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Plant proteins at low concentrations as natural emulsifiers for an effective orange essential oil microencapsulation by spray drying

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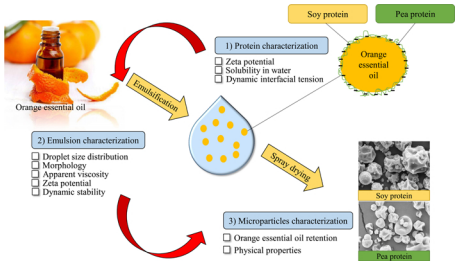
GRAPHICAL ABSTRACT



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ABSTRACT

In this work, pea and soy proteins were used as emulsifiers on the encapsulation of orange essential oil (OEO, rich in d-limonene) by emulsification followed by spray drying. A commercial pea protein concentrate (PPC) was studied regarding its ability to act as an emulsifier, stabilize oil-in-water emulsions containing OEO, and to produce spray-dried microparticles in comparison to a commercial soy protein isolate (SPI), which is an emulsifier that has been already used on the microencapsulation of flavors. Both PPC and SPI presented low solubility in water at their natural pH; however, the even lower solubility of PPC resulted in lower adsorption at the oil-water interface. High solids oil-in-water emulsions stabilized using different concentrations of PPC or SPI (0.6–6.0 wt%) presented different physicochemical properties and physical stability due to the difference between the solubility of PPC and SPI in water. The spray-dried microparticles produced from selected SPI emulsions presented better stability during the drying stage than PPC emulsions, which resulted in higher OEO retention values (84.1–100 wt% and 92.6–97.9 wt% for PPC and SPI, respectively). Overall, the spray-dried microparticles produced using PPC or SPI present similar physical properties that promote the protection of the encapsulated OEO. Based on these results, despite its low solubility, pea protein can be used for encapsulating OEO effectively; therefore, its use as an emulsifier on the encapsulation of other different flavor compounds must be explored even further.

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1. Introduction

In the microencapsulation field of food ingredients, synthetic emulsifiers and animal proteins are the most common sources of surface-active compounds that are used for the production of oil-in-water emulsions [1]. These substances present superior emulsifying properties, but products containing these types of materials have faced some rejection by consumers that have been adopting different food habits (i.e. avoid synthetic ingredients) or vegans. This current scenario has attracted the attention of industry and academia to explore the use of new natural and more sustainable sources of emulsifiers. Plant proteins are some of the most promising natural emulsifiers that have been studied as a substitute for animal proteins and synthetic emulsifiers [2,3]. Plant proteins present inferior emulsifying properties than animal proteins; however, several studies have successfully used plant-based emulsifiers for the encapsulation of lipophilic compounds through different encapsulation techniques [4].

Soy protein is one of the most applied plant proteins in the food industry due to its high availability and good physicochemical and technological properties [5]. The performance of soy protein to stabilize food emulsions and encapsulate lipophilic compounds has already been extensively studied [6–9]. Results have shown that soy protein has a better capacity to act as an emulsifying agent when compared to other plant proteins [10,11]. However, novel alternatives of plant proteins have been studied for the production of emulsions, and some showed good results [12]. In the last few years, pea protein has gained some attention due to its relevant growth in the food sector as a sustainable source compared to conventional protein sources. The interest regarding pea protein is mainly related to the growing vegan population, popularity of plant-based products, and the growing consumers' awareness about nutritional benefits offered by pea and pea-based products [13]. Pea proteins are increasingly used as an alternative for soy protein since the pea cultivars may be grown in more moderate climates than soy. Additionally, pea proteins are less related to GMO questions and are not listed as allergenic [14]. Despite its lower solubility in water, studies have shown that pea protein could be used as an emulsifier for the encapsulation and protection of lipophilic compounds by emulsion production [15–17].

Orange essential oil (OEO) is a complex mixture of low molecular weight compounds [18]. Its primary compound, d-limonene, is a terpene that corresponds to about 90 wt% of the mixture, and it is a valuable volatile compound that is applied in a variety of products in the industry, such as in cleaning products, cosmetics, and foodstuffs [19–22]. Flavors, such as OEO must be microencapsulated in order to retain the volatile compounds and preserve the aroma for a longer time [23]. The spray drying of high solids emulsions containing microencapsulated flavors is the most used technique for the conversion of liquid aromas into powders, which makes it easier to transport and store this kind of products [24]. Moreover, this technique is useful for encapsulating heat-sensitive and volatile materials such as OEO because of its short drying time [25].

In the case of the encapsulation of volatile substances such as OEO through this procedure, the emulsion produced must present small oil droplets, which will promote better emulsion stability and high oil retention during the spray drying stage [26]. In this sense, the performance of a protein as an emulsion stabilizer must be evaluated before the spray drying in order to use only stable systems. Studies involving the evaluation of soy and pea protein adsorption at the oil-water interface, the stability and physicochemical properties of the obtained oil-in-water emulsions have already been performed using different oils as the dispersed phase [27,28]. However, in most of these studies, the oil phase consisted of nonvolatile oils. The adsorption of proteins in the d-limonene-water interface is rarely assessed; only a few studies can be found using whey protein, egg white protein, and soy protein [29–31].

In general, high solids emulsions stabilized by soy and pea protein have been used in the production of spray-dried microparticles containing mainly nonvolatile oils such as soybean oil, fish oil, and saturated fatty acids [32–36]. Only a few studies deal with the use of plant proteins for the encapsulation of volatile compounds [37]. Recently, using soy protein and pectin as stabilizers for the production of simple and double emulsions, Pereira, Cattelan, and Nicoletti [38] were able to encapsulate pink pepper essential oil by spray drying effectively. On the other hand, the use of pea protein as an emulsifier in the encapsulation of flavors by emulsification followed by spray drying has not been explored so far.

In this context, this study aimed to compare the overall performance of small concentrations of a commercial pea protein concentrate (PPC) and a commercial soy protein isolate (SPI) as emulsifiers in the microencapsulation of OEO by emulsification followed by spray drying. The physicochemical properties of PPC and SPI and their adsorption behavior at the OEO-water interface were evaluated and related to the physicochemical properties and physical stability of the emulsions. Selected emulsions were spray-dried using two drying temperatures (150 and 180 °C), and the physicochemical properties and OEO retention of PPC and SPI spray-dried microparticles were compared. For the best of our knowledge, the use of pea protein in the microencapsulation of d-limonene by emulsification followed by spray drying has not been explored yet.

2. Material and methods

2.1. Material

Orange essential oil was kindly donated by Citrosuco (Matão, Brazil). Maltodextrin (MD) MOR-REX® 1910 with a dextrose equivalent (DE) of 10 was kindly donated by Ingredion (Mogi Guaçu, Brazil). Pea protein concentrate (PPC, 69 g of protein/100 g of powder on a wet basis) Pisane® C9 was kindly donated by R&S Blumos Industrial e Comercial Ltda (Cotia, Brazil). Soy Protein Isolate (SPI, 85 g of protein/100 g of powder on a wet basis) SUPRO® 710 IP was kindly donated by Solae (Saint Louis, USA). Deionized water was used in all assays. All other chemicals and solvents were of analytical grade and purchased from common suppliers.

2.2. Protein characterization

2.2.1. Zeta potential

The zeta potential of protein dispersions was determined as a function of pH. For this, protein powders were dispersed in deionized water at 0.01 wt%. The superficial charge was measured for pH values from 2.5 to 8 by the addition of 0.01 M solutions of HCl or NaOH. A Zeta Zetasizer Nano Series (Malvern, UK) device with MPT-2 titrator was used, and measurements were done in triplicate.

2.2.2. Protein solubility

Quantification of the water-soluble protein fraction in SPI and PPC was performed according to Morr [39] with some modifications. About 500 mg of SPI or PPC were weighed into a 150 mL standard flask, and 40 mL of 0.1 M NaCl solution were added carefully under stirring in order to avoid any lumps. The pH was adjusted to 3, 5, or 7 by the addition of 0.1 M HCl or NaOH, and the mixture was kept under stirring for 1 h. The dispersion was then transferred to a 50 mL volumetric flask, diluted to the mark with an additional 0.1 M NaCl solution, and mixed. An aliquot of the dispersion was centrifuged for 30 min at 20,000 g, and the supernatant fraction obtained was filtered through Whatman No. 1 filter paper. The protein content of the filtrate was determined by the micro-Kjeldahl method. Measurements were performed in triplicate.

2.2.3. Dynamic interfacial tension

The capacity of SPI and PPC to decrease the interfacial tension between the aqueous phase and the oil phase of emulsions was measured using a tensiometer Tracker-S (Teclis Scientific, France). Dispersions

Table 1
Amount of each component required for the production of soy protein isolate (SPI) and pea protein concentrate (PPC) emulsions containing orange essential oil (OEO).

Sample	Protein powder (g/100 g of emulsion)	Maltodextrin (g/100 g of emulsion)	OEO (g/100 g of emulsion)	Water (g/100 g of emulsion)
SPI0.6/PPC0.6	0.6	23.4	6	70
SPI1.2/PPC1.2	1.2	22.8	6	70
SPI2.4/PPC2.4	2.4	21.6	6	70
SPI3.6/PPC3.6	3.6	20.4	6	70
SPI4.8/PPC4.8	4.8	19.2	6	70
SPI6.0/PPC6.0	6.0	18.0	6	70

containing different concentrations of SPI or PPC (0.6–6.4 wt%) and MD (19.1–24.9 wt%) were used as the aqueous phase, and OEO was used as the lipid phase. The system was assembled with the aqueous phase in the syringe and the lipid phase in the cuvette. The pendant droplet method was used to evaluate the variation of interfacial tension as a function of time during 2000 s. Measurements were performed in triplicate.

2.3. Emulsion preparation

Emulsions (35 g) were produced with a total solids content of 30 wt %, of which 20 wt% corresponded to OEO and 80 wt% to mixtures of SPI or PPC and MD. Different concentrations of SPI or PPC were tested according to Table 1. SPI or PPC, MD, and deionized water were weighed 18 h before homogenization in order to promote the hydration of the materials. Then the mixture was kept under stirring at 450 rpm for 1 h. Finally, OEO was added to the dispersion, and the homogenization was performed with an Ultra-Turrax IKA T18 basic (Campinas, Brazil) at 11,200 rpm for 90 s. Samples were produced in triplicate.

2.4. Emulsion characterization

2.4.1. Droplet size distribution

The droplet size distribution of the emulsions was measured by light scattering using a Mastersizer 2000 (Malvern Instruments Ltd., Worcestershire, UK). Emulsions were dispersed in distilled water and analyzed immediately after their preparation. Samples were analyzed in triplicate, using a new sample for each replicate.

2.4.2. Morphology

Emulsions were visualized without dilution using a Carl Zeiss Axio Scope A1 microscope (Carl Zeiss Inc., Gottingen, Germany) with 400 and 1000 x magnifications. Images were captured using the software Axio Vision Rel. 4.8. For fluorescence analysis, the same microscope was used, and a sample of the emulsion was stained with a drop of FITC (0.2 g mL^{-1} in ethanol) (Sigma Aldrich, Wicklow, Ireland). Proteins were dyed with FITC (green), and oil droplets were not dyed (black).

2.4.3. Apparent viscosity

Emulsion viscosity was measured through the determination of steady-shear flow curves (shear stress versus shear rate) using a rheometer, AR 1500 ex (TA Instruments, New Castle, USA), with a concentric cylinder measurement cup, and the temperature was controlled at 25 °C by a Peltier system. Three flow ramps (up, down, and up-cycles) were obtained in a range of shear stresses corresponding to shear rates from 0 to 300 s^{-1} in order to eliminate possible thixotropic effects. Samples were analyzed in triplicate using a new sample for each replicate.

2.4.4. Zeta potential

For the determination of zeta potential, 100 μL of the emulsion were diluted in 15 mL of deionized water right after its production. Measurements were performed in the equipment described in section 2.2.1. Samples were analyzed in triplicate, using a new sample for each replicate.

2.4.5. Emulsion stability

Emulsion stability was evaluated using an optical analyzer Turbiscan® MA Classic 2000 (Formulaction, France). The glass test tube (140 mm height, 16 mm diameter) was filled up to 40 mm of height and scanned at 40 μm intervals. Backscattering distance profiles were recorded at 0, 1, 2, 3, 4, 5, and 6 h at 25 °C. Only emulsions with minimal required stability, determined by preliminary tests, were evaluated in this assay (1.2–6.0 wt% of protein). The Turbiscan Stability Index (TSI) was also measured as an indication of the destabilization process of the emulsions. This index is calculated as the sum of all the destabilization processes in the glass tube according to Eq. 1

$$TSI = \sum_{i} |scan_i(h) - scan_{i-1}(h)| \quad (1)$$

Where $scan_i$ and $scan_{i-1}$ are the backscattering value at the time i and the previous backscattering value, respectively, with i = 1, 2, 3, 4, 5, and 6 h of storage; h is the given height in the glass tube, and TSI is the sum of all the scan differences from the bottom to the top of the vial. Samples were analyzed in triplicate using a new sample for each replicate.

2.5. Microparticles production

The best emulsion formulations based on the stability assay (section 2.4.5) were selected for spray drying microencapsulation. The selected emulsions were SPI2.4, SPI4.8, PPC2.4, and PPC4.8. The process was performed in a Mini Spray Dryer Büchi B-290 (Flawil, Switzerland) with a double fluid nozzle atomizer of 0.7 mm diameter. Feeding (300 g of emulsion) was performed according to the operational conditions described in Table 2. Microparticles samples were identified by the amount of protein in the emulsion and the drying temperature used for its production. For instance, PPC2.4_150 is the sample that was dried at 150 °C and was obtained from an emulsion containing 2.4 wt% of PPC. Samples were produced in duplicate.

2.6. Microparticles characterization

2.6.1. Moisture content and water activity

Moisture content was determined by the Karl Fisher titration method with KF Titrando equipment (Metrohm, Herisau, Switzerland) using a mixture of formamide and methanol (1:1) as the dispersion medium. Water activity was determined at 25 ± 0.5 °C in a Decagon Aqualab Series 4TE (Pullman, USA) hygrometer.

2.6.2. Solubility in water

The solubility of the microparticles in water was determined according to Cano-Chauca, Stringheta, Ramos and Cal-Vidal [40] with minor modifications. Samples (0.5 g) were placed in tubes with 50 mL of deionized water and kept under agitation in a shaker for 30 min. After that, this mixture was centrifuged (3000 rpm for 10 min) and an

Table 2
Operational conditions of the spray dryer.

Condition	T_{inlet} (°C)	T_{outlet} (°C)	Feed flow (g.min)	Atomization airflow rate (L. h^{-1})
1	150	83 ± 1	10	600
2	180	95 ± 1	13	600

aliquot of the supernatant was transferred to pre-weighed Petri dishes and oven-dried at 105 °C for 5 h. The solubility (%) was calculated by the weight difference.

2.6.3. Emulsion reconstitution

The reconstituted emulsions with 30 wt% of total solids were produced by the dispersion of microparticles in deionized water in Falcon tubes followed by vortex agitation for 2 min. After this preparation, samples had their mean droplet diameter and size distribution determined by laser light diffraction (Malvern Instruments Ltd., Malvern, UK) using distilled water as a dispersant medium. The morphology of emulsions was observed, as described in section 2.4.2.

2.6.4. Surface morphology

The microstructure of the microparticles was evaluated using scanning electron microscopy (SEM). The powders were attached to SEM stubs using double-sided adhesive tape and coated with gold under vacuum in a Sputter Coater K450 (EMITECH, Kent, UK), which resulted in a coat of 200 Å. The coated samples were observed with a LEO440i scanning electron microscope (LEICA Electron Microscopy Ltd., Oxford, UK). The SEM was operated at 15 kV and 50 pA with 1,500 and 6,000 x magnifications.

2.6.5. Particle size distribution

The mean diameter and the size distribution of the microparticles were obtained by laser diffraction using LV 950-V2 equipment (Horiba, Kyoto, Japan). The samples were previously dispersed in absolute ethanol and added to the LV950-V2 sample bath, with ethanol as the dispersion medium, until achieving transmittance levels suitable to perform the measurements. The mean particle size was expressed as the volume mean diameter $D_{4,3}$ (Eq. 2), and the polydispersity was given by the span index (Eq. 3). The measurements were performed in triplicate.

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

$$\text{Span} = \frac{D_{90} - D_{10}}{D_{50}} \quad (3)$$

Where n_i is the number of particles with diameter D_i, and D_{10}, D_{50}, and D_{90} are the respective diameters at 10 %, 50 %, and 90 % of the accumulated volume.

2.6.6. Oil retention on microparticles

The oil retention on the microparticles was determined by Eq. 4.

$$\text{Oil retention (\%)} = \left[\frac{\text{Actual oil content (Total oil)}}{\text{Theoretical oil content (Initial oil)}} \right] x100 \quad (4)$$

Total oil was determined by hydro-distillation in a Clevenger apparatus according to the method described by Jafari, He, and Bhandari [41] with some modifications. About 5 g of microparticles were dissolved in 150 mL of deionized water in a 500 mL round bottom flask. The flask was manually shaken for 1 min to aid in the dissolution of the microparticles. The Clevenger apparatus was placed on the top of the flask, and a condenser with water circulating at 5 °C was placed on the Clevenger. Distillation was performed for 1 h, and the volume of distilled oil was directly read in the Clevenger and multiplied by the orange essential oil's density (0.843 $g.cm^{-3}$) in order to calculate the mass of recovered oil. The theoretical oil content was taken as the mass of oil expected in the microparticles based on the emulsion formulation (Table 1) on a dry basis. Samples were analyzed in triplicate using a new sample for each replicate.

2.7. Statistical analysis

The data were evaluated by analysis of variance (ANOVA) and a Tukey test (significance level of 5%). The software Minitab® 18.1 (Minitab Inc., State College, PA, USA) was used for data analysis.

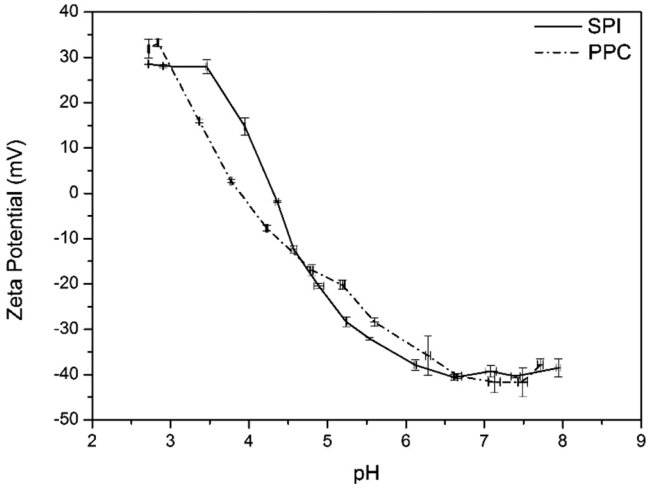

Fig. 1. Zeta potential of soy protein isolate (SPI) and pea protein concentrate (PPC) solutions (0.01 wt%) as a function of pH.

Table 3
Percentage of water-soluble protein of soy protein isolate (SPI) and pea protein concentrate (PPC) at different pH values.

pH	SPI (%)	PPC (%)
3	$63.32^{c} \pm 0.03$	$14.98^{c} \pm 0.01$
5	$73.01^{b} \pm 0.10$	$17.34^{b} \pm 0.03$
7	$76.89^{a} \pm 0.80$	$22.81^{a} \pm 0.51$

Values are expressed as mean ± standard deviation. In each column, different letters correspond to values statistically different ($p < 0.05$).

3. Results and discussion

3.1. Protein characterization

Fig. 1 shows the zeta potential values of SPI and PPC as a function of pH. These proteins presented similar behavior with a net positive charge bellow its isoelectric point (Ip) and a negative charge above it. SPI and PPC presented Ip values of 4.4 ± 0.1 and 3.7 ± 0.1, respectively. The Ip of SPI that we found is similar to the value found by Chang, Tu, Ghosh, and Nickerson [42]. However, the Ip of PPC that we found is below those found by Barac et al. [43] and Ladjal-Ettoumi, Boudris, Chibane and Romero [44], which were around 4.5 − 5.0. In the case of PPC, these differences between the pI values could be inherent to differences in protein composition. At pH 3, both proteins had similar net positive charges (~ 28.0 mV); at pH 5.0, PPC had a lower net charge (~ -18.7 mV) than SPI (~ -23.0 mV); at pH 7.0, PPC had its highest net charge (~ -41.0 mV), whereas SPI presented a similar value (~ - 40.0 mV). Zeta potential gives an estimate of the superficial charges of particles in solution, predicting the stability of the colloidal dispersion. In general, particle solutions with a zeta potential value greater than 30 mV or lower than -30 mV present good stability [45]. Therefore, based on this assumption, the best condition for emulsion production is at pH 7, where both SPI and PPC presented the highest zeta potential values, which are greater than 30 mV.

In order to act as an efficient emulsifier, proteins must present a high solubility in water. This property is necessary because the protein must be able to migrate rapidly towards the oil/water interface [46]. The determination of protein solubility was performed as a complement to the zeta potential assay in order to identify the most appropriate pH value for emulsion production in which the maximum solubilization of protein could be achieved. The percentage of the water-soluble protein of SPI and PPC at different pH values is presented in Table 3. In general,

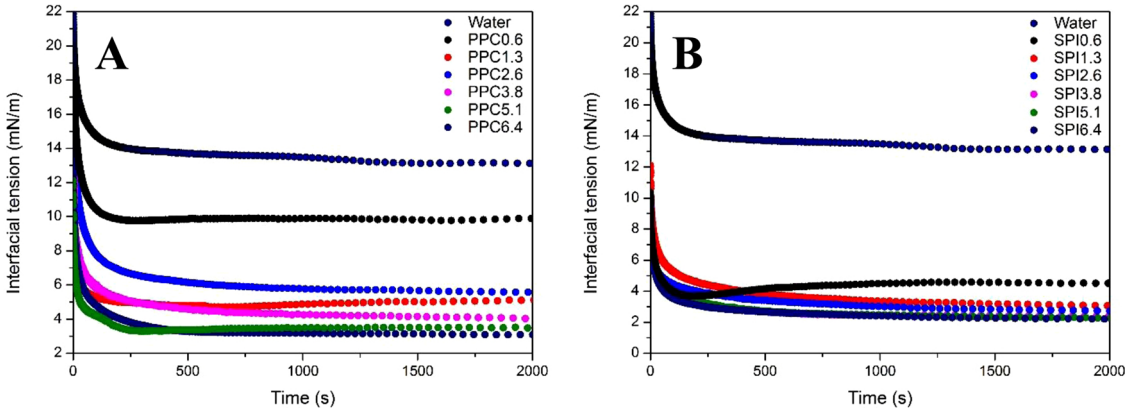

Fig. 2. Dynamic interfacial tension of the system containing water or protein solutions and orange essential oil. A: pea protein concentrate (PPC) samples and B: soy protein isolate (SPI) samples. Concentrations of PPC or SPI in the aqueous phase ranging from 0.6 to 6.4 wt%.

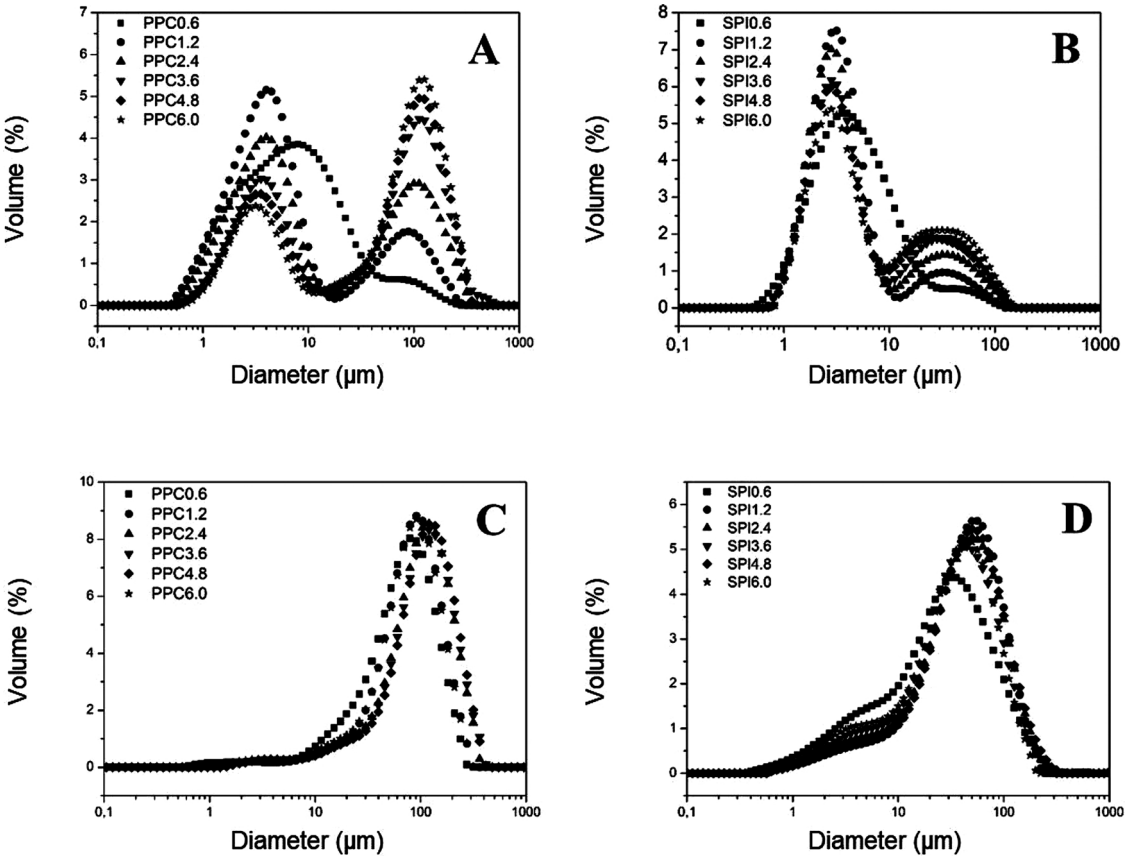

Fig. 3. Droplet size distribution of pea protein concentrate (PPC) and soy protein isolate (SPI) emulsions and particle size distribution of the aqueous phase of the emulsions containing only PPC or SPI and maltodextrin (MD). A: PPC emulsions droplet size distribution; B: SPI emulsions droplet size distribution; C: PPC aqueous phases particle size distribution; D: SPI aqueous phases particle size distribution.

SPI presented higher solubility values than PPC for all pH values tested, and for both proteins, the highest solubility was obtained at pH 7 (77 wt % for SPI and 23 wt% for PPC). This difference of solubility between soy and pea proteins has already been shown in other studies. Chang, Tu, Ghosh, and Nickerson [42] found similar results for soy and pea protein isolates' solubility in water, with the highest values of 70 and 20 wt%, respectively, at pH 7. Karaca, Low, and Nickerson [47] found a positive correlation between plant protein solubility and its superficial charge. In our study, SPI and PPC presented higher solubility at pH 7, in which both of them had higher values of zeta potential. While, at pH 3 and 5, both proteins showed lower solubility and lower zeta potential values, showing a relationship between the solubility and zeta potential.

The dynamic interfacial tension assay was performed in order to verify the kinetics of the decrease in the dynamic interfacial tension (DIT) performed by SPI and PPC; the results are presented in Fig. 2. It was clear that SPI and PPC were able to decrease the interfacial tension between phases, even with the use of the smallest amount of protein. The initial interfacial tension between water and OEO used in this study was around 22 $mN.m^{-1}$, which decreased to an equilibrium interfacial tension (EIT) of 14 $mN.m^{-1}$. Overall, an increase in PPC concentration resulted in a gradual decrease of the EIT of the samples, confirming the capacity of PPC to act as an emulsifying agent (Fig. 2A). The lowest concentration of PPC decreased the EIT between phases to 10 $mN.m^{-1}$;

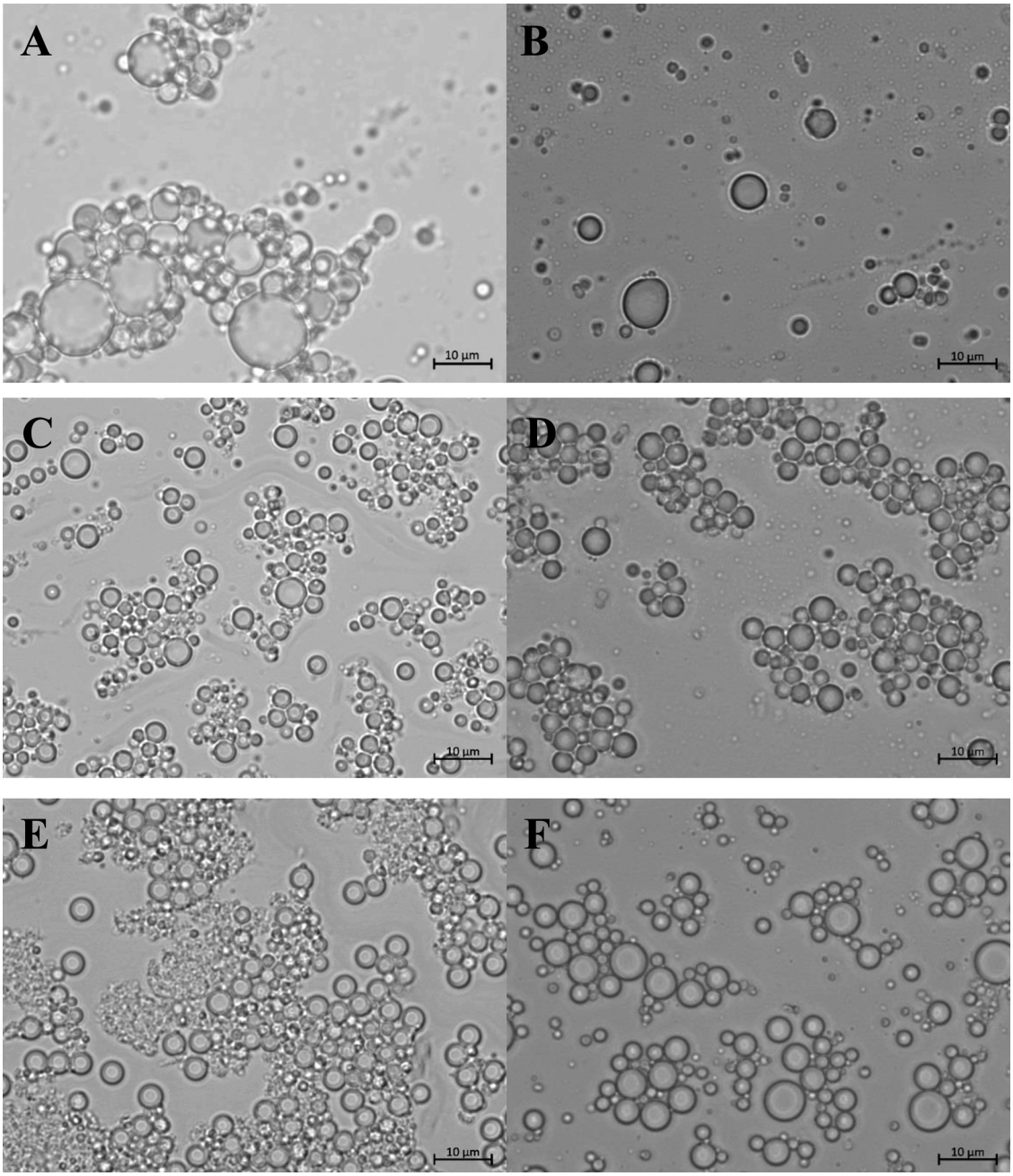

Fig. 4. Optical microscopy of soy protein isolate (SPI) and pea protein concentrate (PPC) emulsions with 1000x of magnification. A: SPI0.6; B: PPC0.6; C: SPI3.6; D: PPC3.6; E: SPI6.0; F: PPC6.0. Scale bar = 10 micrometers.

whereas the highest one resulted in an EIT of around 3 $mN.m^{-1}$. On the other hand, all SPI concentrations decreased the EIT to values around 2–4 $mN.m^{-1}$ (Fig. 2B). The effect of protein concentration on EIT was more evident for PPC than for SPI. At a given time and within the protein concentration of 0.6–5.1 wt% of PPC and 0.6–3.8 wt% of SPI, the higher the protein concentration, the greater was the reduction in interfacial tension. Above 5.1 % and 3.8 wt% of PPC and SPI, respectively, it seems that the interface reaches its saturation since the EIT was no longer affected by the addition of protein. Both SPI and PPC presented a typical behavior of globular proteins, with a decrease on the dynamic interfacial tension as a function of time and they took a long time to reach their equilibrium interfacial tension values [48]. The interfacial behavior of these proteins is a consequence of their tertiary and secondary structures, which need some time to unfold and then to lower the interfacial tension [49]. SPI and PPC presented two different regimes on DIT curves: a fast decrease in DIT (first phase) right after the formation of the interface, followed by a slow decrease in DIT (second phase) towards the equilibrium value. The first phase is related to the diffusion and adsorption of protein molecules into the interface, and the second phase is related to the conformational arrangement of the adsorbed protein in the interface [48]. As can be seen, SPI (Fig. 2B) presents a sharper curve than PPC (Fig. 2A) in the first phase, indicating its faster diffusion towards the oil/water interface. Looking at the EIT at a high protein concentration, both SPI and PPC showed similar values.

3.2. Emulsion characterization

3.2.1. Physicochemical properties

Preliminary tests showed that emulsions stabilized by SPI and PPC had a pH of around 6. Thus, no adjustment of pH was carried out on the

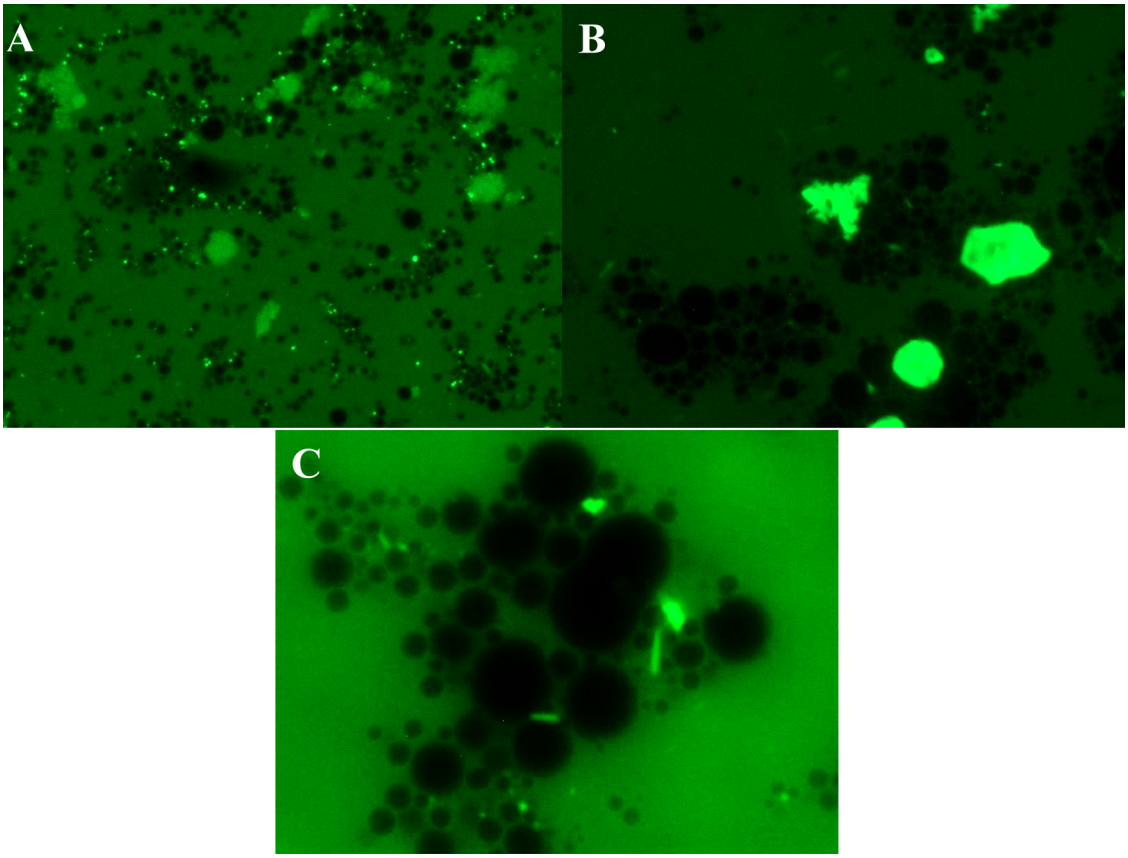

Fig. 5. Fluorescence microscopy of soy protein isolate (SPI) and pea protein concentrate (PPC) emulsions. A: SPI2.4 with 400 x of magnification; B: PPC2.4 with 400 x of magnification; C: PPC2.4 with 1000 x of magnification.

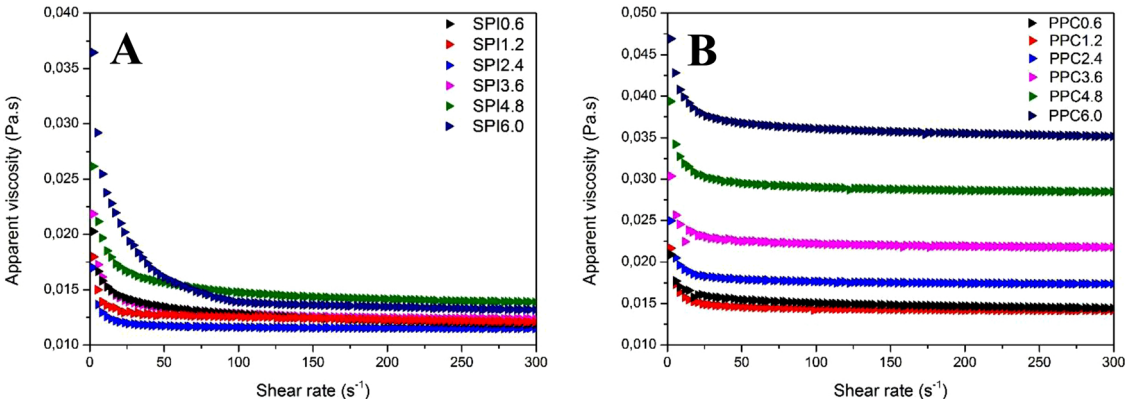

Fig. 6. Representative experimental apparent viscosity values as a function of shear rate of emulsions. A: soy protein isolate (SPI) emulsions and B: pea protein concentrate (PPC) emulsions.

Table 4
Zeta potential of soy protein isolate (SPI) and pea protein concentrate (PPC) emulsions at their original pH.

Sample	Zeta potential (mV)	Sample	Zeta potential (mV)
SPI0.6	$-43^{a} \pm 1$	PPC0.6	$-48^{a} \pm 2$
SPI1.2	$-44^{a} \pm 2$	PPC1.2	$-51^{ac} \pm 2$
SPI2.4	$-50^{bc} \pm 1$	PPC2.4	$-52^{ac} \pm 2$
SPI3.6	$-53^{c} \pm 0$	PPC3.6	$-57^{bc} \pm 1$
SPI4.8	$-46^{ab} \pm 4$	PPC4.8	$-56^{bc} \pm 2$
SPI6.0	$-53^{c} \pm 1$	PPC6.0	$-54^{c} \pm 1$

Values are expressed as mean ± standard deviation. In each column, different letters correspond to values statistically different ($p < 0.05$).

emulsions, since the amount of protein soluble fraction would not suffer a significant change based on the zeta potential and solubility results. Additionally, the emulsions had not their pH adjusted to 7 (pH value where both SPI and PPC presented the highest solubility) in order to avoid the degradation of the OEO due to the addition of an alkali to the emulsions. In the preliminary tests, we also noticed that the high amount of water-insoluble fraction of PPC has a profound impact on the stabilization of the oil-in-water emulsions. We have extracted the water-soluble fraction of PPC and applied it to the stabilization of the emulsions; however, this fraction was not able to stabilize the emulsions on its own, even at high concentrations (data not shown). Therefore, we have used SPI and PPC in the emulsions without any additional physical or chemical treatment.

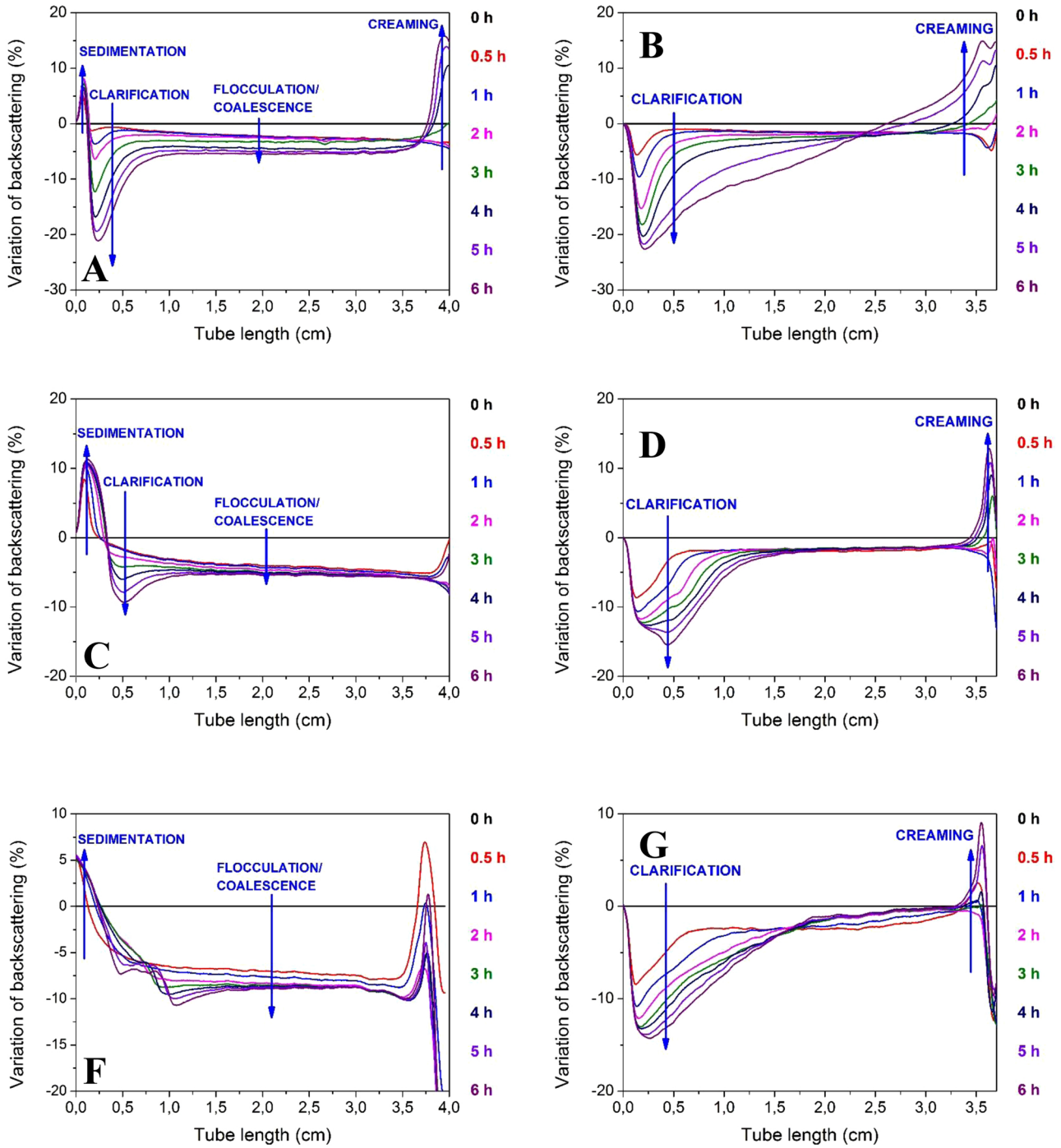

Fig. 7. Variation of backscattering profile of soy protein isolate (SPI) and pea protein concentrate (PPC) emulsions over 6 h after production at 25 °C. A: SPI1.2; B: PPC1.2; C: SPI2.4; D: PPC2.4; F: SPI4.8 G: PPC4.8.

Table 5
Turbiscan Stability Index (TSI) of soy protein isolate (SPI) and pea protein concentrate (PPC) emulsions after 2 h of production.

Sample	TSI	Sample	TSI
SPI1.2	2.1 ± 0.1	PPC1.2	2.4 ± 1.0
SPI2.4	1.8 ± 0.1	PPC2.4	1.5 ± 0.1
SPI3.6	2.4 ± 0.2	PPC3.6	1.5 ± 0.1
SPI4.8	3.0 ± 0.3	PPC4.8	1.5 ± 0.0
SPI6.0	6.2 ± 3.7	PPC6.0	1.7 ± 0.0

Values are expressed as mean ± standard deviation.

The droplet size distributions of the emulsions containing different concentrations of PPC or SPI are presented in Fig. 3. The PPC sample containing less protein (PPC0.6) presented a broad size distribution with a main peak between about 0.5 and 55 μm and a second less pronounced peak between about 55 and 270 μm (Fig. 3A). In this condition of reduced protein content, it is assumed that the amount of protein present is not enough to cover the oil/water interface, resulting in an emulsion with oil droplets of different sizes [50]. The increase of protein content to 1.2 wt% (PPC1.2) resulted in a bimodal distribution, with a more pronounced peak in a smaller droplet size range. This sample presented droplet sizes between about 0.5 and 17 μm, indicating smaller droplets and a more homogeneous distribution as a function of

Table 6
Moisture, water activity and solubility of soy protein isolate (SPI) and pea protein concentrate (PPC) microparticles.

Sample	Moisture (%)	Water activity (-)	Solubility (%, dry basis)
SPI2.4_150	5.6 ± 0.8	0.230^{ab} ± 0.023	85^{ab} ± 1
SPI2.4_180	5.6 ± 0.4	0.204^{abc} ± 0.026	86^{ab} ± 1
SPI4.8_150	6.4 ± 1.0	0.250^{a} ± 0.019	84^{ab} ± 1
SPI4.8_180	5.5 ± 0.3	0.150^{d} ± 0.010	84^{ab} ± 2
PPC2.4_150	5.8 ± 0.4	0.148^{d} ± 0.044	86^{ab} ± 4
PPC2.4_180	5.8 ± 0.2	0.166^{cd} ± 0.007	88^{a} ± 1
PPC4.8_150	5.9 ± 0.5	0.187^{bcd} ± 0.034	84^{ab} ± 1
PPC4.8_180	5.9 ± 0.3	0.165^{cd} ± 0.004	82^{b} ± 2

Values are expressed as mean ± standard deviation. In each column, different letters correspond to values statistically different ($p < 0.05$).

the increase of protein content. At higher protein content (1.2–6.0 wt %), which can be called the protein-rich regime, the protein concentration is sufficient, and the mean droplet size is no longer dependent on the protein concentration, despite the narrowing of the peak at the left due to the protein concentration increase. Similar droplet size distributions were obtained for the emulsions containing SPI (Fig. 3B). However, the oil droplets (peak at left) presented a narrower distribution, indicating that soy protein is more efficient for the production of more homogeneous droplet sizes. These results are in agreement with the dynamic interfacial tension assay since in the range of 1.2–6.0 wt% of SPI and PPC (corresponding to 1.3–6.4 wt% in Fig. 2A and 2B), only small differences were verified for the values of equilibrium interfacial tension among the protein concentrations tested. Besides, the narrower size distributions of the oil droplets from the SPI emulsions are a result of the faster adsorption of SPI on the droplet surface, as verified in Fig. 2A. In general, the increase of protein content (PPC and SPI) resulted in an increase of the peak at the right (Fig. 3A and B). This effect was more evident in PPC emulsions, since this protein presents a higher insoluble fraction in contrast to SPI, as verified on the solubility assay. The evaluation of the particle size distribution of the aqueous phase containing only PPC or SPI and MD confirmed that this peak on the droplet size distributions of the emulsions corresponds only to insoluble fractions of protein. The particle size distributions (Fig. 3C and D) presented a single peak at the exact size range of the peak at the right on the droplet size distribution of the emulsions, which confirms that these peaks correspond to protein agglomerates. Similar particle size distributions were reported by McCarthy et al. [51] using pea protein and by Roesch and Corredig [9] using soy protein as emulsifiers for the production of oil-in-water emulsions. According to Tsumura et al. [52], the poor solubility of plant proteins in water is due to the exposure of hydrophobic amino acid residues, which make particle sub-structures difficult to disintegrate, resulting in a slow release of proteins into the surrounding aqueous phase.

The optical microscopy of SPI and PPC emulsions can be seen in Fig. 4. The use of the smallest concentration (0.6 wt%) of SPI and PPC resulted in emulsions (Fig. 4A and B, respectively) with big oil droplets, indicating that the amount of protein was not enough to cover the droplets' surface and avoid coalescence. An increase in the protein amount to 3.6 wt% resulted in more homogeneous oil droplets; at this protein concentration, the SPI emulsion (Fig. 4C) presented smaller oil droplets than PPC (Fig. 4D). Additionally, an increase in the protein concentration up to 6.0 wt% did not result in a decrease of oil droplets for both protein samples, indicating the saturation of oil droplet surfaces (Fig. 4E and F). At this protein concentration, SPI emulsions showed a more homogeneous droplet size distribution than PPC. All samples presented oil droplet aggregates with a wide range of sizes. SPI samples presented a higher tendency to flocculate because of its bigger oil droplet agglomerates than PPC (Fig. 4E and F). Attractive intermolecular interactions between plant proteins may exist due to their hydrophobic nature. These interactions could be strong enough to overcome electrostatic repulsion leading to the flocculation of oil droplets [53]. Besides, it is possible that due to the presence of non-adsorbed protein, depletion forces also arose, leading to oil droplet flocculation [54].

The reduced adsorption of SPI and PPC on the oil droplets surfaces was confirmed by fluorescence microscopy (Fig. 5), where the green regions are related to protein, and the black regions are related to oil droplets. In SPI (Fig. 5A) and PPC (Fig. 5B) emulsions, the protein was mostly present in the continuous phase as big insoluble agglomerates. The low solubility of plant proteins and their low adsorption at the oil-in-water interface have a profound impact on emulsion stability because the protein must be at the oil droplet surface in order to keep the surrounding droplets away through electrostatic/steric repulsion [4]. Despite the high amount of protein as agglomerates in the continuous phase, it is possible to verify the presence of a protein film around the oil droplets (Fig. 5C), which contributes to the stability of the emulsions.

The apparent viscosity of SPI and PPC emulsions as a function of shear rate is shown in Fig. 6. A linear dependence between shear stress and shear rate was observed for all samples. This behavior characterizes the emulsions as Newtonian fluids [55]. The increase of protein concentration in SPI samples resulted in a minimum increase in the viscosity of the emulsions from 0.011 to 0.014 Pa.s (Fig. 6A). On the other hand, an increase in the protein concentration of PPC samples resulted in a considerable increase in the viscosity from 0.014 to 0.035 Pa.s (Fig. 6B). This difference between the viscosity of SPI and PPC emulsions was verified because SPI and PPC powders also contain different amounts of water-insoluble fractions which are capable of adsorbing water and swelling up, thus promoting a higher resistance to flow. The viscosity of PPC emulsions was more affected by protein concentration than SPI emulsions, probably due to its higher water-insoluble fraction than SPI, which results in higher resistance to flow. Overall, all emulsions presented low values of viscosity. According to Bhandari, Dumolin, Richard, Noleau and Lebert [56], a low viscosity is essential for the production of spherical, dense, and regular spray-dried microparticles.

The zeta potential of an emulsion is commonly related to its stability over a short or long time scale. Emulsions with a high zeta potential value (negative or positive) are considered electrostatically stable, whereas emulsions with low values tend to show flocculation and coalescence of the dispersed phase. In emulsions with a high zeta potential value, repulsion forces exceed attraction forces, resulting in stable dispersed systems [57]. Table 4 shows the zeta potential values of SPI and PPC emulsions. SPI and PPC emulsions presented pH values of around 6.0 and similar zeta potential values, which ranged from −53 to −43 mV and from −57 to −48 mV, respectively. The increase in protein content resulted in a gradual increase of the emulsions' zeta potential values. This trend could be related to an increase in charges in the medium due to the addition of protein. It is recognized that proteins stabilize emulsions mainly by adsorbing at the droplet surface and preventing the surrounding droplets from getting closer through electrostatic repulsion. Although all of the samples presented high zeta potential values, it was possible to verify by preliminary visual tests that a minimal destabilization was occurring one hour after production in all SPI and PPC samples. This result indicates that the high zeta potential values observed probably correspond to the sum of the surface charge of the protein attached to the surface of the oil droplets and the surface charge of the insoluble protein agglomerates dispersed in the continuous phase. Since only the soluble fraction was able to adsorb at the droplet surface and stabilize the droplets by electrostatic repulsion, as was seen by the fluorescence microscopy, this hypothesis seems likely. The zeta potential value provides information on the repulsive electrostatic forces, and it does not consider hydrophobic interactions that commonly happen in plant proteins. Therefore, it is common to find unstable systems with a high zeta potential value [58]. Since SPI and PPC present large insoluble protein fractions, most of the protein

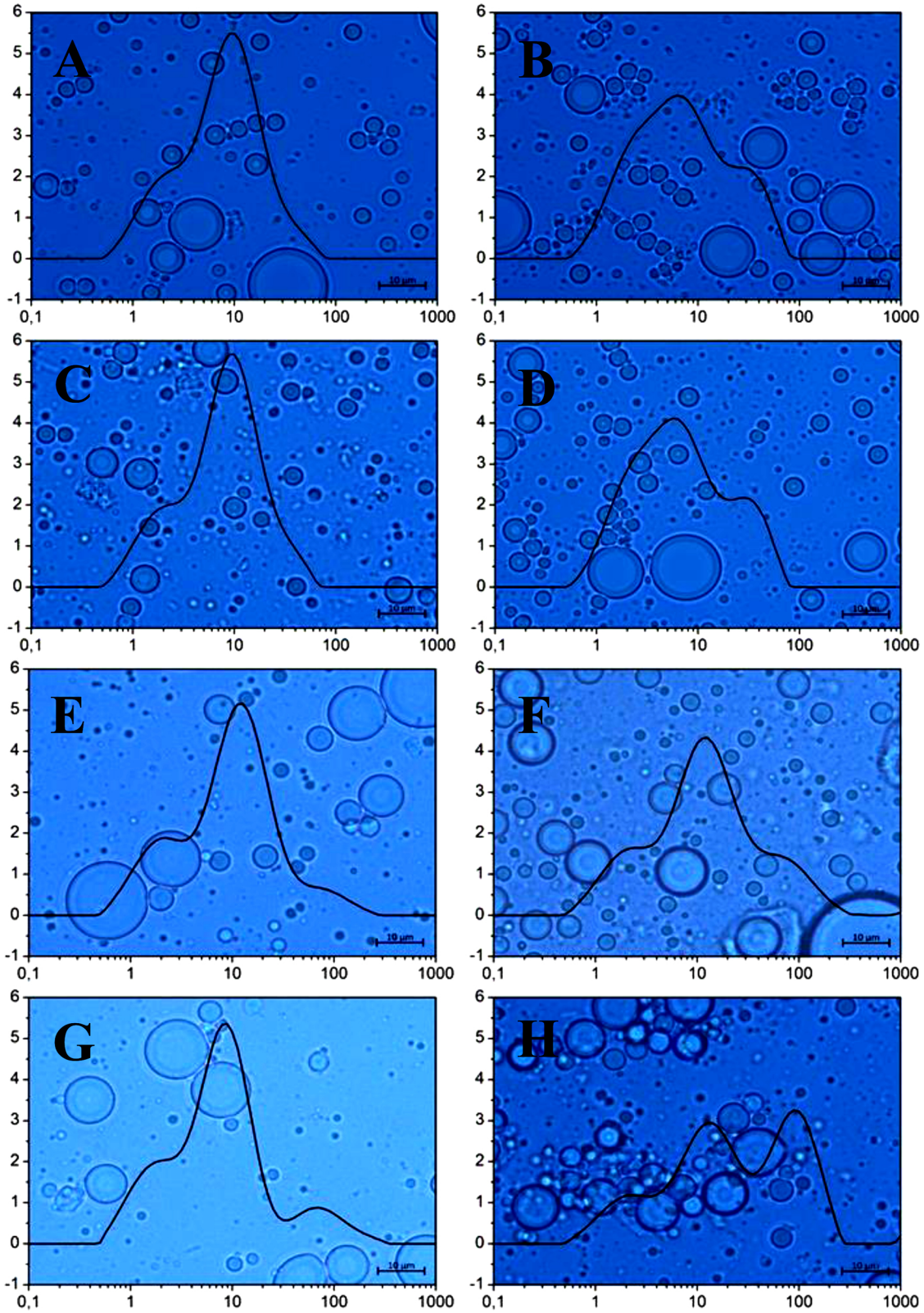

Fig. 8. Droplet size and optical microscopy of soy protein isolate (SPI) and pea protein concentrate (PPC) emulsions obtained from the reconstitution of the microparticles. A: emulsion obtained from SPI2.4_150; B: emulsion obtained from SPI4.8_150; C: emulsion obtained from SPI2.4_180; D: emulsion obtained from SPI4.8_180; E: emulsion obtained from PPC2.4_150; F: emulsion obtained from PPC4.8_150; G: emulsion obtained from PPC2.4_180; H: emulsion obtained from PPC4.8_180. 1000 x of magnification. Scale bar = 10 micrometers.

Table 7
Orange essential oil (OEO) retention values of soy protein isolate (SPI) and pea protein concentrate (PPC) microparticles.

Sample	OEO retention (%)	$D_{4,3}$ (μm)	Span (-)
SPI2.4_150	$94.7^{abc} \pm 4.1$	$15.6^{c} \pm 1.4$	1.94
SPI2.4_180	$92.6^{abc} \pm 0.1$	$18.1^{b} \pm 0.3$	1.76
SPI4.8_150	$97.9^{ab} \pm 4.1$	$19.2^{b} \pm 2.1$	1.96
SPI4.8_180	$94.4^{abc} \pm 2.5$	$20.8^{a} \pm 0.3$	1.87
PPC2.4_150	$88.3^{c} \pm 7.9$	$8.7^{e} \pm 0.9$	1.39
PPC2.4_180	$84.1^{c} \pm 0.2$	$9.6^{de} \pm 0.4$	1.44
PPC4.8_150	$100.0^{a} \pm 2.1$	$8.6^{e} \pm 0.6$	1.38
PPC4.8_180	$89.4^{bc} \pm 2.1$	$10.9^{d} \pm 0.5$	1.77

Values are expressed as mean ± standard deviation. In each column, different letters correspond to values statistically different ($p < 0.05$).

has not contributed to the stability of the emulsions through electrostatic/steric repulsion at the oil droplets surface. Nevertheless, these fractions could absorb water, which increases the continuous phase viscosity and hinders the mobility of the oil droplets, contributing to the stability of the emulsions.

3.2.2. Emulsions stability

Emulsions must be stable throughout the atomization process in order to produce spray-dried microparticles with high oil retention. Therefore, the Turbiscan assay was performed to identify the minimal concentration of protein that results in an emulsion that is stable for at least 2 h. When working on a bench scale, this is the maximum time necessary for spray drying the emulsions. Fig. 7 shows the variation of backscattering (ΔBS) profiles of SPI and PPC emulsions as a function of tube length. SPI and PPC emulsions presented two main destabilization phenomena, flocculation/coalescence, and creaming. Flocculation seems to play a more critical role in SPI emulsion destabilization. Flocculation is identified by how ΔBS profiles changed with time (Fig. 7A). ΔBS diminished over the tube length, which indicates an increase in emulsion particle size with time, meaning that Turbiscan detected particle aggregation. Furthermore, flocculation increased due to the increase in protein concentration (Fig. 7A, C and F), as was also seen by optical microscopy. Creaming was the dominant destabilization mechanism in PPC emulsions and can be seen as the decrease in ΔBS at the bottom of the tube (clarification) and a concomitant increase in ΔBS in the upper zone, attributed to the formation of a cream layer (Fig. 7B). The increase in protein content (Fig. 7B, D and G) showed an improvement in the emulsion stability by the mitigation of creaming. ΔBS profiles of SPI emulsions presented sedimentation of insoluble aggregates (Fig. 7A, C and F). This process was not observed in the PPC emulsions, probably due to the overlap with the clarification profile (Fig. 7B, D and G).

The Turbiscan Stabilization Index (TSI) takes into account all the destabilization phenomena that take place in the sample; the higher the TSI value, the more unstable is the sample at the given time. The increase of PPC up to 2.4 wt% increased emulsion stability. Above this concentration, an increase in protein content did not affect the stability of the emulsions (Table 5). For SPI samples, an increase of protein up to 2.4 wt% also improved emulsion stability; however, the increase in protein concentration above this point negatively affected the stability of the emulsions, probably due to an increase of flocculation, as seen in the ΔBS profiles.

Based on the TSI values (Table 5), two protein concentrations were selected to be used for the production of spray-dried microparticles. Since SPI1.2 and PPC1.2 presented the lowest TSI values and no improvement in emulsion stability was observed above these protein concentrations, these samples were selected for spray drying. In order to compare the effect of protein concentration on oil retention of spray-dried microparticles, emulsions with 4.8 wt% of protein were also used for the production of microparticles containing SPI or PPC.

3.3. Microparticles characterization

3.3.1. Moisture, water activity, and solubility

The spray drying process produced microparticles with moisture content (5.5–6.4 %) and water activity (0.148 − 0.250) values (Table 6) below of the values recommended for powders and microparticle stability, which are 6 % (wet basis) and 0.3, respectively [24,59]. Overall, SPI microparticles presented higher water activity values than PPC samples, while the solubilities of all samples were close to each other. The solubility of SPI and PPC microparticles in water was around 85 % (Table 6). In previous work on microencapsulation of essential oils by spray drying, a solubility of 70 % was observed for pink pepper essential oil particles produced with SPI and pectin, whereas cocoa aroma microparticles produced with maltodextrin showed solubility ranging from 73.8 to 82.7 % [37,60]. The high solubility of SPI and PPC microparticles allows the application of OEO in products in which this oil is not soluble in its pure form.

3.3.2. Emulsions reconstitution

The droplet size distribution and optical microscopy of the reconstituted emulsions are shown in Fig. 8. Small differences in the droplet size distributions were noted in all reconstituted emulsions when compared to the original emulsions. The SPI and PPC reconstituted emulsions containing 2.4 wt% of protein (Fig. 8A, C, E and G) presented an increase in the mean diameter of the oil droplets due to coalescence since bigger oil droplets were observed instead of small oil droplet agglomerates. The reconstituted emulsions containing 4.8 wt% of SPI (Fig. 8B and D) presented a droplet size distribution that was more similar to the original emulsions, but they still showed an increase in the oil droplets' size. The reconstituted emulsions containing 4.8 wt% of PPC (Fig. 8F and H) presented a similar droplet size distribution when compared to the reconstituted emulsions containing 2.4 wt% of PPC (Fig. 8E and G). However, the emulsion obtained from the microparticles produced at 180 °C (Fig. 8H) showed big oil droplet agglomerates. In general, the reconstituted emulsions presented fewer oil droplet agglomerates than the original emulsions. Probably, the atomization process, which promoted high shear in the emulsions, could have disrupted the flocculated oil droplets. Overall, SPI emulsions presented narrower droplet size distributions than PPC emulsions, which shows the better emulsifying capacity of SPI.

3.3.3. Orange essential oil retention

Table 7 shows the values of oil retention in SPI and PPC microparticles. The use of both plant proteins as emulsifiers resulted in microparticles with high oil retention values. In general, SPI samples presented slightly better performance, resulting in microparticles that preserved 92.6–97.9 wt% of the oil initially added to the emulsion in contrast to PPC samples, which presented values ranging from 84.1 to 100.0 wt%. These results are promising because, despite their lower solubility, the use of PPC and SPI as stabilizing agents for emulsions proved to be a good strategy for the production of microparticles with a high load of the OEO. The oil retention values observed in our study are much higher than those found by Pereira, Cattelan, & Nicoletti [37] using SPI and pectin for the encapsulation of pink pepper essential oil, which were around 42.1 and 49.4 wt% and close to the values found by Carmona, Tonon, Cunha and Hubinger [61] and Garcia, Tonon and Hubinger [62] on the microencapsulation of orange and basil essential oils, respectively. The better overall oil retention promoted by SPI may be related to the better stability of SPI emulsions when compared to PPC, which was verified during the atomization process. As seen in the stability assay, SPI samples presented a significantly higher flocculation process than PPC samples; however, this destabilization phenomenon did not evolve to creaming and phase separation in SPI emulsions, as was seen in the PPC emulsions. During the atomization processes, even using magnetic stirring, only PPC emulsions presented visible oil coalescence and oil separation, which can explain the lower oil retention in

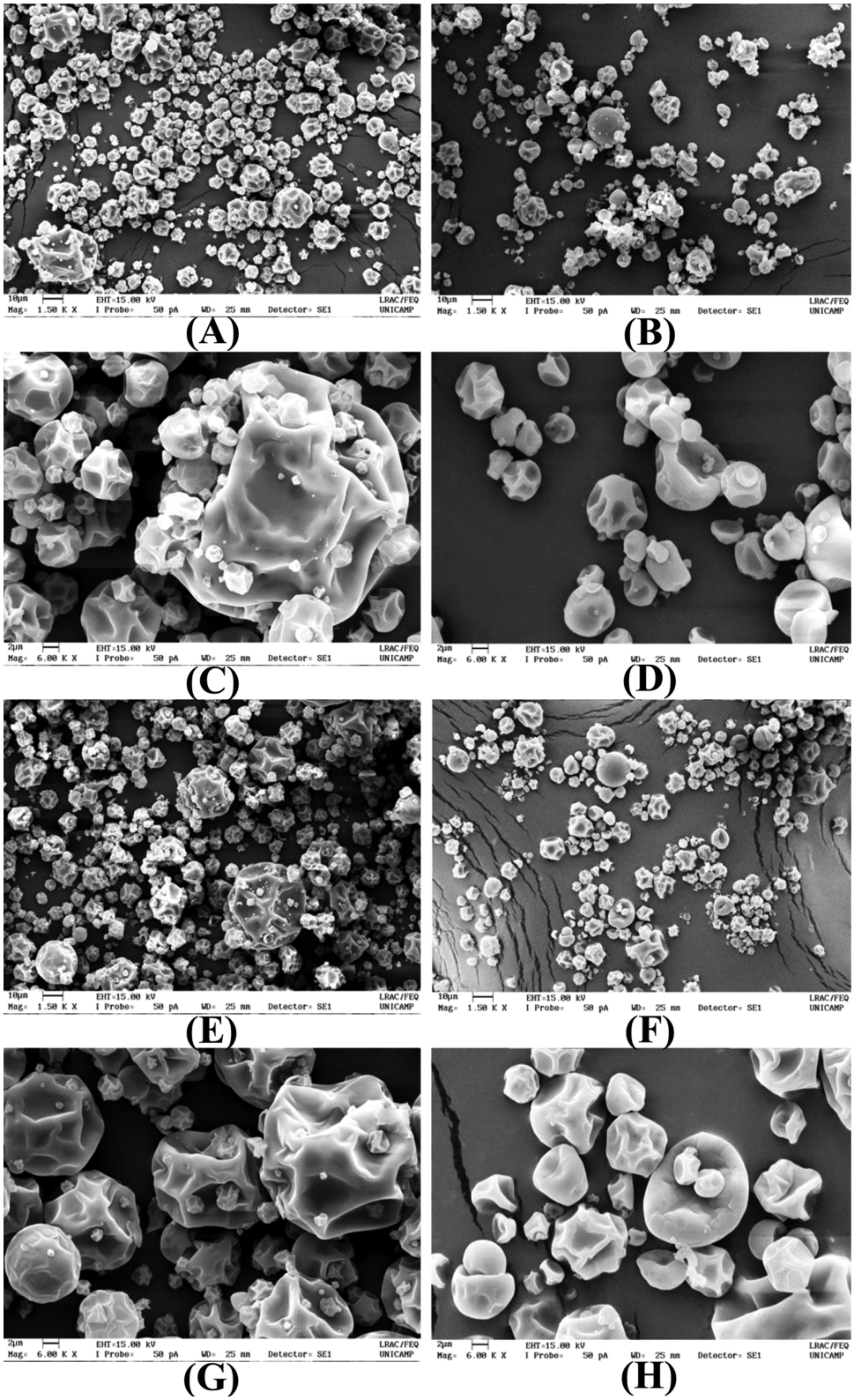

Fig. 9. SEM of orange essential oil microparticles produced at 150 °C by spray drying of soy protein isolate (SPI) and pea protein concentrate (PPC) emulsions. A and C: SPI2.4_150; B and D: PPC2.4_150; E and G: SPI4.8_150; F and H: PPC4.8_150.

PPC microparticles.

Looking at the mean oil retention values of PPC samples, it seems that an increase in the protein content promotes an increase in oil retention. This result may be related to the higher viscosity of the emulsions containing 4.8 wt% of PPC. By increasing the emulsion viscosity, the oil droplet mobilization within the emulsion droplet reduces, and a rapid formation of a semipermeable membrane occurs around it, which limits the loss of volatiles during the drying stage [26]. Some studies involving the encapsulation of volatile compounds have shown that the use of higher drying temperatures also may favor a

lower loss of the oil due to the quicker formation of a membrane around the atomized droplet, which limits oil volatilization during the drying stage [63]. In our study, the use of a lower drying temperature (150 °C) resulted in higher oil retention. Probably, the temperature effect may have been less evident in this case because of the small difference between the drying temperatures used.

In the case of SPI, an increase in protein content did not result in significant differences in oil retention. Once the viscosity of these emulsions was slightly affected by protein concentration, the formation of emulsion droplets during the atomization and drying steps probably occurred similarly, resulting in microparticles with a similar oil load. The variation of the drying temperature has not affected the oil retention in the SPI emulsions, confirming that emulsion viscosity played a more significant role in oil retention as verified for the PPC samples.

3.3.4. Particle size

The mean diameter ($D_{4,3}$) and the span value of the microparticles are shown in Table 7. In general, SPI microparticles presented a significantly ($p < 0.05$) higher mean diameter than all the PPC samples. This is probably related to the difference in the water-soluble protein fractions of SPI and PPC. The higher amount of soluble protein in SPI could have promoted higher viscoelasticity of the atomized emulsion droplets. This could allow greater ballooning during the drying stage, which resulted in bigger microparticles. Apart from that, the larger size of the SPI microparticles can be related to the higher oil retention of SPI formulations, since higher encapsulation values of volatile compounds have been reported for particles with larger diameters [24]. Among SPI microparticles, the biggest sample was SPI4.8_180, and among PPC microparticles, the biggest sample was PPC4.8_180, showing that the highest protein concentration and the highest drying temperature resulted in bigger microparticles using both proteins.

3.3.5. Surface morphology

Scanning Electron Microscopy (SEM) of the SPI and PPC microparticles obtained at 150 °C are shown in Fig. 9. The images of the microparticles obtained at 180 °C were not shown because these did not present visual differences from the ones produced at 150 °C. In general, the particles presented a spherical shape and a great extent of surface depressions, which is typical of spray-dried material containing carbohydrates. These depressions are related to fast water loss and shrinkage at the initial stage of drying [63]. Furthermore, microparticles presented a continuous wall without cracks or apparent pores. These are excellent features that promote the preservation of the OEO because the wall will be able to limit oxygen entry and also the release of the volatile compounds. SPI microparticles (Fig. 9A, C, E, and G) were bigger than PPC microparticles (Fig. 9B, D, F, and H) and also presented greater agglomeration. The increase in protein concentration did not seem to have impacted the structural characteristics of SPI microparticles (Fig. 9C and G) or PPC microparticles (Fig. 9D and H).

4. Conclusions

In this study, the encapsulation of orange essential oil (OEO, rich in d-limonene) through emulsification followed by spray drying using pea protein at low concentration as emulsifier was described for the first time, which constitutes a novelty in the present context. The capacity of a commercial pea protein concentrate (PPC) to act as an emulsifier and stabilize high solids emulsions containing OEO was compared to soy protein isolate (SPI), which is an emulsifier that has been already used on the microencapsulation of flavors. Even at low concentrations, both PPC and SPI were able to reduce the interfacial tension between OEO and water and produce oil-in-water emulsions; however, the lower solubility in water and the lower adsorption rate at the oil-water interface of PPC compared to SPI resulted in emulsions with slightly different physicochemical properties and physical stability. During the spray drying of the selected emulsions, SPI emulsions were more stable than PPC ones, which overall resulted in microparticles with a higher OEO retention. PPC and SPI spray-dried microparticles were produced using two protein concentrations (2.4 and 4.8 wt%) and two drying temperatures (150 and 180 °C), but no great differences were verified among the samples regarding the microparticles' physicochemical properties. Both PPC and SPI microparticles presented morphologies without cracks and pores, which allows the retention and protection of the encapsulated OEO. Pea protein was used for the first time for the encapsulation of d-limonene and proved to be a suitable emulsifier for this purpose. These results contribute to the advance on the use of pea and soy protein as emulsifiers for the encapsulation of flavors by spray drying and help to meet the demand of the food industry for plant-based ingredients.

CRediT authorship contribution statement

Cristhian Rafael Lopes Francisco: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Writing - original draft, Visualization, Funding acquisition. **Fernando Divino de Oliveira Júnior:** Methodology, Formal analysis. **Gabrieli Marin:** Methodology, Formal analysis. **Izabela Dutra Alvim:** Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition. **Miriam Dupas Hubinger:** Conceptualization, Methodology, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors report no declarations of interest.

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