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Stability and *in vitro* digestibility of emulsions containing lecithin and whey proteins

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The effect of pH and high-pressure homogenization on the properties of oil-in-water (O/W) emulsions stabilized by lecithin and/or whey proteins (WPI) was evaluated. For this purpose, emulsions were characterized by visual analysis, droplet size distribution, zeta potential, electrophoresis, rheological measurements and their response to *in vitro* digestion. Lecithin emulsions were stable even after 7 days of storage and WPI emulsions were unstable only at pH values close to the isoelectric point (pI) of proteins. Systems containing the mixture of lecithin and WPI showed high kinetic instability at pH 3, which was attributed to the electrostatic interaction between the emulsifiers oppositely charged at this pH value. At pH 5.5 and 7, the mixture led to reduction of the droplet size with enhanced emulsion stability compared to the systems with WPI or lecithin. The stability of WPI emulsions after the addition of lecithin, especially at pH 5.5, was associated with the increase of droplet surface charge density. The *in vitro* digestion evaluation showed that WPI emulsion was more stable against gastrointestinal conditions.

1 Introduction

Oil-in-water (O/W) emulsions have significant potential for encapsulation of bioactive compounds, despite being thermodynamically unstable systems, which can lead to phase separation.[1] Emulsions can be kinetically stabilized with addition of emulsifying agents[1] and by homogenization processes at high pressures, which enable the formation of a small droplet size.[2] There are two great classes of emulsifying agents considered as food-grade [generally recognized as safe (GRAS)]: low molecular weight (LMW) (*e.g.* lecithin) and macromolecular emulsifiers (*e.g.* proteins).[3]

Whey proteins are widely used as a emulsifying/stabilizing agent[4,5] due to their ability to form a thick protective layer at the interface of oil droplets, promoting electrostatic repulsion between charged droplets which increases emulsion stability to the creaming process.[6,7] However, this stability is reduced when the pH approaches the isoelectric point (pI) ($\approx$5.1) and/or ionic strength becomes high.[8] Thus, the pH value can modify the behavior of surface activity and the hydrodynamic interactions between oil droplets in emulsions stabilized by proteins.[1,9]

In this way, interactions with the lecithin, a zwitterionic surfactant, can lead to changes in the surface activity of the protein, and modify the emulsion structure and surface charge.[9] Furthermore, lecithin is also considered an important natural emulsifier[10] and very efficient in reducing interfacial tension.[6] However, a mixture of compounds with surface active properties can compete for the same space or interact synergistically in the interface, depending on their molecular characteristics.[6,11] Ionic surfactants and proteins can interact directly through hydrophobic or electrostatic interactions, which can lead to favorable or unfavorable changes in protein conformation.[12] Thus, it is important to verify the origin and nature of surfactant–protein interactions and their influence on protein functionality, in order to produce stable emulsions with desirable characteristics.

Besides stability, another challenge in emulsion applications is its use as an encapsulation system which promotes the appropriate bioavailability of the lipid components. The bioavailability is related to the characteristics of lipid components (*e.g.* chemical structure, physicochemical properties) and the nature of the food matrix that surrounds them.[13] The lipid digestion process consists of an interfacial reaction since its occurrence depends on the lipase adsorption onto the oil droplet surface.[14] In this case, with different size and composition of the droplets, as well as the structure and physicochemical properties of the interfacial layer, it would be possible to produce emulsions with desired sensory properties besides controlling the conditions of oil release.[12] Thus, the understanding of the response of the interfacial composition to the

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gastrointestinal tract conditions is important for better control of lipid bioavailability[15,16] and the results are fundamental to define the final application of the encapsulation system.

Therefore, this work aimed to produce O/W emulsions stabilized by different natural ingredients with surface activity (lecithin and/or WPI), evaluating the interaction effect between them on emulsion properties. The influence of high-pressure homogenization (250–600 bar) and emulsion pH (3–7) was studied. For this purpose, creaming, droplet size distribution, surface charge density, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), rheological measurements and *in vitro* digestibility were evaluated.

2 Materials and methods

2.1 Materials

Whey protein isolate (WPI) (protein content of 90.6 ± 0.5% w/w) was obtained from New Zealand Milk Products (ALACEN 895, New Zealand). Soybean lecithin (Lec) (SOLEC AE IP) was purchased from Solae of Brazil (Esteio, Brazil). Soybean oil was obtained from the local market and other analytical grade reagents were purchased from Sigma Aldrich Co. (St. Louis, USA).

2.2 Emulsion preparation

WPI stock solution (3% w/w) was prepared by dispersing the powder in deionized water using magnetic stirring at room temperature for 2 h, ensuring complete dissolution of the protein. Lecithin was dispersed in oil (7% w/w) using magnetic stirring at room temperature until complete dissolution prior to use in emulsion preparation. O/W emulsions were prepared at 25 °C by homogenizing the oil phase in the aqueous phase with a rotor stator Ultra-Turrax model T18 (IKA, Germany) at 14 000 rpm for 5 minutes. Afterwards, these systems were homogenized at high pressure in a Panda 2K NS1001L double-stage homogenizer (Niro Soave, Italy). The pressure homogenization was 250, 450 and 600 bar in the first step and 50 bar in the second. For all systems, the oil phase content was fixed at 30% (w/w). The aqueous phase pH was adjusted to 3, 5.5 or 7 with 1 N HCl or 1 N NaOH, immediately before the emulsion preparation. The following systems were evaluated: (i) emulsions stabilized by 2% (w/w) WPI, (ii) emulsions stabilized by 2% (w/w) lecithin and (iii) emulsions stabilized by the mixture of 1% (w/w) WPI and 1% (w/w) lecithin. Sodium azide (0.01% w/w) was added to the aqueous phase of the emulsion as an antimicrobial agent. Creaming stability, droplet size distribution, zeta potential, electrophoresis, rheological measurements and response to *in vitro* digestion of these emulsions were evaluated.

2.3 *In vitro* digestion

Emulsions containing whey proteins and/or lecithin prepared at 450 bar were tested in a simulated *in vitro* digestion by the static method. Firstly, emulsions were mixed with a phosphate buffer (5 mM, pH 6.9, 0.04% NaCl, 0.004 M $CaCl_2$) at a ratio of 1 g of each sample to 4 mL of buffer.[17] The gastric digestion was simulated by the addition of a simulated gastric fluid (SGF) which consisted of porcine pepsin (40 mg mL^{-1} in 0.1 M HCl) to the initial mixtures in a ratio of 0.5 g of pepsin per 100 g of sample[18,19] and the pH was adjusted to 2 with 6 M HCl. After one hour of incubation, the enteric digestion was carried out. In this step, the resulting mixture of gastric digestion was neutralized to pH 5.3 with 0.9 M sodium bicarbonate before addition of 9 mL of the simulated intestinal fluid (SIF) composed of a mixture containing bile extract and pancreatin (2 mg per mL pancreatin + 12 mg per mL porcine bile extract, 0.1 M sodium bicarbonate). The pH of the system was adjusted to 7 with 0.1 M NaOH, prior to incubation of the samples for 2 hours. Incubation was performed in an orbital shaking incubator ("shaker") Model ET-420 (Tecnal, Brazil), at 100 rpm and 37 °C. Variations of the simulated fluid compositions were evaluated in order to identify the cause of the emulsion destabilization. The following "control simulated fluids" were prepared: SGF-control (pH 2, without pepsin), SIF-control (pH 7, without bile extract and pancreatin) and SIF-BE (pH 7, bile extract, without pancreatin). The droplet size and optical microscopy of the emulsions were evaluated after the gastric and enteric digestion steps. All measurements after gastric digestion were carried out after 1 hour of exposure on SGF at pH 2 followed by the pH adjustment to 5.3 in order to reduce the pepsin activity. On the other hand, measurements after enteric digestion were carried out immediately after exposure for 2 hours on SIF at pH 7.

2.4 Evaluation of emulsion stability

Immediately after preparation, 30 mL of each emulsion was poured into a cylindrical glass tube (internal diameter = 25 mm, height = 95 mm), sealed with a plastic cap and stored at 25 °C for one week. The volume of the serum phase (H) was quantified and the creaming index (CI) was reported as CI (%) = $(H/H_o) \times 100$, where H_o represents the initial height of the emulsion.[20]

2.5 Optical microscopy

Emulsions were evaluated after 7 days of storage. The microstructure of the cream phase was also analysed for systems that showed phase separation. Samples were poured onto microscope slides, covered with glass cover slips and observed using a Carl Zeiss Model Axio Scope.A1 optical microscope (Zeiss, Germany). Objective lenses (40× and 100×) were used to visualize the microstructure of the emulsions.

2.6 Determination of average droplet size

A Mastersizer 2000 (Malvern Instruments Ltd., UK) was used to determine the average diameter of the particles or emulsion droplets. The mean size was determined as the volume–surface mean diameter (d_{32}) (eqn (1)).

$$d_{32} = \frac{\sum n_i d_i^3}{\sum n_i d_i^3} \quad (1)$$

where n_i is the number of particles with diameter d_i.

The emulsions were analysed after preparation (t0) and 7 days of storage (t7). Each sample was measured in triplicate at 25 °C.

2.7 ζ-Potential measurements

To determine the surface electric charge density of the particles, the emulsions were diluted to a concentration of about 0.005% (w/w) in a citrate–phosphate buffer solution (pH 3, 5.5 or 7) before being placed in the measuring chamber of microelectrophoresis (Zetasizer Nano-ZS, Malvern Instruments Ltd., UK). The Smoluchowski mathematical model was used to convert the electrophoretic mobility measurement into ζ-potential values. The samples were measured after 7 days of storage in triplicate at 25 °C.

2.8 Rheological measurements

Rheological measurements of the emulsions were performed with a Physica MCR301 modular compact rheometer (Anton Paar, Germany). A 5 cm rough plate geometry was used to analyse the separated phases and emulsions with no phase separation after 7 days of preparation. Flow curves were obtained by an up–down–up step program using different shear stresses range for each sample, in order to give a maximum shear rate value of 300 s^{-1}.[21,22] The samples were measured in duplicate at 25 °C and the apparent viscosity of the systems was evaluated at a shear rate of 100 s^{-1}. This shear rate value is typical for food processes such as flow through a pipe, stirring or mastication.[1]

2.9 SDS-PAGE electrophoresis

Samples were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing and non-reducing conditions, using a slab gel of 1.5 mm thickness with 15% acrylamide running gel and 5% stacking gel. A Mini-Protean electrophoresis system (Bio-Rad Laboratories, USA) was used for the measurements at a constant voltage of 120 V.

Protein solutions (2% w/w) and emulsions were diluted in deionized water (2 mg protein per mL) and mixed at 1 : 1 ratio with an electrophoresis sample buffer (containing 50 mM Tris–HCl, 2% SDS, 10% (v/v) glycerol, 0.1% (w/v) Coomassie Brilliant Blue) to perform non-reducing conditions. Then, 0.001 M β-mercaptoethanol was added to the buffer in order to evaluate reducing conditions. These mixtures were heated for 5 min at 70 °C and then 15 μg of aliquots were loaded onto the polyacrylamide gels. Whey protein solution at the same emulsion pH was used as a reference and a commercial molecular weight marker (Pre-stained Invitrogen™ Bench Marker Protein Ladder, Bioagency International, USA) was used for the control of molecular weight. After each run, the gels were immediately stained using 0.25% (w/v) Coomassie Brilliant Blue for 2 hours and destained with a solution composed of methanol–glacial acetic acid–water (4.5 : 1 : 4.5) for 3 hours with five changes.

2.10 Statistical analysis

Significant differences were determined by the Tukey test. Statistical analyses were performed using the software STATISTICA 7.0 (Statsoft Inc., Tulsa, USA) and the level of confidence was 95%.

3 Results

3.1 Emulsion stability

The creaming index of emulsions after 7 days is shown in Table 1. The emulsions stabilized by WPI did not show phase separation at pH 3 and 7. At pH 5.5, the emulsions containing WPI and homogenized at lower pressure (250 and 450 bar) showed a slight phase separation initiated only in the sixth day of storage (creaming index < 1.5% v/v). Such separation can be attributed to the proximity of the pH to WPI pI, leading to an increase of the attractive forces and self-association between the non-adsorbed and the adsorbed proteins to the interface, which promoted destabilization of the emulsion.[23] However, such an effect was less pronounced increasing the homogenization pressure, because of the probable exposure of hydrophobic groups of protein that became more available for interaction with the oil. Due to the small volume of the serum phase observed in this emulsion (WPI at pH 5.5), only the cream phase was evaluated. The emulsions containing only lecithin did not show any phase separation independent of pH. Systems containing the mixture of emulsifiers at pH 3 showed fast phase separation, exhibiting almost 50% (v/v) of an equilibrium creaming index (after around 1 hour), independent of the homogenization pressure. At other pH values, no visual phase separation was observed in the emulsions containing the mixture of emulsifiers.

3.2 Mean droplet size

Emulsions stabilized with only WPI showed smaller droplet sizes by increasing the homogenization pressure and most of the systems presented very small or no variations in the mean diameter (d_{32}) during the storage (Table 2), indicating that there was no coalescence or flocculation of the droplets. However, the emulsions stabilized only by WPI at pH 5.5 showed an increase in droplet size, which was more significant at higher pressure. Emulsions stabilized with lecithin showed a slight reduction in droplet size when the pressure was increased from 250 to 450 bar. However, a large droplet size was observed by changing the homogenization pressure from 450 to 600 bar, indicating a tendency to re-coalescence. The mean diameter of the systems containing the mixture of emulsifiers at pH 3 corresponded to

Table 1 Creaming index (% v/v) of emulsions with 30% (w/w) of soybean oil containing lecithin and/or WPI[a]

Emulsion	Pressure (bar)	pH 3	pH 5.5	pH 7
WPI	250	—	1.3	—
	450	—	0.3	—
	600	—	—	—
WPI + Lec	250	50.7	—	—
	450	54.3	—	—
	600	52.3	—	—
Lec	250	—	—	—
	450	—	—	—
	600	—	—	—

[a] (—) No phase separation.

Table 2 Mean droplet diameter, d_{32} (μm), of emulsions with 30% (w/w) of soybean oil containing lecithin and/or WPI after preparation (t0) and 7 days of storage (t7)[a]

Emulsion	Pressure (bar)	pH 3		pH 5.5		pH 7	
		t0	t7	t0	t7	t0	t7
WPI	250	1.76^{Bc}	1.70^{Ac}	1.49^{Ac}	2.25^{Bc}	1.71^{Ac}	1.75^{Bc}
	450	1.59^{Ab}	1.56^{Ab}	1.08^{Ab}	1.85^{Ba}	1.48^{Ab}	1.47^{Ab}
	600	1.46^{Aa}	1.47^{Aa}	0.91^{Aa}	2.07^{Bb}	1.29^{Ba}	1.23^{Aa}
WPI + Lec	250	109.9^{Aa}	120.7^{Ba}	1.55^{Bc}	1.49^{Ac}	1.55^{Ac}	1.54^{Ac}
	450	122.4^{Aa}	129.1^{Aa}	1.27^{Bb}	1.24^{Ab}	1.35^{Bb}	1.31^{Ab}
	600	121.6^{Aa}	110.3^{Aa}	1.10^{Ba}	1.09^{Aa}	1.19^{Aa}	1.20^{Ba}
Lec	250	1.57^{Ab}	1.59^{Bb}	1.43^{Aa}	1.43^{Aab}	1.53^{Ac}	1.58^{Ab}
	450	1.52^{Aa}	1.53^{Aa}	1.40^{Ba}	1.39^{Aa}	1.41^{Aa}	1.39^{Aa}
	600	1.62^{Ac}	1.60^{Ac}	1.53^{Bb}	1.49^{Ab}	1.51^{Ab}	1.52^{Ab}

[a] Different letters indicate statistical differences ($p < 0.05$). Capital letters: the differences between the storage time for the same system. Small letters: differences within the same column at the same pH.

the aggregate size formed in the cream phase (Fig. 1). At pH 5.5 and 7, the mixture of emulsifiers led to a gradual decrease of d_{32} with the increase of homogenization pressure in a similar way of WPI emulsions. Moreover, the droplet size observed for higher pressures was lower than those observed for emulsions containing only lecithin or WPI, which would contribute to the higher stability of the emulsions. Specially, at pH close to the pI, the interaction between proteins and lecithin was favorable since emulsions did not present variation in droplet diameter during storage, differently from that occurred for those stabilized only by WPI.

3.3 Surface charge density

ζ-Potential values of the different systems are described in Table 3. Emulsions stabilized only by WPI showed droplets with high positive surface charge density (near +39 mV) at pH 3, resulting from the protonation of the amino groups as the pH is below and away from the pI of the protein. At pH 5.5 (close to pI) and 7 (above the pI), the ζ-potential became negative, but the emulsions at pH 7 showed greater magnitude than those found at pH 5.5, since the pH was above and away from the pI of the protein. The emulsions stabilized by lecithin showed negatively charged droplets at all pH values. In addition, ζ-potential absolute values were relatively high, which probably favored the stability due to the electrostatic repulsion between the droplets preventing destabilization of the systems during storage.

For all pH values, ζ-potential of the system stabilized by the mixture of lecithin and WPI was between the values of the emulsions containing only WPI or lecithin. Specially at pH 3, these systems showed particles with a very low ζ-potential value (around +10 mV). This result corroborates that the high kinetic instability observed at pH 3 can be attributed to the formation of electrostatic complexes with low ζ-potential, resulting in an electrostatic repulsion insufficient to maintain the particles dispersed in only one phase. At pH 5.5, the values were around −20 mV. Although a very large absolute value was not presented, the systems were stable during the storage period, which did not occur in the emulsions stabilized only by WPI, suggesting that lecithin was fundamental to the emulsion stability mainly due to its charge contribution at the interface.[9] In mixed systems at pH 7, as well as to emulsions containing only lecithin or WPI, the droplets formed presented relatively high absolute values of surface charge density. As a consequence, the systems were stabilized due to the electrostatic repulsion which avoided the coalescence or flocculation processes of the droplets.

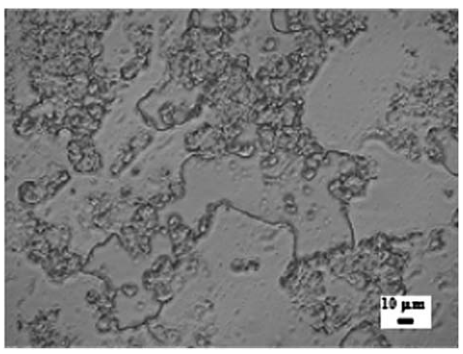

Fig. 1 Typical microstructure of the cream phase of systems containing lecithin and WPI at pH 3. Scale bar = 10 μm.

3.4 Rheology

Rheological measurements were carried out in order to understand the effect of different conditions of the process and composition on emulsion stability. The cream phase of unstable systems exhibited a shear-thinning behavior. The stable emulsions behaved as Newtonian fluids, except for the emulsion containing WPI at pH 5.5 and homogenized at 600 bar which also showed shear-thinning behavior.

Apparent viscosity values at a shear rate of 100 s^{-1} are described in Table 4. For the emulsions stabilized by WPI, it was observed that the increase of homogenization pressure led to an increase in viscosity, but this difference was greater in the emulsions at pH 5.5 which led to phase separation inhibition at 600 bar. Other studies have also reported an increase in

Table 3 ζ-Potential (mV) of emulsions with 30% (w/w) of soybean oil containing lecithin and/or WPI[a]

Emulsion	Pressure (bar)	pH 3	pH 5.5	pH 7
WPI	250	$+39 \pm 1^{a}$	-12.7 ± 0.7^{a}	-29.6 ± 0.4^{a}
	450	$+39 \pm 1^{a}$	-12 ± 1^{ab}	-33 ± 1^{b}
	600	$+39.4 \pm 0.7^{a}$	-11.2 ± 0.4^{b}	-30 ± 1^{a}
WPI + Lec	250	$+11 \pm 1^{a}$	-23.6 ± 0.9^{a}	-33 ± 2^{b}
	450	$+10 \pm 1^{a}$	-18 ± 1^{b}	-28.3 ± 0.5^{a}
	600	$+14 \pm 1^{b}$	-22 ± 2^{a}	-27.4 ± 0.5^{a}
Lec	250	-50 ± 2^{a}	-40 ± 1^{a}	-26 ± 1^{a}
	450	-50 ± 2^{a}	-39.3 ± 0.9^{a}	-37 ± 1^{b}
	600	-50 ± 2^{a}	-37.8 ± 0.4^{b}	-35 ± 2^{b}

[a] The different letters represent significant difference ($p < 0.05$) in the same column and the same emulsifier ratio.

Table 4 Viscosity at a shear rate of 100 s^{-1} (mPa s) of emulsion containing lecithin and/or WPI[a]

Emulsion	Pressure (bar)	pH 3	pH 5.5	pH 7
WPI	250	1.59[a]	25.45[a]*	1.92[a]
	450	2.20[b]	42.00[b]*	2.67[bc]
	600	2.65[d]	74.50[c]	2.70[bc]
WPI + Lec	250	88.00[f]*	1.98[d]	2.15[a]
	450	89.55[f]*	2.46[e]	2.46[b]
	600	73.70[e]*	2.48[e]	2.72[c]
Lec	250	2.47[c]	2.47[e]	2.79[c]
	450	2.11[b]	2.21[e]	2.82[c]
	600	2.21[b]	2.31[e]	2.79[c]

[a] The different letters represent significant difference ($p < 0.05$) in the same column. * Cream phase.

viscosity due to the increase of homogenization pressure in emulsions stabilized by protein.[24,25] Such behavior could be related to a decrease of the mean separation distance between the droplets due to droplet size reduction, which leads to an increase in hydrodynamic interactions between the droplets and consequently to a higher viscosity.[26] Moreover, during and after the emulsification process, emulsion droplets are subjected to Brownian motion and mainly a high-intensity turbulence that leads to collision between droplets.[2] In this way, higher viscosity with increasing high-pressure homogenization could also be attributed to the greater collision frequency due to the increase of the energy supplied during the emulsification, leading to enhanced interaction between the droplets. Kuhn and Cunha (2012) associated the increase of viscosity values with a higher protein aggregation due to the high-pressure effect primarily related to the rupture of non-covalent interactions (hydrophobic and electrostatic), followed by the partial protein unfolding and exposure of reactive sulfhydryl groups under more severe conditions (*e.g.* higher homogenization pressure), increasing the emulsion stability.[27] On the other hand, the protein unfolding, exposure and interaction between hydrophobic groups could also result in a decrease in emulsifying capacity and droplet coalescence as occurred with WPI emulsions at pH close to the pI. Emulsions stabilized by lecithin, in general, did not show significant differences between the values of viscosity with the increase of homogenization pressure, which could be related to its low sensibility to the high-pressure effects, in agreement with their mean droplet size.

The cream phase of systems containing lecithin and WPI at pH 3 showed the highest viscosity values that decreased by increasing the homogenization pressure. Emulsions containing the mixture of emulsifiers with no phase separation (pH 5.5 and 7) presented higher viscosity with increasing pressure homogenization in the same way of WPI emulsions. Furthermore, similar and much lower viscosity at these pH values showed that no electrostatic interactions occurred different from the systems at pH 3. In general, the viscosity values obtained for the systems containing only lecithin or the mixture of emulsifiers at pH 5.5 were not significantly different, while the viscosity observed in systems containing only WPI was much higher. In this case, the lower viscosity may be related to the decrease of protein interactions due to its reduced concentration in emulsions stabilized by the mixture of lecithin and WPI.

3.5 Polyacrylamide gel electrophoresis (SDS-PAGE)

The effect of different pH and homogenization pressure on distribution of the WPI proteins in aqueous systems (Fig. 2A and B) and in emulsions (Fig. 2C–H) was evaluated through electrophoretic profiles. In general, bands of β-lactoglobulin (β-lg) and α-lactalbumin (α-la), which are the most abundant fractions in WPI,[28] and minor bands related to the fraction of bovine serum albumin (BSA) and β-lg dimers were clearly visualized. Under non-reducing conditions, the profiles of the WPI solutions treated with different pressures (Fig. 2A) were very similar regardless of the pH value, with no aggregates of high molecular weight. In the reducing gel (Fig. 2B) bands were visualized in the region between the fractions of BSA and β-lg dimers, which can suggest that some protein aggregation occurred and were disrupted under reducing conditions.

For emulsions containing only WPI, the formation of protein aggregates of molecular weight higher than 180 kDa was observed in the stacking gel of non-reducing polyacrylamide gels (except pH 3) (Fig. 2C–E). At pH 5.5 and 7, the aggregation was intensified with the increase of homogenization pressure. However, these bands disappeared when the emulsions were dissolved in reducing buffer, suggesting that disulfide bonds, formation of which is not favorable at low pH, were important for the stabilization of the aggregates formed at higher pH values. The formation of the aggregates might be related to the adsorption of the proteins at the interface, which was facilitated by the opening of the protein structure at higher pressures, since protein aggregates were not visualized in WPI solution (Fig. 2A). Hunt and Dalgleish (1994) reported the pH dependence of surface activity of the main WPI fractions. They observed that the β-lg adsorption is favored at higher pH, while α-la is preferentially adsorbed at lower pH due to changes in WPI conformation and quaternary structure.[29] Despite aggregate formation in both cases, emulsions at pH 5.5 and 7 showed different stability properties. Weaker repulsion forces due to the low surface charge density in the systems at pH 5.5 (around pI) could lead to the droplets proximity, allowing more hydrophobic interactions between proteins which committed the emulsion stability.

The emulsions containing a mixture of lecithin and WPI did not show the formation of aggregates of high molecular weight (>180 kDa). In systems containing both emulsifiers at pH below the pI under non-reducing conditions, marked bands of β-lg were observed in both phases being more intense in the serum phase, while α-la bands were seen only in the cream phase, showing the preferential adsorption of its protein fraction at the interface at lower pH.[29] Moreover, the scattering of the α-la and β-lg bands suggests the formation of electrostatic complexes between lecithin and these proteins, which was highly favorable at pH 3 in which they are oppositely charged. When the cream and serum phases were dissolved in reducing buffer, bands were observed in the region below the bands of β-lg and α-la,

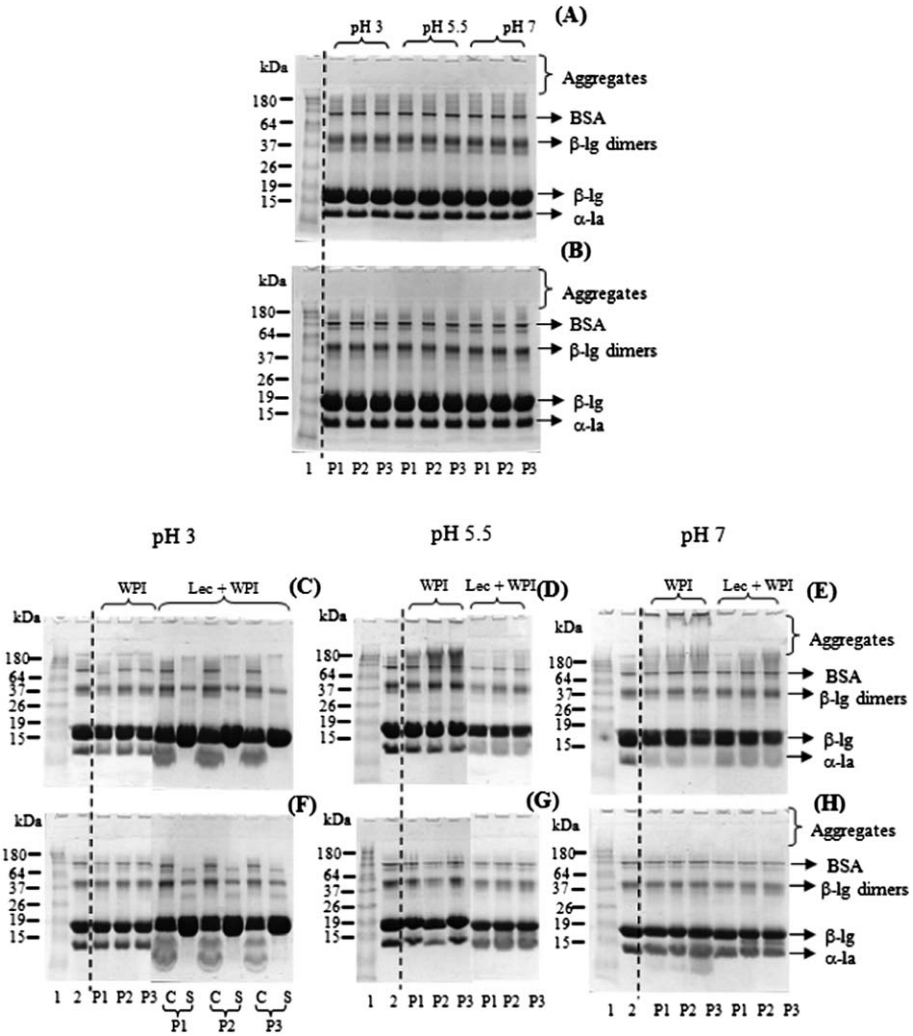

Fig. 2 Polyacrylamide gel electrophoresis (SDS-PAGE) of the (A and B) solutions containing 2% (w/w) of WPI and emulsions containing WPI or WPI and lecithin at (C and F) pH 3, (D and G) pH 5.5 and (E and H) pH 7 homogenized at high-pressure. (A, C, D and E) SDS-PAGE under non-reducing conditions and (B, F, G and H) SDS-PAGE under reducing conditions. Lanes (1) commercial molecular weight marker, (2) native WPI solution (non-homogenized), (P1) 250 bar, (P2) 450 bar, (P3) 600 bar, (C) cream phase and (S) serum phase.

suggesting that an interaction between lecithin and the protein at this pH changed the electrophoretic mobility of these proteins.

On the other hand, in emulsions containing WPI and lecithin at pH 5.5 and 7, bands of protein aggregates (180–64 kDa) were observed in the non-reducing polyacrylamide gel and were slightly intensified with the increase of homogenization pressure. When these emulsions were dissolved in reducing buffer, these bands disappeared and the band of α-la became more marked, suggesting that this protein participated of the aggregates. According to Monahan *et al.* (1993), in a model system containing equal concentrations of both α-la and β-lg, α-la was shown to participate in the polymerization reaction and in doing so reduced the frequency of β-lg–β-lg interactions.[30] Thiol–disulfide interchange between free –SH of β-lg and –S–S– bonds of α-la can also explain the involvement of α-la in the polymerization reaction in emulsions containing both α-la and β-lg. Furthermore, diffuse bands located between β-lg and α-la were also observed which may be related to the presence of lecithin, since the same was not observed for the emulsions containing only WPI.

3.6 *In vitro* digestion

The simulated digestion was performed only to emulsions homogenized at 450 bar and pH 7, since emulsions under this initial pH condition did not show any phase separation after 7 days of storage for none of the compositions evaluated, which allowed the comparison between the different systems.

The visual appearance (Table 5) after simulated gastric digestion varied with the composition. Emulsion stabilized by WPI was the most stable since it did not show phase separation or free oil released after exposure to SGF or SIF. Emulsion containing lecithin showed an opaque bottom phase and an upper cream phase when mixed with SGF-control and in the presence of pepsin. On the other hand, a free oil phase could be observed in lecithin emulsion immediately after incubation in SIF containing only bile extract or bile extract and lipase.

Table 5 Description of visual appearance of emulsions containing WPI and/or lecithin after incubation in simulated gastrointestinal fluids

	Simulated gastrointestinal fluids				
Emulsion	SGF-control	SGF	SIF-control	SIF-BE	SIF
WPI	Stable	Stable	Stable	Stable	Stable
WPI + Lec	Phase separation (translucid bottom phase/upper cream phase)	Phase separation (translucid bottom phase/upper cream phase)	Phase separation (opaque bottom phase/upper cream phase)	Oil release	Oil release
Lec	Phase separation (opaque bottom phase/upper cream phase)	Phase separation (opaque bottom phase/upper cream phase)	Phase separation (opaque bottom phase/upper cream phase)	Oil release	Oil release

Emulsion stabilized by both emulsifiers also showed phase separation after incubation in SGF-control and in the presence of pepsin and oil release after incubation in SIF-BE or SIF, similarly to emulsions containing only lecithin. However, for the emulsion stabilized by both emulsifiers the phase separation after gastric digestion occurred during pH adjustment from 2 to 5.3 and not immediately after incubation under acidic conditions.

The mean droplet diameter (d_{32}) and optical microscopy of the emulsions after the steps of gastric and enteric *in vitro* digestion are shown in Fig. 3 and 4. Emulsion containing only WPI showed extensive flocculation upon exposure to SGF (Fig. 4A(ii) and (iii)) resulting in an increase of mean particle size (Fig. 3). Flocs or aggregates were visualized with the emulsion mixed with acid gastric fluid alone (Fig. 4A(ii)) and in the presence of pepsin (Fig. 4A(iii)), showing that the flocculation could be attributed to the proteolysis of the proteinaceous interfacial layer by pepsin in SGF[31] or even to the proximity of the medium pH (5.3) to the WPI pI. However, after exposition to SIF, WPI-stabilized emulsion showed a decrease of the mean droplet diameter (Fig. 3) to similar values of the original emulsion (before simulated digestion) independent of the SIF composition (Fig. 4A(iv)–(vi)).

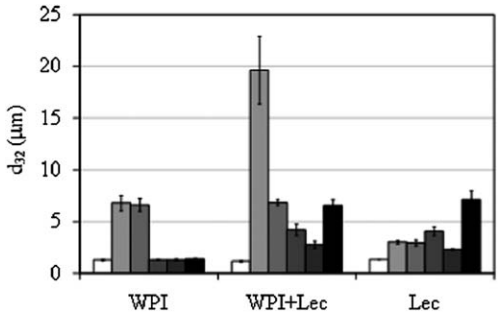

Fig. 3 Mean droplet diameter (d_{32}) of emulsions containing lecithin and/or WPI at various stages of *in vitro* digestion: (□) initial emulsion (before *in vitro* digestion), (▩) SGF-control/1 h (▩) SGF/1 h, (▩) SIF-control/2 h, (■) SIF-BE/2 h and (■) SIF/2 h.

Lecithin-stabilized emulsions underwent a slight increase of mean droplet size (Fig. 3) after exposure on SGF related to a coalescence process (Fig. 4C(ii) and (iii)). The proximity of acidic conditions to the pK_a (around 1.5) of the phospholipid[32] became the droplets closer due to a smaller electrostatic repulsion which favoured the coalescence. Lecithin emulsion exposed to SIF control (Fig. 4C(iv)) showed an increase of the mean droplet size, indicating that the system remained unstable and droplet coalescence kept occurring even after the pH value is restored at 7. The droplet size reduction after incubation in SIF with bile extract alone (Fig. 4C(v)) followed by an increase of droplet size with lipase addition (Fig. 4C(vi))

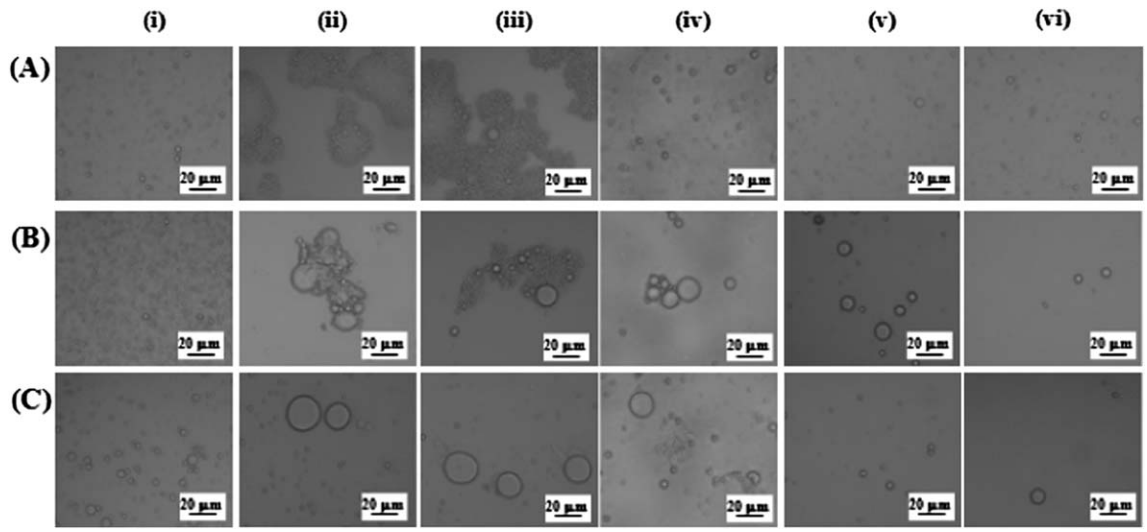

Fig. 4 Microstructure of emulsions containing (A) WPI, (B)WPI with lecithin and (C) lecithin at various stages of *in vitro* digestion: (i) initial emulsion (before *in vitro* digestion), (ii) SGF-control/1 h, (iii) SGF/1 h, (iv) SIF-control/2 h, (v) SIF-BE/2 h and (vi) SIF/2 h. Scale bar = 20 μm.

indicated that bile salts adsorbed onto the droplet surface, favouring lipid hydrolysis by pancreatic lipase. According to Mun *et al.* (2007), the formation of free fatty acids and monoacylglycerols as products of lipolysis by pancreatic lipase leads to droplet coalescence due to the low HLB number of these surface active substances which are ineffective by stabilizing oil-in-water emulsions against coalescence.[15]

Regarding emulsions containing WPI and lecithin, it was observed an increase of the mean droplet size (Fig. 3) under SGF exposure due to flocculation and coalescence mechanisms (Fig. 4B(ii) and (iii)). When exposed to SGF-control (in the absence of pepsin) (Fig. 4B(ii)) a great increase of droplet size was observed due to the formation of great electrostatic complexes between WPI and lecithin and free oil was not observed. On the other hand, the addition of pepsin (Fig. 4B(iii)) led to a smaller increase of droplet size and oil release, indicating that the electrostatic complexes were adsorbed at the interface and broken (Fig. 2C and F) preferentially by pepsin.[33] After exposure on SIF (Fig. 4B(iv)–(vi)), emulsions containing both emulsifiers presented a slight decrease of mean droplet size. The mean droplet size of emulsion stabilized by both emulsifiers was similar to lecithin emulsion after incubation in SIF of different compositions, suggesting that only lecithin remained adsorbed at the interface after proteolysis. Different emulsion structuring behaviour when stabilized by WPI or the mixture of WPI and lecithin could be attributed to the lower content of protein which was insufficient to entirely recover the interface in the second case or even to the preferentially lecithin adsorption due to its predominance of hydrophobic features when compared to WPI.

4 Discussion

The influence of the interface composition and pH on the emulsion stability response under simulated digestive conditions was evaluated.

O/W emulsions stabilized by WPI showed high resistance against creaming, low viscosity far from the pI and smaller droplet size by increasing the homogenization pressure. On the other hand, emulsions stabilized by WPI at pH 5.5 were kinetically unstable with high viscosity showing phase separation even if they are homogenized at high pressure. Weak repulsion forces due to the low surface charge density in these systems led to the droplet proximity, allowing more hydrophobic interactions between proteins which committed the emulsion stability.

Emulsions stabilized by lecithin were stable, with low viscosity and small droplet size independent of pH, which could be related to the high surface charge density of the droplets recovered by this emulsifier. A tendency to re-coalescence was observed with increasing homogenization pressure, suggesting that the droplets were not completely covered by lecithin or a less resistant interfacial film was formed. Therefore the increased size would be a consequence of the collision frequency between the droplets enhanced by the high energy supplied during the emulsification.[2]

Emulsions stabilized by WPI and lecithin showed phase separation only at pH 3 due to the formation of electrostatic complexes with low ζ-potential. However, at pH 5.5 and 7, these emulsions were stable against creaming, showed low viscosity and small mean droplet size. The presence of different compounds with surface-active properties leads the molecules to 'compete' for space to adsorb at the interface. Important factors that would affect the interface composition are: (i) binding capacity, (ii) concentration and (iii) size ratio of the surface-active substances. Because of its simpler structure and size 100 times smaller than protein molecules, low molecular weight (LMW) surfactants such as lecithin tend to arrange more efficiently at the interface adsorbing a much larger number of molecules within the same surface area. Moreover, if the LMW surfactant content is high enough, the adsorbed protein molecules are displaced by the smaller surfactant molecules.[6] This information added to electrophoretic profiles (Fig. 2) give evidence that lecithin is probably adsorbed to the interface. However, both emulsifiers still remained bound by hydrophobic interactions at pH 5.5 and 7 and hydrophobic and electrostatic interactions at pH 3.

Exposing the emulsions to the simulated gastric and enteric fluids led to a macro and microstructural changes, depending on the composition of the droplet interface. WPI stabilized emulsions were the most stable after simulated digestion, while emulsions containing lecithin (with WPI or not) showed phase separation after SGF incubation. However, emulsions containing WPI with or without lecithin showed agglomeration of droplets after exposure to SGF, which could be attributed to flocculation (reversible) or coalescence (irreversible) mechanisms. Droplet flocculation is characterized by the redispersion of emulsion aggregates after electrostatic stabilization at pH 7, while droplet coalescence occurs when there is irreversible change of emulsion droplet size even after the pH increase.[31] Therefore, only for WPI emulsions the flocculation mechanism was predominant since the agglomerates broke after the pH increase resulting in droplet size very similar to the droplet size of the initial emulsion (before *in vitro* digestion). For emulsions stabilized by lecithin or the mixture of lecithin and WPI, the coalescence process was predominant. It is important to emphasize that the stability under simulated gastrointestinal conditions does not necessarily mean that there is no occurrence of lipolysis. The rate of fat digestion is controlled by surface accessibility, which is controlled by surfactant chemistry and mainly the emulsion interfacial area.[31] Thus, further analyses would be necessary in order to determine the capability of these systems to protect the emulsified lipid against lipolysis.

5 Conclusion

The use of WPI led to emulsion stability at pH values away from the pI even at lower homogenization pressure, although WPI emulsions near pI showed reduced stability. Lecithin emulsions showed stability independent of pH, but exhibited less resistance under gastric conditions different from WPI emulsion. Results also showed that the addition of a second emulsifier improves the stability of WPI emulsion near pI. Moreover, despite the lower content of lecithin, emulsions stabilized by

both emulsifiers maintained a resistance against the conditions of the gastrointestinal tract quite similar to lecithin emulsion.

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