, 2018). Due to their amphiphilic nature and film forming abilities, proteins are widely used as functional ingredients for the formation and stabilisation of emulsions (Lam & Nickerson, 2013). While small molecular weight emulsifiers diffuse rapidly to the interface during emulsion formation, proteins diffuse at a much slower rate due to their higher molecular weight (McClements, 1999). Once adhering to the interface, proteins form viscoelastic films at the surface of the oil droplets providing electrostatic and steric stabilization which are largely influenced by protein type and environmental conditions (Tcholakova, Denkov, Ivanov, & Campbell, 2006a). At the isoelectric pH value, where the net charge of the protein molecules is neutral, protein

aggregation occurs, leading to emulsion instability (Foegeding & Davis, 2011; Guzey & McClements, 2007). Beyond the isoelectric point, pH conditions influence the overall charge and functional behaviour of the protein. For example, improved emulsion stability and viscoelastic properties of interfaces of amaranth proteins were observed at pH 2.0, in contrast to pH 8.0; this was related to the denatured state of the proteins at pH 2.0, where proteins formed a harder interfacial film, as compared to pH 8.0 (Ventureira et al., 2012). Moreover, additional emulsion stabilization is achieved by proteins present within the continuous phase which act as bulking agents, increasing emulsion viscosity, and thus reducing the mobility and coalescence of oil droplets within the emulsion (Jafari, Beheshti, & Assadpoor, 2012).

The physico-chemical properties of proteins play an important role in determining their ability to form and stabilize the emulsion (Papalamprou, Doxastakis, & Kiosseoglou, 2010). Solubility is one of the most important features of proteins, since many functional properties of protein depend upon their capacity to initially go into solution (Radha & Prakash, 2009). In general, hemp protein exhibits low solubility in comparison to other vegetable proteins, which is attributed to edestin (11S globulin) aggregation at pH below 7.0 (Malomo et al., 2014). The employed protein isolation technique can influence protein solubility. It was shown that alkali extracted hemp protein (HPI) has minimum solubility at pH 5.0, which increased as pH was decreased or increased. In contrast, salt extracted hemp protein (HMI) has minimum solubility at pH 6.0. The higher solubility of HMI at lower pHs suggests that HMI may be in a more native state than HPI (Hadnađev et al., 2018).

An emulsifying activity index (EAI) and emulsifying stability index (ESI) of hemp stabilized emulsions has been reported by Tang et al. (2006), which has shown that EAI profiles of HPI at different pH values have the similar pattern to the protein solubility (PS) profiles, suggesting a possible relation between those two characteristics. Lower EAI and ESI of hemp protein stabilized emulsions, when compared with those of soy protein, were attributed to the propensity of hemp protein to form covalent disulfide bonds between individual proteins and its subsequent aggregation at neutral or acidic pH (Tang et al., 2006). The decreased solubility of canola (CaPI) and flax seed (FlPI) protein isolates, relative to whey protein isolate (WPI), were responsible for their lower EAI and ESI, although emulsion capacity of CaPI was comparable to WPI (Karaca Low, & Nickerson, 2011b). Karaca et al. (2011b) also reported that CaPI and FlPI produced by salt extraction showed improved solubility and interfacial activity compared to those produced by isoelectric precipitation. Although salt extracted CaPI exhibited better EAI than isoelectric precipitated CaPI, the former was characterized with a higher mean emulsion droplet diameter and rapid creaming. On the contrary, the isolation method did not influence significantly the EAI and creaming stability of FlPI. The different response of CaPI and FlFI to isolation technique, however, remains unclear.

In order to corroborate deeply the effect of protein isolation technique on emulsion formation as well as the ability of hemp protein to stabilize emulsions, the aim of this work was to

compare the emulsifying properties of alkali extracted/isoelectric precipitated hemp protein (HPI) and micellar (salt extracted) hemp protein (HMI).

In general, emulsifying properties of hemp proteins are poorly investigated, mostly due to their low solubility. Therefore, enzymatically hydrolysed version of hemp protein was mostly investigated for its emulsifying ability (Yin et al., 2008), while hemp protein isolate stabilized emulsions were only characterized in terms of emulsifying activity index (EAI) and emulsifying stability index (ESI) in comparison to soy protein isolate. However, there is a lack of knowledge in the hemp protein emulsions morphology and structure, rheological behaviour, stability mechanism, effect of solubility and denaturation degree on emulsifying ability and stability, etc.

In this paper, emulsifying properties of two hemp proteins (HPI and HMI), isolated using different techniques, were compared at different protein concentrations and pH condition far from the isoelectric point (pH = 3.0). These conditions were chosen based on previous studies investigating the influence of pH on protein emulsifying properties. These studies confirmed improved emulsification and stability against coalescence with protein-stabilized emulsions at pHs below the isoelectric point (pI), due to extensive protein unfolding induced by the extreme pH conditions (Liang & Tang, 2013; Ventureira et al., 2012). According to Gharsallaoui, Cases, Chambin, & Saurel (2009) better stability of pea protein-based emulsions at acidic conditions in comparison to neutral or alkali pH, was influenced by the ability of pea protein to form a stronger and denser viscoelastic network when adsorbed at the interface.

The emulsions prepared in this paper were characterized in terms of their droplet size distribution, microstructural and morphological properties, rheological behaviour and stability and related to protein solubility, denaturation degree and surface/interfacial activity.

2 Materials and methods

2.1 Materials

Hemp pellets obtained as a by-product in cold oil processing were provided by the local company Svet Konoplje (Kisač, Serbia). Refined sunflower oil was purchased in local market. Nile blue was procured from Sigma-Aldrich. All chemicals used were of reagent grade and deionized water was used to prepare all solutions used in the experimental work.

2.2 Preparation of protein isolates

Hemp pellets were ground in a Foss Knifetec 1095 laboratory mill (Foss, Hillerød, Denmark) equipped with water circulation, to ensure sample cooling and avoid overheating during milling procedure. The obtained ground hemp meal was separated into two fractions using a universal laboratory sifter (Bühler AG, Uzwil, Switzerland): a coarse fraction (particle size ≥250μm) and fine fraction (particle size <250 μm). The fine hemp meal fraction contained significantly higher

protein content than the course fraction, and thus, was employed for further protein extraction (Pojić et al., 2014).

Prior to the hemp protein extraction process, fine hemp meal was defatted using a triple hexane extraction at 1:3 hemp meal to hexane ratio for 2 h each. Subsequently, the defatted hempseed meal was air dried in fume hood at room temperature.

After drying, defatted hempseed meal was used, as a starting material, to isolate proteins with the aid of two different extraction techniques: alkali extraction/isoelectric precipitation and micellization, both techniques are described in detailed by Hadnađev et al. (2018). Both alkali extracted hemp protein, assigned as HPI (91.44±0.34% protein content on dry matter basis), and micelle protein referred to as HMI (98.87±0.41% protein content on dry matter basis) were prepared in triplicates and compared for their emulsifying ability.

2.3 Protein solubility determination (at pH 3.0)

Under constant stirring, 1% (w/w) suspensions of hemp protein isolates were adjusted to pH 3.0 with the aid of 1 M HCl followed by further stirring for 1 h at room temperature (23 °C) with pH maintenance throughout. Afterwards, centrifugation was applied to the protein solution for 30 min at 7500xg. The obtained supernatant was filtered, diluted accordingly and the protein content was determined by the Lowry method (Lowry, Rosenbrough, Fair, & Randall, 1951). Protein solubility was determined as the ratio of protein content in supernatant of the suspension as compared to the total protein content. The measurements were performed in triplicates.

2.4 Protein suspension/solution preparation

HMI and HPI protein solutions were prepared by dispersing 0.25, 0.75 and 1.5% (w/w) of proteins isolates (calculated on protein content) in deionized water. Under constant stirring, pH was adjusted to 3.0 with 1.0 M HCl and stirred for additional 2 h at room temperature. Subsequently, sodium azide (0.02% w/v), which was used as an antimicrobial agent, was added and solutions were stored overnight at 4 °C in order to allow complete protein hydration. Two separate batches of suspensions were prepared for analysis.

2.5 Protein solutions surface and interfacial tension measurements

The surface and interfacial tension measurements were carried out on a digital Krüss Easy Dyne tensiometer (Hamburg, Germany) using a du Noüy ring method. The surface tension values were corrected by Harkins and Jordan method, which is integrated into the electronic Krüsstensiometer (Milanović, Krstonošić, Dokić, Hadnađev, & Dapčević Hadnađev, 2015). Prior to surface tension (air-protein solution) measurements, the ring was immersed in the liquid and left for 10 min in order to allow the surface to equilibrate. For interfacial tension measurements (oil-protein solution), the ring was firstly immersed in the solution, and afterwards, sunflower

oil was poured on the top of the solution surface followed by system equilibration for 10 min. The reported surface/interfacial tension values were the average of five measurements per batch at constant temperature of 25 ± 0.1 °C. Surface tension of the solvent (water adjusted to pH = 3.0) was recorded as 72.3±0.09 mN/m, while its interfacial tension was 23.3±0.17 mN/m. Surface activity was expressed as surface/interfacial pressure:

$$\pi = \sigma_0 - \sigma \quad (1)$$

where σ_0 and σ are surface/interfacial tensions of solvent and protein solution, respectively (Bučko et al., 2015).

2.6 Emulsion preparation

Six different hemp protein isolate solutions were prepared (0.25, 0.75 and 1.5% (w/w) HPI and HMI), as described in section 2.4, to act as the continuous phases in emulsion preparation. Oil-in-water emulsions were prepared by blending 90% (w/w) hemp protein isolate solution (0.25 - 1.5% protein content per continuous phase) with 10% (w/w) sunflower oil. The emulsions were pre-homogenized using an Ultraturrax T-25 (IKA®Werke GmbH & Co. KG, Germany) under continuous mixing at a speed of 12000 rpm for 60 s. Afterwards, pre-homogenized emulsions were homogenized at 400 bar using an APV Lab 1000 homogenizer (SPX flow, Germany). Two separate batches of emulsion were prepared for analysis.

2.7 Determination of droplet-size distribution

Particle size distribution of the freshly prepared and stored (24 h) emulsions was measured at room temperature utilizing a Malvern Mastersizer 3000 equipped with a Hydro R cell (Malvern Instruments Ltd, Worcestershire, UK). An obscuration between 4-10% was employed and deionized water or 2.0% (w/v) SDS solution was used as the dispersant. Refractive index values of 1.33 for continuous phase and 1.471 for sunflower oil were used as the optical parameters, while emulsion droplet absorbance was set to 0.1. Relative refractive index, which represents the ratio of the dispersed refractive index to that of the dispersant, was set at 1.106. Droplet size distribution was reported as distribution profile as well as the mean volume diameter ($d_{4,3}$):

$$d_{4,3} = \frac{\sum n_i d_i^4}{\sum n_i d_i^3} \quad (2)$$

where n_i is the number of droplets with diameter d_i.

Droplet size distribution parameters were presented as the mean value of three measurements of each batch.

2.8 Flocculation and coalescence quantification

Flocculation and coalescence indices were evaluated as described by Liang & Tang (2013). Mean volume diameter ($d_{4,3}$) determined as described above for freshly prepared (0 h) and stored (24 h) emulsions diluted in deionized water with and without 2.0% (w/v) SDS was used to calculate the percentage of flocculation index (%Fl) and percentage of coalescence index (%Cl):

$$Fl(\%) = \left[\frac{d_{4,3}\ in\ water}{d_{4,3}\ in\ 2\%\ SDS} - 1\right] \cdot 100 \quad (3)$$

$$Cl(\%) = \left[\frac{d_{4,3}\ (24\ h)}{d_{4,3}\ (0\ h)} - 1\right] \cdot 100 \quad (4)$$

2.9 Confocal laser scanning microscopy

Confocal laser scanning microscopy (CLSM) was performed using a Leica TCS SP5 Confocal Laser Scanning Microscope (CLSM; Leica Microsystems CMS GmbH, Wetzlar, Germany) using 63x oil immersion objective at 3 times zoom factor. Emulsions were imaged used dual confocal illumination through the use of an Argon laser at 488 nm and a Helium/Neon laser at 633 nm to show lipids and proteins, respectively. To prepare the samples for imaging 10 µL of Nile Blue (0.1 g/100 µL) was added into 1 mL of emulsion and vortexed for 10 s. After vortexing, approximately 50 µL of labelled emulsion was pipetted onto a microscope slide and a coverslip was placed on top. Images were taken using simultaneous dual-channel imaging and were pseudo-coloured to show protein (red) and lipids (green). Emulsions were evaluated 24 h after preparation to qualify the microstructure of the droplets after equilibration.

2.10 Rheological properties of emulsions

Rheological measurements were carried out using a Haake MARS rheometer (Thermo Scientific, Germany) equipped with DG41 Ti cylinder measuring geometry. For each emulsion, 6.3 ml of liquid was carefully loaded into measuring cup. The steady-state flow measurements were recorded at 25 ± 0.1 °C in the range of 0-100 1/s. Shear rate was increased linearly for 3 min, then it was held on maximum shear rate of 100 1/s for 2 min, afterwards the shear rate was decreased linearly from 100 to 0 1/s for 3 min. The obtained data were described using power law model:

$$\tau = K\dot{\gamma}^{n} \quad (5)$$

where τ is the shear stress (Pa), K is the consistency index (Pa s^n), and n is the flow behaviour index. All the rheological measurements were performed in triplicates for each batch 24 h after preparation.

2.11 Emulsion stability measurements

Stability of previously prepared emulsions was estimated using both simple (under gravitational forces) and rapid (under centrifugal forces) creaming methods. Gravitational separation in emulsions was followed by visual observation. The tested emulsions were placed in 10 ml sealed transparent glass-cylinders, gently agitated to ensure initial homogeneity and left for 14 days at room temperature. The extent of creaming was characterized as the bottom (serum) layer height to the total height of the emulsion.

Rapid stability method was conducted using a LUMiSizer Dispersion Analyser (L.U.M. GmbH, Germany). The measurements were performed by applying centrifugal sedimentation in order to accelerate the instability phenomena of tested emulsions such as sedimentation, creaming or flocculation (Petzold, Goltzsche, Mende, Schwarz, & Jaeger, 2009; Sobisch & Lerche, 2008). The obtained recordings refer to intensities of transmitted light as a function of time i.e. percentage of light absorbance per hour which is related to "creaming rate". Creaming rate is inversely related to emulsion stability, and thus, the LUMiSizer can be employed to judge the performance of an emulsion system. The parameters on the LUMiSizer were as follows: 600 µL of emulsion, 1500 rpm, time 3600 s, time interval 60 s and temperature 25 °C. Measurements were conducted in duplicates for each batch 24 h after preparation.

2.12 Statistical analysis

The obtained results were expressed as mean values of replicate analyses. Analysis of variance (ANOVA) and Tukey's test were used to assess significant differences among the mean values at significance level of 0.05.The statistical analyses were performed by software TIBCO Statistica™ 13.3.0 (TIBCO Software Inc., Palo Alto, CA, USA).

3 Results and discussion

3.1 Protein isolates characterization

Protein isolates from hemp seed meal were prepared using two different isolation techniques: alkaline extraction/isoelectric precipitation (HPI) and micellization (a "salting in - salting out" process; HMI). Both techniques resulted in the extraction of the same protein fraction, i.e. edestin (11S globulin) as identified by protein electrophoretic profiles (Hadnađev et al., 2018). However, according to FTIR spectra and differential scanning calorimetry, HPI and HMI differ in their secondary structure, since highly alkaline conditions during HPI extraction leads to partial

protein denaturation, thus HMI is generally found in a more preserved native state (Hadnađev et al., 2018).

The influence of isolation technique on the solubility of two hemp proteins, prepared in this study (HPI and HMI), is presented in Table 1. In general, under the chosen conditions (pH = 3.0), both protein isolates had solubility higher than 70%, which is an important prerequisite for a protein to be an effective emulsifier (Liang & Tang, 2013). Comparing the isolates prepared in this study, it can be noticed that HMI possessed significantly better solubility at pH 3.0 than HPI, which is in agreement with previous studies using the employed isolation techniques on canola and flaxseed protein isolates (Karaca et al., 2011b; Krause, Schultz, & Dudek, 2002). The observed lower solubility of HPI could be the consequence of partial denaturation during its preparation (Hadnađev et al., 2018). On the contrary, due to mild isolation conditions, hydrophobic part of HMI was placed in the core region of the molecule.

The effect of protein preparation procedure as well as proteins solution concentration on surface/interfacial pressure is summarized in Table 1.

By increasing the protein concentration in solution an increase in surface/interfacial pressure was observed, i.e. more proteins were adsorbed at the interface. Niño, Sánchez, Fernández, & Patino (2001) reported that the protein concentration dependence on surface pressure expresses classical sigmoidal behaviour, i.e. increase in surface pressure with protein concentration and reaching of a plateau at the maximum protein concentration in the bulk phase. Only a slight increase in the surface pressure was observed with higher concentrations for both HPI and HMI, suggesting that the examined concentration range (0.25 – 1.50% w/w) was close to the plateau value. In general, the decrease in interfacial tension between air/water and oil/water due to hemp protein adsorption was 24.93 – 32.61 mN/m and 9.13 – 15.73 mN/m, respectively, which is comparable to the values recorded for other plant protein isolates, such as pumpkinseed protein (Bučko et al., 2015), lentil protein isolate (Joshi et al., 2012), amaranth protein (Ventureira et al., 2012).

Comparing the two proteins, HMI expressed slightly higher surface/interfacial activity than HPI. This was probably influenced by higher solubility of HMI since higher protein solubility led to increased biopolymer mobility and greater diffusion rates to the interface (Lam & Nickerson, 2013; Karaca Low, & Nickerson, 2011a). Liang & Tang (2013) also reported that the pea protein adsorption at the interface at pH 3.0 was closely associated with its solubility. Onsaard, Vittayanont, Srigam, & McClements (2006) also noticed that freeze–thaw treated coconut cream protein (CCP2), which was characterized with more than two times higher solubility than isoelectric precipitated protein (CCP1), attained higher surface pressure at the air–water interface.

According to the solubility values (at pH = 3.0) of hemp proteins prepared in this study, more pronounced differences in their surface activities could be expected.

The results of Liang & Tang (2013) indicated that protein behaviour at the interface is also highly correlated with its surface hydrophobicity. Although it could be expected that increase in protein denaturation degree due to isolation conditions would lead to increase in its hydrophobicity, study on the effect of extraction pH on the properties of the pigeonpea and cowpea protein isolates has demonstrated that increase in the degree of protein denaturation resulted in an increase in the % of hydrophobic amino acids, while the exposed hydrophobicities decreased (Mwasaru et al., 1999).

The results obtained in this study indicated that, besides solubility and surface hydrophobicity, the amount of protein adsorption at the surface/interface is also affected by the unfolding and conformational flexibility of the protein at the interface (Liang & Tang, 2013), i.e. changes in the protein secondary structure and agglomeration. According to FTIR spectra HPI protein secondary structure was characterized with lower intensity of the peaks corresponding to native protein structural elements such as intramolecular β-sheets and α-helices, as well as higher intensities of "aggregation peaks" indicating enhanced protein aggregation compared to HMI (Hadnađev et al., 2018). The results of the protein dispersions (30% by weight) microstructure have also revealed that HMI behaviour was governed by electrostatic repulsions, while hydrophobic interactions were noted between the HPI molecules (Dapčević-Hadnađev, Hadnađev, Lazaridou, Moschakis, & Biliaderis, 2018). Therefore, increased hydrophobic interactions forced the HPI protein molecules to the interface in order to minimize unfavorable interactions (Papalamprou et al., 2010), although it was characterized with lower solubility in comparison to HMI. Bučko et al. (2015) have reported that the largest surface pressure of pumpkin protein solution was detected at pH = 5.0 where it exhibited the lowest solubility and ascribed that to lower hydration of proteins and the increased hydrophobic interactions.

3.2 Protein isolates stabilized emulsions characterization

Emulsifying properties of hemp proteins (HPI and HMI) at pH 3.0 were evaluated at various protein concentrations (0.25, 0.75 and 1.5%, w/v) via emulsion droplet-size analysis, microscopy, rheological and stability measurements.

3.2.1 Emulsifying ability

The droplet size distributions of emulsions in the absence of SDS are shown in Figure 1 and were found to be mostly bimodal. Emulsions containing HPI were characterized with two droplets populations: i) smaller droplets (diameter 0.5 – 1 μm) which size did not change with protein concentration and ii) larger droplets (diameters 10 – 50 μm, 4 – 20 μm and 1 – 5 μm for 0.25, 0.75 and 1.5%, w/v HPI, respectively) which size decreased with increase in the protein concentration. Emulsions stabilized with HMI had one distribution peak at d = 0.4 – 1 μm which

height increased with increase in protein concentration and the second peak at d = 1 – 5 µm which slightly shifted toward larger sizes with the decrease in the protein concentration.

In order to measure the effect of droplet flocculation, droplet-size distribution was also determined in emulsions diluted in 2% SDS. The volume mean droplet diameter ($d_{4,3}$) of emulsions with deionized water and 2% SDS as dispersant was calculated and summarized in Table 2.

As the concentration of the HPI protein increased, the volume mean droplet diameter of the emulsion significantly decreased (Table 2). The same increase in the concentration of HMI protein was found to have less progressive influence on the $d_{4,3}$ as compared to HPI stabilized emulsions. Thus HMI was deemed to have better emulsifying properties than the HPI protein, since addition of HPI in lower concentrations was not enough to completely saturate the total surface of emulsion droplets, which resulted in larger droplets and lower emulsifying activity. The better emulsifying ability of HMI might be associated with its higher solubility at pH 3.0 and, consequently, faster adsorption rate at the interface. A similar relationship between protein solubility and emulsifying ability at pH 3.0 was observed by Liang & Tang (2013). Moreover, for HPI emulsions $d_{4,3}$ in water was significantly higher than $d_{4,3}$ in 2% SDS indicating flocculation of oil droplets. Oil droplets flocculation was quantified by calculating flocculation index (Fl) which is also listed in Table 2. For HPI, the Fl progressively increased with the increase in protein concentration from 0.25 to 0.75 %, while further increase in protein concentration to 1.5% led to decline in Fl value. On the contrary, HMI emulsions were characterized with small flocculation indexes.

3.2.2 Microstructure

As both HPI and HMI were characterized with a similar surface activity but created emulsions with different droplet sizes, especially at lower protein concentrations, and exhibited differences in flocculation behaviour, confocal laser scanning microscopy (CLSM) was employed to understand the possible mechanism involved (Fig. 2). Images capture of the HPI emulsions demonstrated a visible decrease in the average droplet size with a subsequent increase in protein concentration (Fig. 2 a-c), as confirmed by particle size measurements (Fig. 1 and Table 2).

As expected from Fl index values (Table 2), CLSM images of the HPI stabilized emulsions showed flocculation of the droplets, which appeared to be connected by protein aggregates (Fig. 2a-c).

In general, in biopolymer stabilized emulsions, two types of droplet-droplet interactions could be distinguished: i) depletion and ii) bridging flocculation (Dickinson, 2003). Depletion flocculation occurs when the biopolymer in the continuous phase of the emulsion is unadsorbed or poorly adsorbed and is driven by an osmotic pressure gradient associated with the exclusion of biopolymer from a narrow region surrounding the droplet, which results in droplets attraction toward each other (McClements, 2000). On the contrary, bridging

flocculation occurs when biopolymers, added at lower levels, adsorbs onto the surface of more than one droplet leading to droplets connection via bridges and subsequently their flocculation (Dickinson, 2003). The interactions between the interfacial layer and the emulsion droplet influence the prevailing flocculation mechanism.

According to Fig. 2 (a-c) bridging flocculation prevailed over depletion flocculation since the droplets (green coloured) were surrounded and interconnected with protein molecules (red coloured).

Formation of droplets aggregates was also reported in other hemp protein stabilized emulsions. In one study, hemp protein was enzymatically hydrolysed; while this increased the solubility of the protein, the emulsifying properties of the protein decreased; this was also hypothesized to be due to presence of aggregates within the continuous aqueous phase which inhibited the formation of viscoelastic film on the droplet surface (Yin et al., 2008). Furthermore, in a study by Wang, Jiang, & Xiong (2018), hemp milk emulsions were studied for its stability using a native and alkali adjusted version of the system. As seen within this study, the protein present in its native state was able to create emulsions with enough droplet-droplet static repulsion to generate a homogenous distribution of oil droplets. However, in the alkali adjusted solution large aggregates of oil droplets were found and the droplet size of the emulsion subsequently increased.

The differences in emulsifying ability of HPI and HMI could have been because they were characterized with different denaturation degrees prior to emulsion preparation. According to Fig. 2 (d-f), HMI had the ability to generate repulsive interactions (steric and/or electrostatic) between the oil droplets which took prevalence over hydrophobic or covalent interactions between proteins. Some protein-protein aggregates observed in Fig. 2 (e-f), might originate from HMI exposure to extreme pH (3.0) and subsequent high pressure homogenization process which led to protein structural unfolding and exposure of its hydrophobic core (Tcholakova, Denkov, Sidzhakova, & Campbell, 2006b; Wang et al., 2018). On the contrary, the isolation process of HPI provoked earlier protein conformational changes which allowed the formation of hydrophobic bonds between proteins at different droplet interfaces, thus causing droplets aggregation. Moreover, since hemp proteins possess a high concentration of sulfur-containing amino acids (Hadnađev et al., 2018), exposure of previously hidden sulfhydryl groups upon protein unravelling, and thus, consequent disulfide bond formation between protein molecules must be considered. Different HPI and HMI emulsifying activities were probably the result of different ratio of bonds formed at the interface and those formed between two droplet interfaces. Similarly, Wang et al. (2012) reported that temperature (90 °C) induced denaturation of soy proteins resulted in enhanced emulsion stability due to formation of disulfide bonds with neighbouring proteins. However, increased heat treatment (120 °C) caused a reduction in the emulsifying capabilities of the soy proteins due to excessive bonding among the proteins and formation of aggregates.

Moreover, flocculation of the droplets covered with HPI could also be related to the fact that, at pH 3.0, HPI contained higher amount of non-soluble proteins (Table 1). According to Ventureira et al. (2012) the emulsions made with total proteins show more flocculation that those made with soluble protein, since non-soluble proteins could be adsorbed in two different interfaces at the same time thus leading to bridging flocculation.

3.2.3 Rheological behaviour

The rheological properties of emulsions were also monitored since they represent a useful analytical measurement to provide insights into the structural organization and interactions of the components within emulsions. Moreover, they influence emulsion shelf life and sensory properties (McClements, 1999). According to flow behaviour indexes (*n*) obtained from power law model (Eq. 5) all the emulsions showed near-Newtonian behaviour (n=0.9004-1.0130), i.e. their viscosities were nearly independent on applied shear rates. The similar behaviour was noticed by Demetriades, Coupland & McClements (1997) for 20 wt% corn oil-in-water emulsions stabilized by 2 wt% whey protein isolate. In their study flow behaviour index was close to 1 at high and low pH values. Emulsions (20 vol. %) stabilized with 2 wt% soy 11S globulin obtained at low homogenizing pressure have also shown Newtonian flow behaviour with quite low viscosities (Floury, Desrumaux & Legrand, 2002).

In general, rheological measurements (Fig. 3) revealed that HPI stabilized emulsions had slightly higher viscosities than HMI stabilized emulsions.

The higher apparent viscosity of the emulsion prepared with HPI can be explained by the occurrence of the bridging flocculation and enlargements of apparent droplet size. These findings were in accordance with other studies which also reported that flocculated emulsions were more viscous (Sun, Gunasekaran, & Richards, 2007; Wang et al., 2018; Xu, Wang, Jiang, Yuan, & Gao, 2012). According to McClements (1999), an emulsion containing flocculated droplets has a higher viscosity than an emulsion containing the same number of isolated droplets due to ability of the flocs to trap some of the continuous phase within their structure thus exhibiting a higher effective volume fraction than the actual volume fraction of the individual droplets. Moreover, differences in the viscosities of continuous phase also contributed to differences in the viscosity of final emulsion. As it can be noticed in Fig. 4, HPI dispersion (1.5% w/w) expressed a higher viscosity than HMI dispersion of the same concentration due to more intensive HPI protein-protein interactions and aggregation and consequent increase in hydrodynamic volume size (Wang et al., 2018).

Moreover, rheological behaviour of HMI stabilized emulsions was not influenced with increase in protein concentration, indicating that emulsion viscosity was insensitive to the amount of unadsorbed HMI in the continuous phase. On the contrary, HPI stabilized emulsions have exhibited an increase in apparent viscosity with the decrease in emulsifier concentration. Higher viscosity of 0.25% HPI emulsions could be ascribed to the presence of large flocs

485 composed of large droplets (Fig. 2a) that were characterized with increased hydrodynamic
486 volume in comparison to 0.75% and 1.50% HPI emulsions, which consequently led to the
487 increase in resistance to flow and then of emulsion viscosity.
488
489 **3.2.4 Emulsion stability**
490 The stability of HPI and HMI emulsions upon storage of 24 h was evaluated in terms of
491 flocculation (FI) and coalescence (CI) indexes.
492 The coalescence index of droplets was determined as the relative change in $d_{4,3}$ (in 2% SDS) of
493 droplets, before and after storage for 24 h and summarized in Table 2. The flocculation stability
494 of the fresh and stored (24 h) emulsions was also evaluated as the relative change in $d_{4,3}$
495 determined in water and 2% SDS as dispersants and the results are also included in Table 2.
496 As expected, the CI for both HPI and HMI was much lower at higher protein concentrations than
497 that at lower concentration values (Table 2), confirming the results of other studies (Liang &
498 Tang, 2014). The highest coalescence stability was noticed in 1.5% HPI emulsions, which was
499 also characterized by the highest content of non-soluble proteins (Table 1). According to Wang
500 et al. (2018), interactions between the adsorbed and unadsorbed proteins at droplet interface
501 could impart a steric stabilization against further coalescence during storage, contributing to
502 the enhancement of emulsion stability. HPI emulsions prepared with low protein
503 concentrations (0.25 – 0.75%) were unstable against coalescence, as well as susceptible to
504 droplet flocculation. Moreover, in these emulsions FI increased after the storage of 24 h. On the
505 contrary, 1.5% HPI emulsions were more stable against flocculation in comparison to 0.25% and
506 0.75% HPI emulsions.
507 Liang & Tang (2014) also reported progressive increase in the flocculation index of pea protein
508 stabilized emulsions, as the concentration increased to 1.0 g/100 ml, followed by gradual
509 decrease in flocculation index with further increase in protein concentration. This was
510 explained by the dominance of the inter-droplet attractive interactions between the adsorbed
511 proteins on individual droplets at low protein concentrations and the prevalence of the
512 interactions between the adsorbed and unadsorbed proteins over the inter-droplet interactions
513 at high protein concentrations (Liang & Tang, 2014).
514 The similar phenomenon was observed in emulsions stabilized with protein-polysaccharide
515 mixtures, where low concentration of polysaccharide caused polymeric linkages between
516 proteins adsorbed on emulsion droplets leading to bridging flocculation. Higher concentrations
517 of the same polysaccharide were sufficient to completely cover the droplet interface and
518 stabilize the emulsion via steric and electrostatic forces (Dickinson & Eriksson, 1991; Dickinson
519 & Galazka, 1991).
520 Comparing the flocculation stability of emulsions prepared in this study, HMI emulsions were
521 characterized with significantly lower Fl in comparison to HPI.

Thus it can be concluded that the mechanism for the emulsion instability vary with the technique used to isolate the hemp protein and consequently protein denaturation degree. Droplet coalescence was the underlying mechanism of the HMI emulsion instability, while instability of HPI emulsions was governed by bridging flocculation.

The creaming stability of all the tested emulsions was also evaluated. In general, all emulsions are thermodynamically unstable systems and if stored long enough, they will separate into an opaque layer consisting of oil droplets ("cream"') at the top and a slightly turbid or transparent aqueous layer ("serum") at the bottom (Liang & Tang, 2013).

In this study, creaming stability was monitored under gravitational and centrifugal forces. The gravitational separation of 0.25% HPI emulsion was the largest among all the tested emulsions since this emulsion developed into two layers after 1 day of storage (Figure 4). The creaming index at the first day of 0.25% HPI emulsion storage was 78% and it slightly increased over the storage period. The other tested emulsions did not exhibited visible creaming under gravitational forces during the tested storage period (Figure 4).

Therefore, accelerated destabilization test performed with the aid of Lumisizer measurements was also used to determine the effect of HPI and HMI concentration on emulsions stability. The measurement is based on the monitoring of changes in intensity of the parallel transmitted NIR light over the entire sample length during centrifugation at different time intervals (Lim & Roos, 2015). At the beginning of the centrifugation, only a small amount of light is transmitted along the sample cell, while, during centrifugation, the heavier and more transparent aqueous phase moves to the bottom. This accelerated creaming process promotes transmission as the less transparent oil phase moves to the top (Xiang, Liu, Fan, & Gao, 2015). The larger changes in the transmission with centrifugation, i.e. higher slope of the integral transmission vs. time, indicate emulsions with lower stability (Lim & Roos, 2015; Xiang, Liu, Fan, & Gao, 2015).

Integral transmission (%) against time (s) for all systems is shown in Fig. 5. As expected, the creaming behaviour of emulsions in accelerated test was highly related to the applied protein concentration. Emulsions with lower protein concentrations creamed more readily as compared to systems with 0.75 and 1.5% (w/w) emulsifier. For the 0.25% (w/w) HPI emulsion, the creaming rate was high at the beginning, and after that, it gradually increased (Fig. 5). In contrast, at the beginning, the creaming instability of the HMI emulsion at concentration of 0.25% (w/w) was lower than that of 0.25% (w/w) HPI emulsion, but it progressively increased over a prolonged period and after 3000 s centrifugation it was even higher. This indicates that creaming stability under accelerated mode is associated with the combined effect of droplet size as well as the flocculation and coalescence stability.

In general, creaming stability of emulsions can be enhanced with decreased droplet size and increased continuous phase viscosity (McClements, 1999). In this study, the creaming instability of HMI emulsions was the consequence of decreased stability against coalescence (Table 2) and low continuous phase viscosity (Fig. 3) which led to increased movement of droplets due to

gravity or Brownian motion; however, fast creaming in 0.25 and 0.75% (w/w) HPI emulsions was influenced with large droplet sizes and protein induced droplet bridging flocculation. Interestingly, emulsions with 1.5% (w/w) HPI exhibited the best stability against creaming, despite the bridging flocculation of droplets observed (Fig. 2 f).

In contrast to 1.5% (w/w) HMI emulsions, HPI emulsion prepared with the same amount of protein exhibited bridging flocculation (Fig. 2 c) and higher continuous phase viscosity (Fig. 3). In general, at low or intermediate droplet concentrations, flocculation tends to increase the creaming rate because the floccules have a larger effective size than the individual droplets, while in concentrated emulsions flocculation retards creaming due to formation of a three-dimensional network of aggregated floccules that prevents the individual droplets from moving (McClements, 1999). However, according to McClements (1999), a network can form at lower disperse phase volume fractions when the droplets in a floccule are more openly packed, and therefore creaming is prevented at lower droplet concentrations. Since, in this paper, droplets in floccules were openly packed (Fig. 2), the possible reason for higher creaming stability of 1.5% (w/w) HPI emulsion could be attribute to formation of a weak transient network of floccules that suppressed the movements of the droplets. Ventureira, Martinez, & Anon, (2010) also noticed that emulsion prepared at pH 2.0 with non-hydrolized amaranth proteins showed lower creaming rate in comparison to other emulsions although it was characterized with the highest degree of flocculation. Moreover, aggregation of HPI protein molecules in the continuous phase influenced an increase in the viscosity (Fig. 3), which suppressed droplets movement within the emulsion (Frelichowska, Bolzinger, & Chevalier, 2010; Liang & Tang, 2014).

4 Conclusions

Alkali extracted/isoelectric precipitated hemp protein (HPI) and micellar (salt extracted) hemp protein (HMI) possess different emulsification and emulsion stabilization mechanisms. The two proteins differed in their denaturation state which contributed to the observed changes in their solubility and surface/interfacial activity. HMI protein, which had preserved native state after isolation process, formed emulsions with small droplets ($d_{4,3}$ = 1.92 - 3.42 µm in 2% SDS) and enough droplet-droplet static repulsion. However, low viscosity of HMI stabilized emulsions enabled fast droplet movement and led to increased creaming and coalescence at lower protein concentrations (0.25 – 0.75% w/w). In the HPI stabilized emulsions, isolation technique favoured pH-induced structural unfolding of protein molecules, exposure of hydrophobic sites and sulfhydryl groups and subsequent formation of protein connected droplet aggregates during emulsification. At lower HPI concentrations (0.25 - 0.75 % w/w), the dominant type of interactions were the ones between proteins adsorbed at the droplets which influenced bridging flocculation, while at higher HPI concentrations (1.5% w/w), there was the prevalence of the interactions between the adsorbed and unadsorbed proteins at droplet interface as well

as the protein-protein interactions in the continuous phase. While the latter influenced formation of transient network of floccules and increased continuous phase viscosity thus contributing to the emulsion stability, the former led to increase in emulsion droplet size and instability.

Acknowledgement

This work was financially supported by the Provincial Secretariat for Higher Education and Scientific Research, Republic of Serbia [grant number 142-451-2458/2018-01/02] as a part of the project entitled "Techno-functionality of proteins from alternative plant sources from Vojvodina region".

The experimental work on this paper is supported by project that has received funding from the European Union's Horizon 2020 Spreading Excellence and Widening Participation programme [under grant agreement number 692276].

References

Bučko, S., Katona, J., Popović, L., Vaštag, Ž.,Petrović, L., & Vučinić–Vasić, M. (2015). Investigation on solubility, interfacial and emulsifying properties of pumpkin (Cucurbita pepo) seed protein isolate. *LWT-Food Science and Technology*, *64*, 609-615.

Callaway, J. C. (2004). Hempseed as a nutritional resource: an overview. *Euphytica*, *140*, 65-72.

Dapčević-Hadnađev, T., Hadnađev, M., Lazaridou, A., Moschakis, T., & Biliaderis, C. G. (2018). Hempseed meal protein isolates prepared by different isolation techniques. Part II. gelation properties at different ionic strengths. Food Hydrocolloids, 81, 481-489.

Demetriades, K., Coupland, J. N., & McClements, D. J. (1997). Physical properties of whey protein stabilized emulsions as related to pH and NaCl. *Journal of Food Science*, 62(2), 342-347.

Dickinson, E. (2003). Hydrocolloids at interfaces and the influence on the properties of dispersed systems. *Food Hydrocolloids*, 17(1), 25-39.

Dickinson, E., & Eriksson, L. (1991). Particle flocculation by adsorbing polymers. *Advances in Colloid and Interface Science*, *34*, 1-29.

Dickinson, E., & Galazka, V. B. (1991). Emulsion stabilization by ionic and covalent complexes of β-lactoglobulin with polysaccharides. *Food Hydrocolloids*, *5*, 281-296.

Foegeding, E. A., & Davis, J. P. (2011). Food protein functionality: A comprehensive approach. *Food Hydrocolloids*, *25*, 1853–1864.

Frelichowska, J., Bolzinger, M. A., & Chevalier, Y. (2010). Effects of solid particle content on properties of o/w Pickering emulsions. *Journal of colloid and interface science*, *351*, 348-356.

Gharsallaoui, A., Cases, E., Chambin, O., & Saurel, R. (2009). Interfacial and emulsifying characteristics of acid-treated pea protein. *Food Biophysics*, *4*, 273-280.

634 Guzey, D., & McClements, D. J. (2007). Impact of electrostatic interactions on formation and
635 stability of emulsions containing oil droplets coated by β-lactoglobulin–pectin complexes.
636 *Journal of Agricultural and Food Chemistry*, *55*, 475–485.

637 Hadnađev, M. S., Dapčević-Hadnađev, T. R., Pojić, M. M., Šarić, B. M., Mišan, A. Č., Jovanov, P.
638 T., & Sakač, M. B. (2017). Progress in vegetable proteins isolation techniques: A review. *Food*
639 *and Feed Research*, *44*, 11-21.

640 Hadnađev, M., Dapčević-Hadnađev, T., Lazaridou, A., Moschakis, T., Michaelidou, A.-M.,
641 Popović, S., & Biliaderis, C.G. (2018). Hempseed meal protein isolates prepared by different
642 isolation techniques. Part I. physicochemical properties. *Food Hydrocolloids*, *79*, 526-533.

643 Jafari, S. M., Beheshti, P., & Assadpoor, E. (2012). Rheological behavior and stability of D-
644 limonene emulsions made by a novel hydrocolloid (Angum gum) compared with Arabic gum.
645 *Journal of Food Engineering*, *109*, 1–8.

646 Joshi, M., Adhikari, B., Aldred, P., Panozzo, J. F., Kasapis, S., & Barrow, C. J. (2012). Interfacial
647 and emulsifying properties of lentil protein isolate. *Food Chemistry*, 134(3), 1343-1353.

648 Karaca, A. C., Low, N., & Nickerson, M. (2011a). Emulsifying properties of chickpea, faba bean,
649 lentil and pea proteins produced by isoelectric precipitation and salt extraction. *Food Research*
650 *International*, *44*, 2742–2750.

651 Karaca, A. C., Low, N., & Nickerson, M. (2011b). Emulsifying properties of canola and flaxseed
652 protein isolates produced by isoelectric precipitation and salt extraction. *Food Research*
653 *International*, *44*, 2991-2998.

654 Krause, J. P., Schultz, M., & Dudek, S. (2002). Effect of extraction conditions on composition,
655 surface activity and rheological properties of protein isolates from flaxseed
656 (*Linumusitativissimum* L). *Journal of the Science of Food and Agriculture*, *82*, 970-976.

657 Lam, R. S., & Nickerson, M. T. (2013). Food proteins: a review on their emulsifying properties
658 using a structure–function approach. *Food Chemistry*, *141*, 975-984.

659 Liang, H. N., & Tang, C. H. (2013). pH-dependent emulsifying properties of pea [Pisumsativum
660 (L.)] proteins. *Food Hydrocolloids*, *33*, 309-319.

661 Liang, H. N., & Tang, C. H. (2014). Pea protein exhibits a novel Pickering stabilization for oil-in-
662 water emulsions at pH 3.0. *LWT-Food Science and Technology*, *58*, 463-469.

663 Lim, A. S., & Roos, Y. H. (2015). Stability of flocculated particles in concentrated and high
664 hydrophilic solid layer-by-layer (LBL) emulsions formed using whey proteins and gum Arabic.
665 *Food Research International*, *74*, 160-167.

666 Lowry, O. H., Rosenbrough, N. J., Fair, A. L., & Randall, R. J. (1951). Protein measurement with
667 the Folin-phenol reagent. *Journal of Biological Chemistry*, *193*, 265-275.

668 Malomo, S. A., & Aluko, R. E. (2015).A comparative study of the structural and functional
669 properties of isolated hemp seed (*Cannabis sativa* L.) albumin and globulin fractions. *Food*
670 *Hydrocolloids*, *43*, 743-752.

671 Malomo, S. A., He, R., & Aluko, R. E. (2014). Structural and functional properties of hemp seed
672 protein products. *Journal of Food Science*, *79*, C1512-C1521.

673 McClements, D. J. (1999). *Food emulsions: Principles, practice and techniques*. Boca Raton: CRC
674 Press LLC, (Chapter 5 and 7).

675 McClements, D. J. (2000). Comments on viscosity enhancement and depletion flocculation by
676 polysaccharides. *Food Hydrocolloids*, 14(2), 173-177.

677 Milanović, M., Krstonošić, V., Dokić, Lj., Hadnađev, M., & Dapčević Hadnađev, T. (2015). Insight
678 into the interaction between Carbopol® 940 and ionic/nonionic surfactant. *Journal of*
679 *Surfactants and Detergents*, *18*, 505-516.

680 Mwasaru, M. A., Muhammad, K., Bakar, J., & Man, Y. B. C. (1999). Effects of isolation technique
681 and conditions on the extractability, physicochemical and functional properties of pigeonpea
682 (Cajanus cajan) and cowpea (Vigna unguiculata) protein isolates. I. Physicochemical properties.
683 *Food Chemistry*, 67(4), 435-443.

684 Niño, M. R. R., Sánchez, C. C., Fernández, M. C., & Patino, J. M. R. (2001). Protein and lipid films
685 at equilibrium at air-water interface. *Journal of the American Oil Chemists' Society*, *78*, 873-879.

686 Onsaard, E., Vittayanont, M., Srigam, S., & McClements, D. J. (2006). Comparison of properties
687 of oil-in-water emulsions stabilized by coconut cream proteins with those stabilized by whey
688 protein isolate. *Food Research International*, *39*, 78-86.

689 Papalamprou, E. M., Doxastakis, G. I., & Kiosseoglou, V. (2010). Chickpea protein isolates
690 obtained by wet extraction as emulsifying agents. *Journal of the Science of Food Agriculture*, *90*,
691 304-313.

692 Petzold, G., Goltzsche, C., Mende, M., Schwarz, S., & Jaeger, W. (2009). Monitoring the stability
693 of nanosized silica dispersions in presence of polycations by a novel centrifugal sedimentation
694 method. *Journal of applied polymer science*, *114*, 696-704.

695 Pojić, M., Mišan, A., Sakač, M., Dapčević Hadnađev, T., Šarić, B., Milovanović, I., &Hadnađev, M.
696 (2014). Characterization of by products originating from hemp oil processing. *Journal of*
697 *Agricultural and Food Chemistry*, *62*, 12436-12442.

698 Pojić, M., Mišan, A., & Tiwari, B. (2018).Eco-innovative technologies for extraction of proteins
699 for human consumption from renewable protein sources of plant origin. *Trends in Food Science*
700 *& Technology*, *75*, 93-104.

Radha, C., & Prakash, V. (2009). Structural and functional properties of heat-processed soyabean flour: Effect of proteolytic modification. *Food Science and Technology International*, *15*, 453-463.

Sobisch, T., &Lerche, D. (2008). Thickener performance traced by multi sample analytical centrifugation. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, *331*, 114-118.

Sun, C., Gunasekaran, S., & Richards, M. P. (2007). Effect of xanthan gum on physicochemical properties of whey protein isolate stabilized oil-in-water emulsions. *Food Hydrocolloids*, *21*, 555-564.

Tang, C. H., Ten, Z., Wang, X. S., & Yang, X. Q. (2006). Physicochemical and functional properties of hemp (*Cannabis sativa* L.) protein isolate. *Journal of Agricultural and Food Chemistry*, *54*, 8945-8950.

Tcholakova, S., Denkov, N. D., Ivanov, I. B., & Campbell, B. (2006a).Coalescence stability of emulsions containing globular milk proteins. *Advances in Colloid and Interface Science*, *123–126*, 259–293.

Tcholakova, S., Denkov, N. D., Sidzhakova, D., & Campbell, B. (2006b).Effect of thermal treatment, ionic strength, and pH on the short-term and long-term coalescence stability of β-lactoglobulin emulsions. *Langmuir*, *22*, 6042-6052.

Ventureira, J. L., Bolontrade, A. J., Speroni, F., David-Briand, E., Scilingo, A. A., Ropers, M. H., Boury, F., Añón, M.C. & Anton, M. (2012). Interfacial and emulsifying properties of amaranth (Amaranthus hypochondriacus) protein isolates under different conditions of pH. *LWT-Food Science and Technology*, *45*, 1-7.

Ventureira, J., Martinez, E. N., & Anon, M. C. (2010). Stability of oil: Water emulsions of amaranth proteins. Effect of hydrolysis and pH. *Food Hydrocolloids*, 24(6-7), 551-559.

Wang, Q., Jiang, J., & Xiong, Y. L. (2018). High pressure homogenization combined with pH shift treatment: A process to produce physically and oxidatively stable hemp milk. *Food Research International*, *106*, 487-494.

Wang, J. M., Xia, N., Yang, X. Q., Yin, S. W., Qi, J. R., He, X. T., Yuan, D.B. & Wang, L. J. (2012). Adsorption and dilatational rheology of heat-treated soy protein at the oil–water interface: Relationship to structural properties. *Journal of Agricultural and Food Chemistry*, *60*, 3302-3310.

Xiang, J., Liu, F., Fan, R., & Gao, Y. (2015). Physicochemical stability of citral emulsions stabilized by milk proteins (lactoferrin, α-lactalbumin, β-lactoglobulin) and beet pectin. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, *487*, 104-112.

Xu, D., Wang, X., Jiang, J., Yuan, F., & Gao, Y. (2012). Impact of whey protein–Beet pectin conjugation on the physicochemical stability of β-carotene emulsions. *Food Hydrocolloids*, *28*, 258-266.

Yin, S. W., Tang, C. H., Cao, J. S., Hu, E. K., Wen, Q. B., & Yang, X. Q. (2008). Effects of limited enzymatic hydrolysis with trypsin on the functional properties of hemp (*Cannabis sativa* L.) protein isolate. *Food Chemistry*, *106*, 1004-1013.

Figure captions

Figure 1. The size distribution profiles of alkali extracted hemp protein (HPI) and micellar hemp protein (HMI) stabilized emulsion (fresh emulsions, diluted in water) at different protein concentrations (0.25-1.5% w/w)

Figure 2. Confocal laser scanning micrographs of hemp protein stabilized emulsions at pH 3.0. Emulsions stabilized with alkali extracted hemp protein (HPI) are shown in images at (a) 0.25%, (b) 0.75% and (c) 1.50% w/w in the aqueous phase. Native micellar hemp protein (HMI) emulsions are also shown with various concentrations of HMI: (d) 0.25%; (e) 0.75% and (f) 1.50%. Sunflower oil acting as the oil phase in the emulsion is labelled as green with hemp protein in red. Scale bar = 25µm

Figure 3. Flow curves of 10% O/W emulsions stabilized by alkali extracted hemp protein (HPI) and micellar hemp protein (HMI) at various levels (0.25-1.5% w/w) and 1.5% w/w HPI and HMI dispersions (continuous phases)

Figure 4. Images of the alkali extracted hemp protein (HPI) and micellar hemp protein (HMI) stabilized emulsion at different protein concentrations (0.25-1.5% w/w) after storage for 14 days

Figure 5. Integral transmission profiles as a measure of stability of 10% O/W emulsions stabilized by alkali extracted hemp protein (HPI) and micellar hemp protein (HMI) at various concentrations (0.25-1.5% w/w)

Table 1. Solubility at pH = 3.0, surface and interfacial pressure of hemp protein isolate dispersions of different concentrations prepared by different extraction techniques

Sample	**Solubility at pH = 3.0 (%)**	
HPI	71.2±3.56[a]	
HMI	88.2±0.73[b]	
Sample concentration	**Surface pressure π (mN/m)**	**Interfacial pressure π (mN/m)**
0% HPI/HMI	0[a]	0[a]
0.25% HPI	24.93±0.259[b]	9.13±0.115[b]
0.75% HPI	27.67±0.148[c]	11.07±0.059[c]
1.50% HPI	30.47±0.148[d]	12.63±0.503[d]
0.25% HMI	28.21±0.167[c]	9.47±0.252[b]
0.75% HMI	30.95±0.122[d]	13.60±0.557[d]
1.50% HMI	32.61±0.26[e]	15.73±0.416[e]

Values followed by different letters indicate significant differences ($p \leq 0.05$)

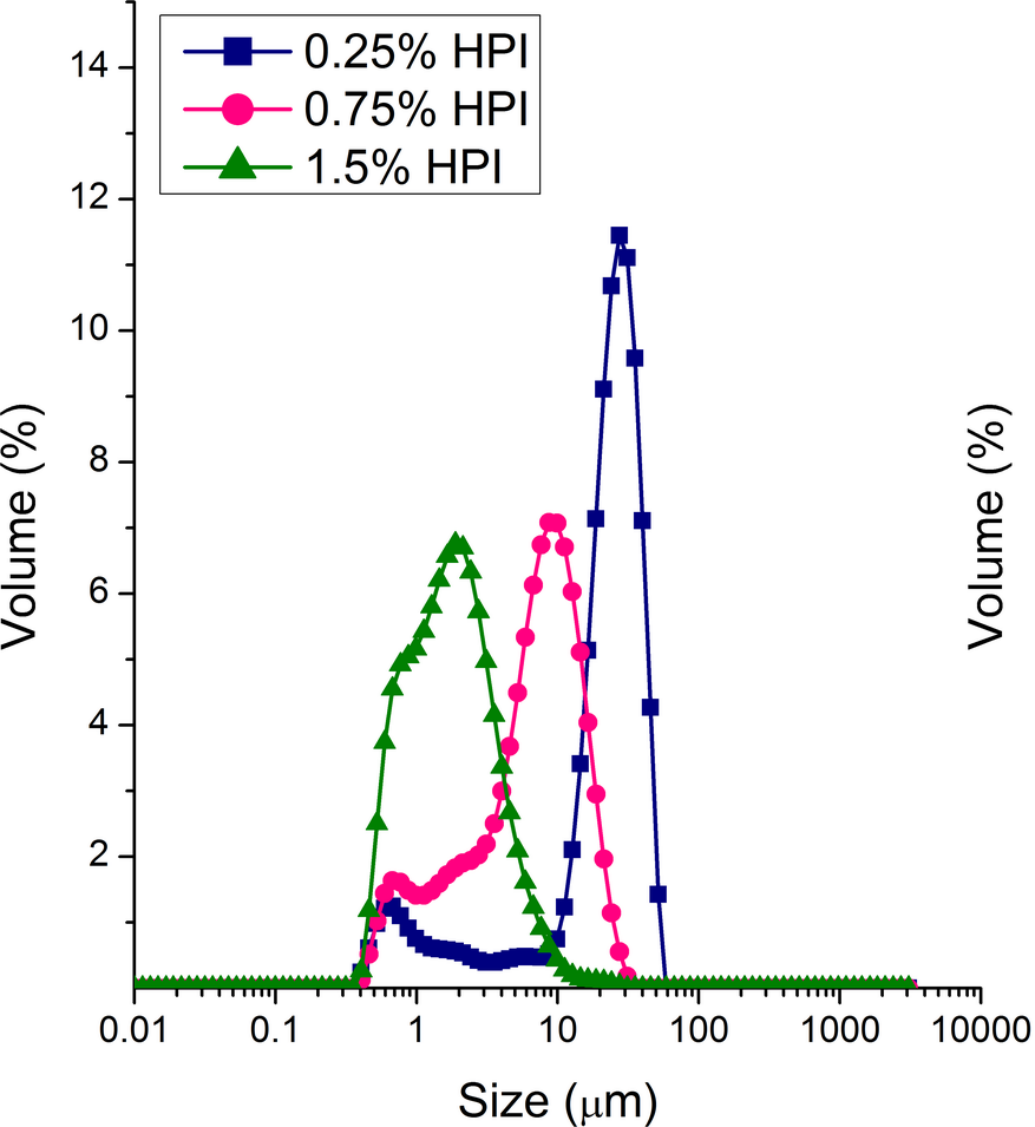
Table 2. Volume mean droplet diameter ($d_{4,3}$), flocculation (FI) and coalescence (CI) indices of HPI and HMI hemp protein stabilized emulsion, for different protein concentrations (0.25-1.5% w/w) immediately after preparation (0 h) and after 24 h of storage

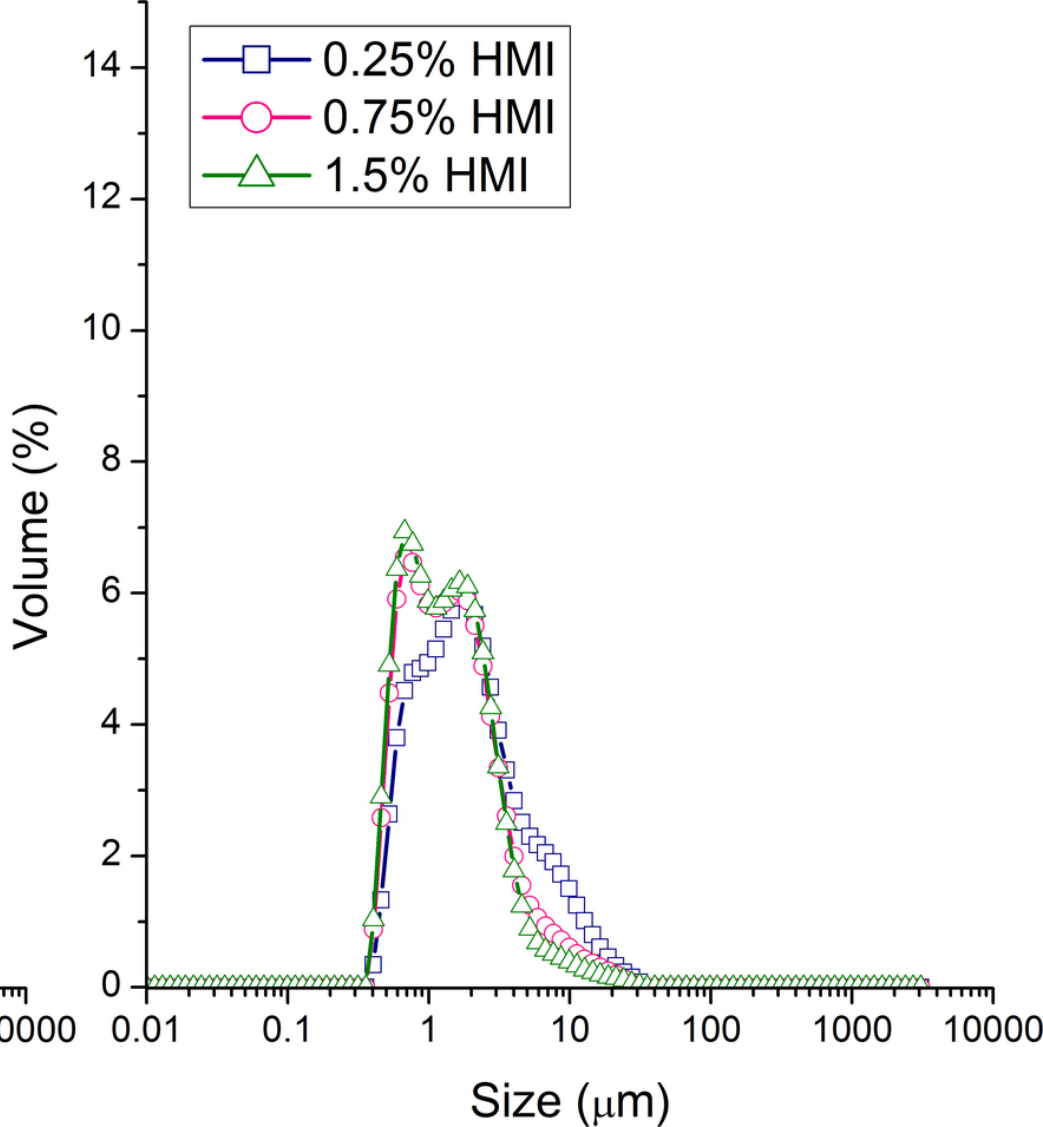
Sample	$d_{4,3}$ (µm)				FI (%)		CI (%)
	0 h		24 h		0 h	24 h	24 h
	Water	2% SDS	Water	2% SDS			2% SDS
0.25% HPI	24.17±0.814^{c}	15.77±0.404^{c}	39.80±1.670^{d}	24.13±1.320^{c}	53.3±2.56^{c}	65.0±3.46^{c}	53.1±7.43^{d}
0.75% HPI	8.76±0.431^{b}	3.80±0.141^{b}	12.15±0.495^{c}	5.10±0.283^{b}	130.6±2.77^{d}	138.3±3.51^{d}	34.2±2.45^{c}
1.5% HPI	2.71±0.260^{a}	2.25±0.160^{a}	2.86±0.290^{ab}	2.37±0.178^{a}	20.1±3.00^{b}	20.7±3.82^{b}	5.0±0.90^{a}
0.25% HMI	3.54±0.403^{a}	3.42±0.375^{b}	5.40±0.311^{b}	5.28±0.332^{b}	3.5±0.44^{a}	2.2±0.54^{a}	54.7±7.22^{d}
0.75% HMI	2.31±0.083^{a}	2.18±0.070^{a}	2.76±0.070^{ab}	2.63±0.090^{a}	5.8±0.56^{a}	5.0±0.93^{a}	20.5±1.31^{bc}
1.5% HMI	2.01±0.042^{a}	1.92±0.043^{a}	2.20±0.020^{a}	2.12±0.028^{a}	4.7±0.10^{a}	3.5±0.38^{a}	10.4±0.97^{ab}

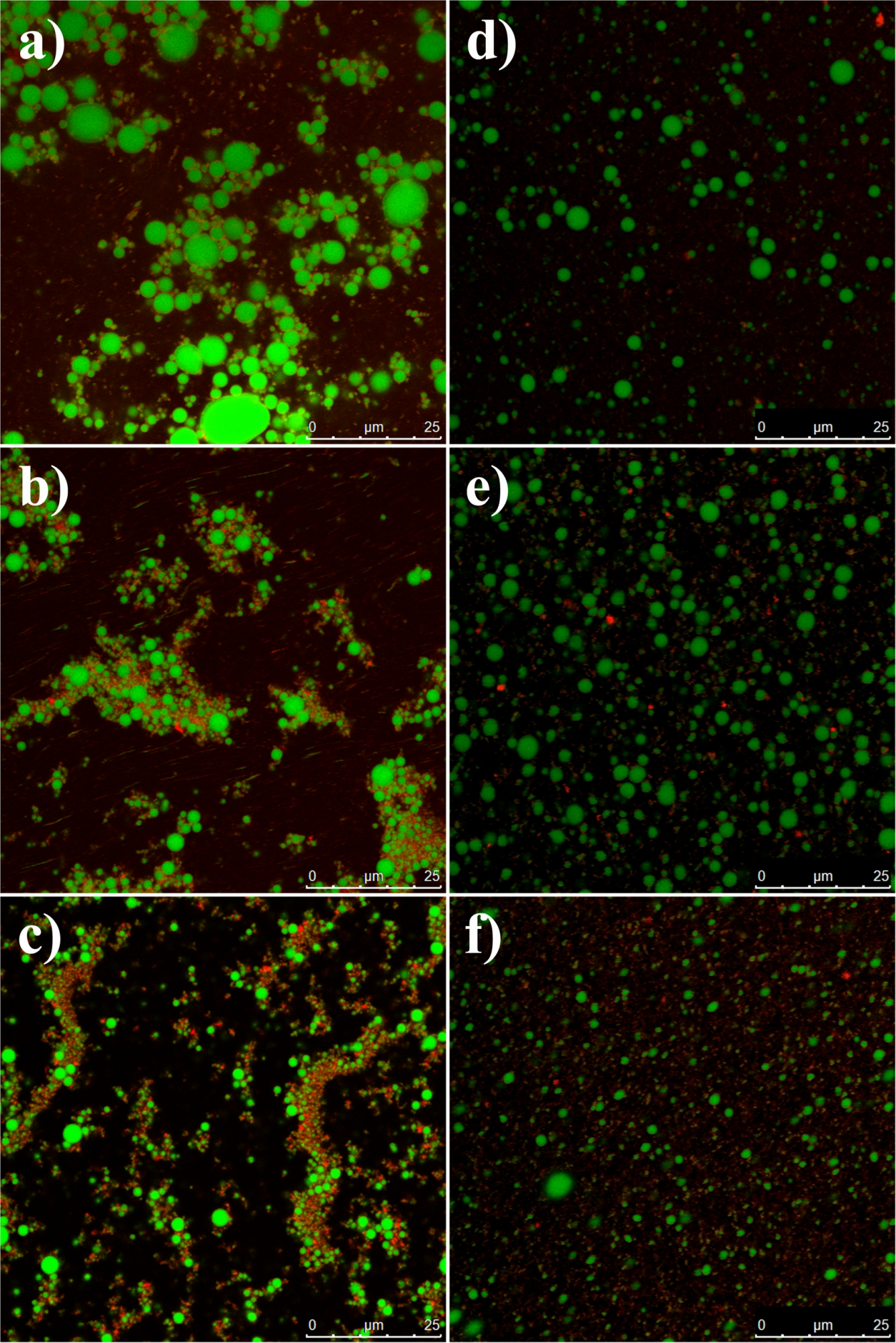
Values followed by different letters indicate significant differences ($p \leq 0.05$)

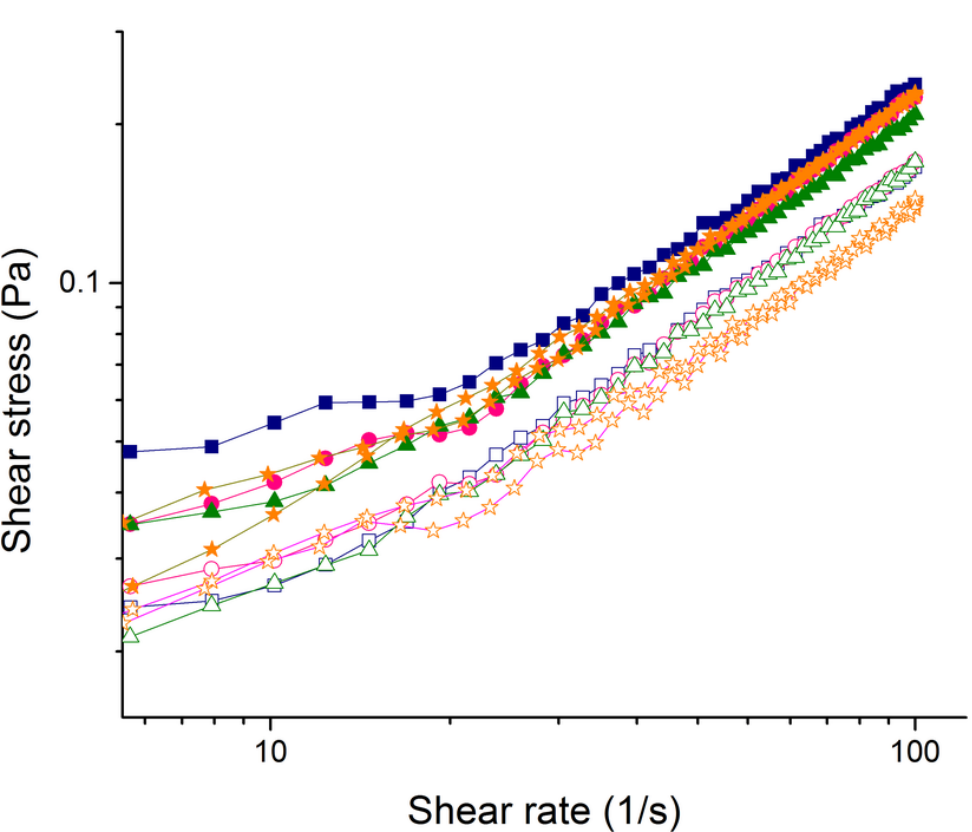
Highlights

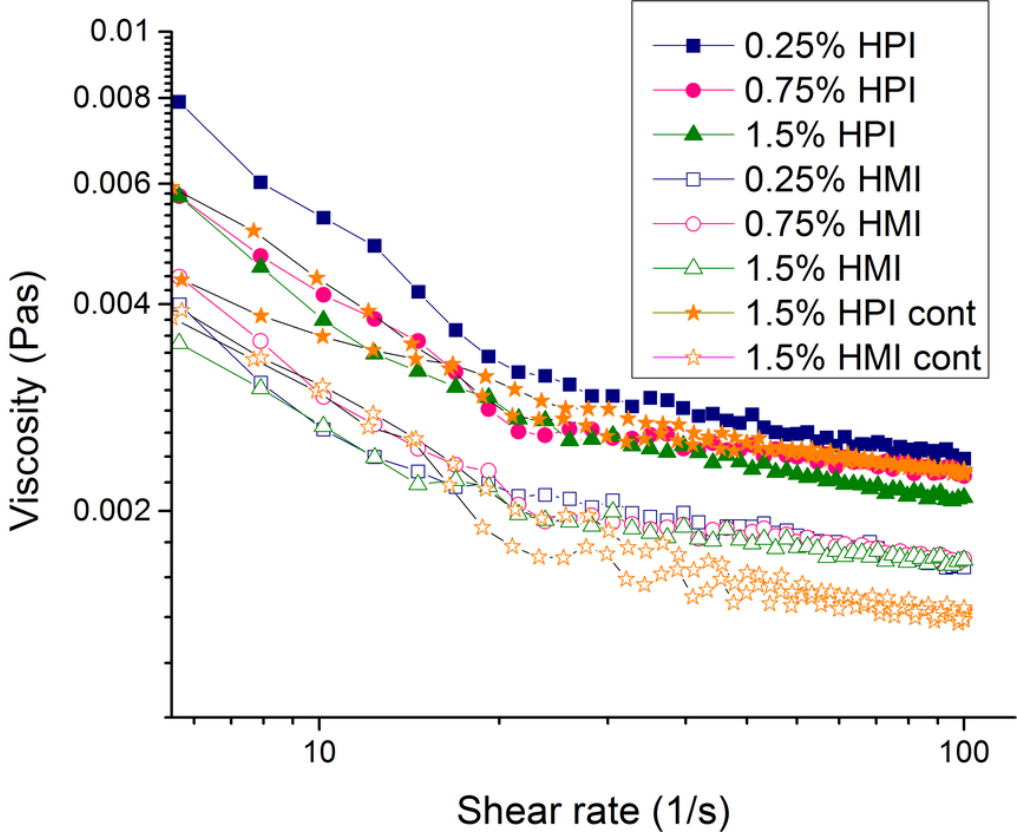
- Hemp proteins were isolated using two different isolation procedures
- Alkali (HPI) and salt (HMI) extracted proteins differ in emulsifying ability
- HMI emulsions had smaller volume mean droplet diameter than HPI emulsions
- Microscopy indicated occurrence of bridging flocculation in HPI emulsions
- 1.5% HPI emulsions had the best stability due to formation of floccules network

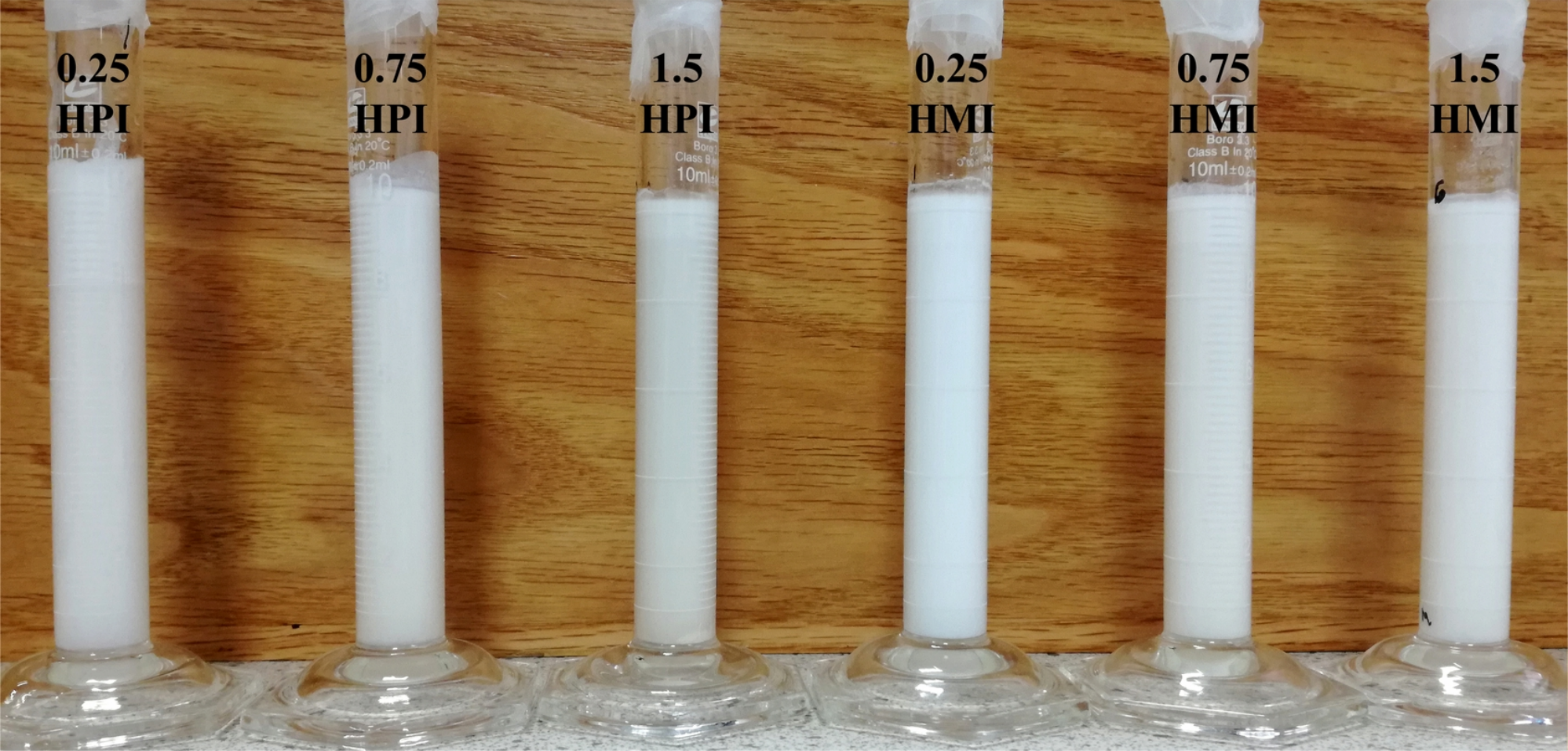

0.25% HPI
0.75% HPI
1.5% HPI
Volume (%)
Size (μm)
0.01
0.1
1
10
100
1000
10000
2
4
6
8
10
12
14

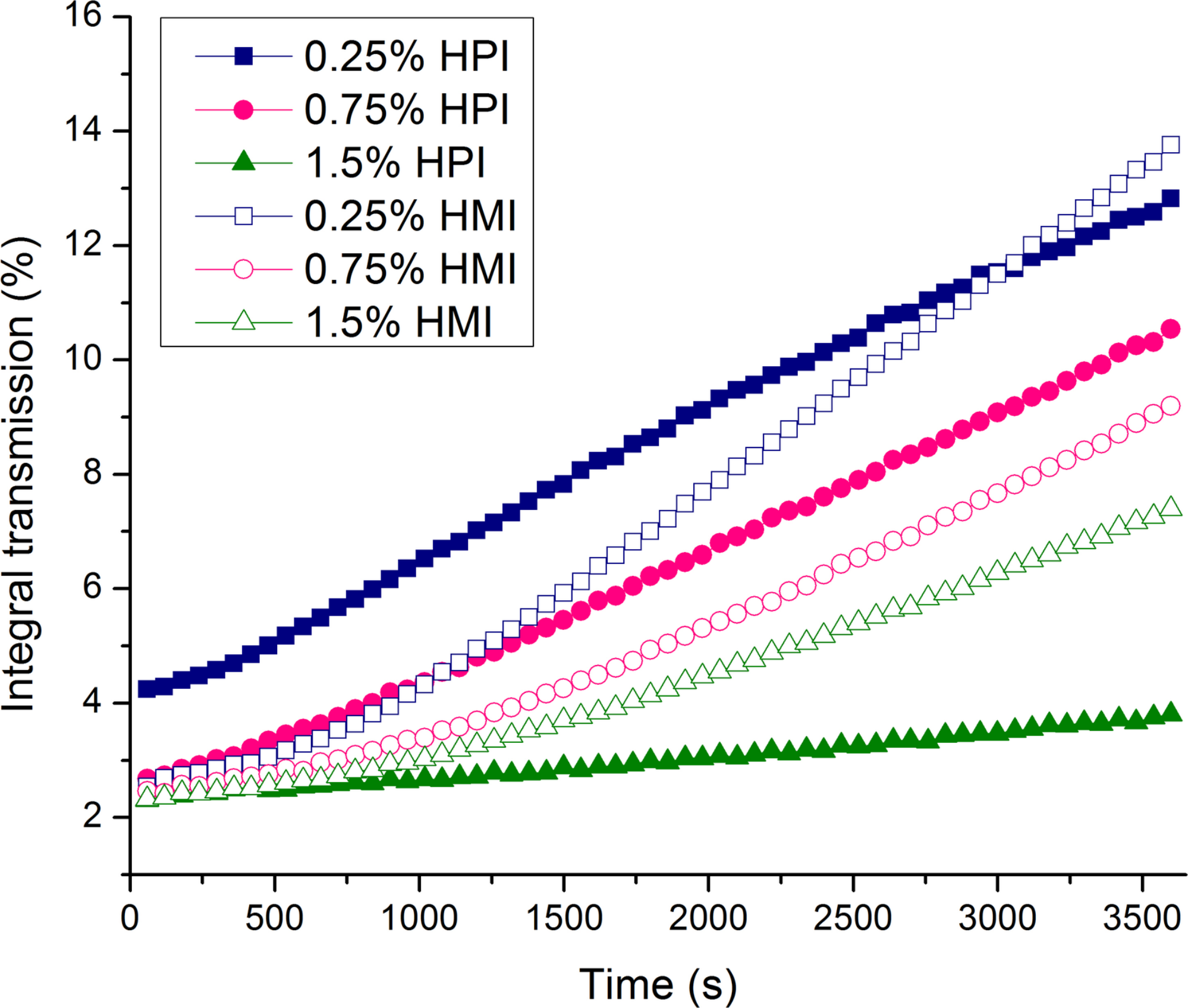

0.25% HMI
0.75% HMI
1.5% HMI
Volume (%)
Size (μm)
0.01
0.1
1
10
100
1000
10000
0
2
4
6
8
10
12
14

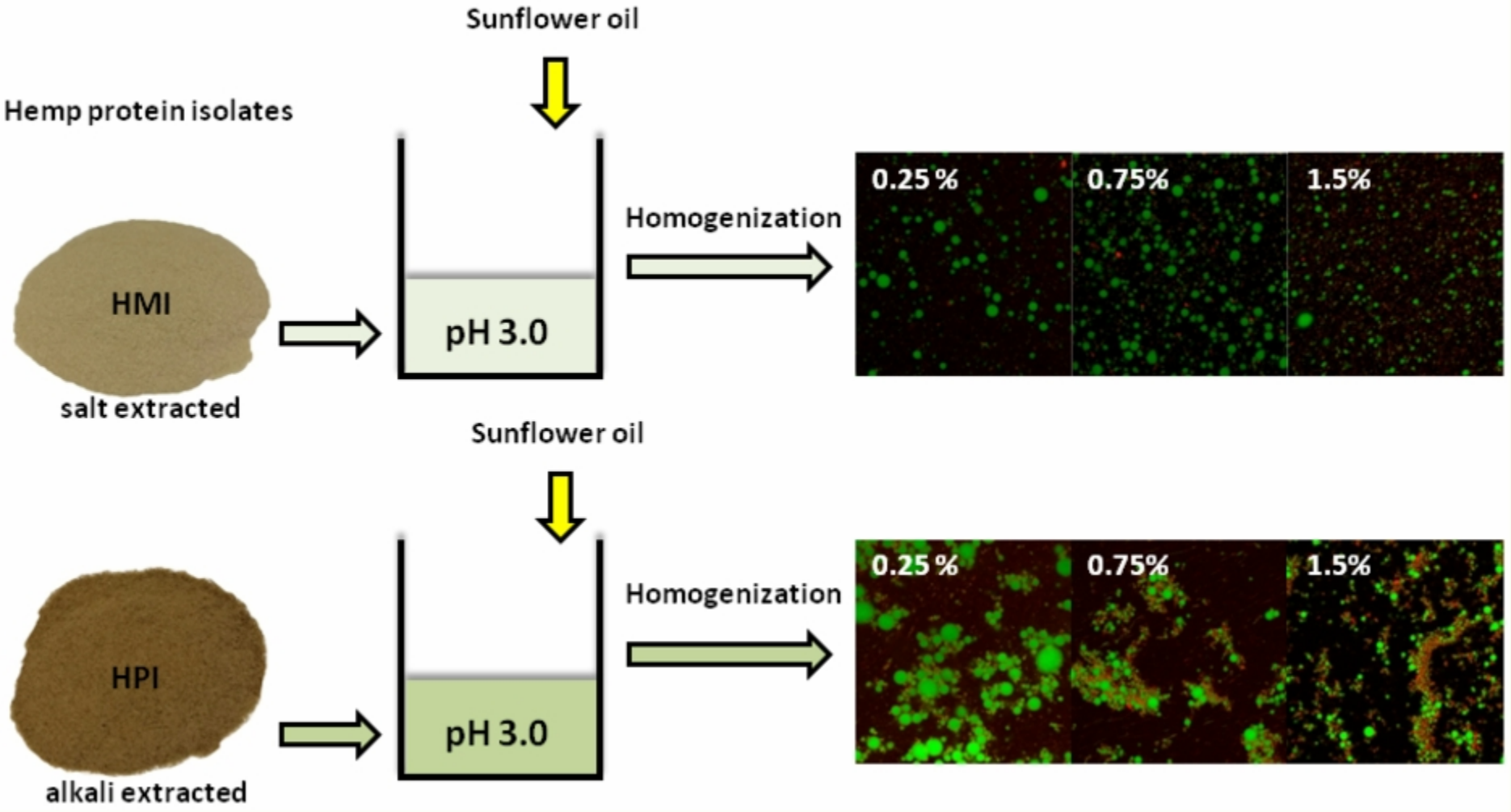

a)
0 µm 25
d)
0 µm 25
b)
0 µm 25
e)
0 µm 25
c)
0 µm 25
f)
0 µm 25


Shear stress (Pa)
0.1
10
100
Shear rate (1/s)


Viscosity (Pas)
0.01
0.008
0.006
0.004
0.002
10
100
Shear rate (1/s)
0.25% HPI
0.75% HPI
1.5% HPI
0.25% HMI
0.75% HMI
1.5% HMI
1.5% HPI cont
1.5% HMI cont


0.25
HPI
0.75
HPI
1.5
HPI
0.25
HMI
0.75
HMI
1.5
HMI


0.25% HPI
0.75% HPI
1.5% HPI
0.25% HMI
0.75% HMI
1.5% HMI
Integral transmission (%)
Time (s)
16
14
12
10
8
6
4
2
0
500
1000
1500
2000
2500
3000
3500


Sunflower oil
Hemp protein isolates
HMI
salt extracted
pH 3.0
Homogenization
0.25 %
0.75%
1.5%
Sunflower oil
HPI
alkali extracted
pH 3.0
Homogenization
0.25 %
0.75%
1.5%