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Comparative behavior of protein or polysaccharide stabilized emulsion under in vitro gastrointestinal conditions

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ABSTRACT

In the present work, an in vitro model digestion was used to compare the behavior of emulsions stabilized by proteins or polysaccharide upon digestion and to analyze its relationship with the kinetics and extent of lipid digestion. Oil/water emulsions were prepared using different emulsifiers (β-lactoglobulin, soy protein isolate and hydroxypropylmethylcellulose (HPMC)). The emulsion digestion was carried out in two continuous stages at 37 °C: 1) under simulated gastric conditions (1 h) using pepsin and phosphatidylcholine (simulated gastric fluid: pH 2.5, NaCl, NaH_2PO_4, KCl and $CaCl_2$) and 2) under simulated intestinal conditions (1 h) with bile salts, pancreatic lipase, trypsin and chymotrypsin (simulated intestinal fluid: pH 7.0, K_2HPO_4, NaCl and $CaCl_2$). The changes in the particle size distributions, the interfacial area and their microstructures were analyzed as a function of the digestion time. The free fatty acid release during the simulated intestinal stage was also determined and an empirical model was fitted to estimate different kinetic parameters. Irrespective of the composition/structure of emulsions, the initial surface area was found to determine the initial rate of lipolysis. Soy protein was the protein that forms the most resistant emulsion to digestion, showing a degree of free fatty acid release similar to HPMC, which is a non digestible emulsifier. The results are discussed on the basis of the role of bile salts and its effect on oil/water interfaces.

1. Introduction

The capacity to control the lipid digestion within the gastrointestinal human tract could facilitate the development of functional foods that could result in decreasing the risk of suffering some diseases associated with the high lipid absorption such as obesity and cardiovascular diseases (Golding et al., 2011; Li, Hu, & McClements, 2011; Li & McClements, 2011; Lowe, 1994; Singh, Ye, & Horne, 2009; Wooster et al., 2014). Moreover, this knowledge is important to develop lipid based delivery systems that would facilitate the incorporation of bioactive substances that could have healthy benefits (McClements, Decker, Park, & Weiss, 2008; Salvia-Trujillo, Qian, Martín-Belloso, & McClements, 2013).

An important part of the lipids in processed foods are consumed in the form of oil–water (o/w) emulsions, in which they are embedded in form of droplets in an aqueous medium. The surface of these droplets is coated by a layer of interfacial active molecules, such as proteins or polysaccharides. These molecules adsorb at fluid interfaces playing an important role not only in the formation and stability of emulsions but also in the rate of the digestion process (Bouyer et al., 2011; Qian & McClements, 2011; Wan et al., 2014). It has been reported in recent works that the characteristics of the interfacial layers surrounding the fat droplets could play a significant role in the extent of lipid digestion, as well as the release rate of any entrapped lipophilic components (Bellesi, Pizones Ruiz-Henestrosa, & Pilosof, 2014; Malaki Nik, Wright, & Corredig, 2011; Ye, Cui, Zhu, & Singh, 2013).

During the digestion process, the emulsion is exposed to physical and biochemical changes that result in flocculation, coalescence, aggregation, droplet disruption, etc. (McClements & Li, 2010; Singh et al., 2009). In humans, the lipid digestion process takes place firstly in the stomach but it is more important in the small intestine through the action of pancreatic lipase (70–90% of the fat

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digestion) (Bauer, Jakob, & Mosenthin, 2005; Maldonado-Valderrama et al., 2008; Mun, Decker, Park, Weiss, & McClements, 2006).

The emulsion is mixed in the stomach with acidic digestive juices. These juices contain gastric components such as pepsin and surface active components such as phosphatidylcholine (PC) that alter the interfacial composition of oil droplets. It has been reported that the pH of the human stomach is between 1 and 3 and the meal could remain for 1–3 h, depending on the ingested food (composition, initial pH, buffering capacity, quantity, etc.). The partially digested food in the stomach, called chyme (Ekmekcioglu, 2002; Kalantzi et al., 2006; Lindahl, Ungell, Knutson, & Lennernäs, 1997), is mixed in the duodenum with sodium bicarbonate, bile salts (BS), phospholipids, and enzymes secreted by the liver, pancreas and gall bladder. The sodium bicarbonate secreted in the small intestine causes the pH increment from 1 to 3 (gastric conditions) to 6–7 where the pancreatic enzymes present maximum activity (Bauer et al., 2005). Trypsin and chymotrypsin are the main proteases in the intestinal juice and catalyze the breaking of specific peptides bonds (Ma, Tang, & Lai, 2005; McClements et al., 2008).

The lipid hydrolysis is an interfacial phenomenon that requires the adsorption of pancreatic lipase at the lipid droplet surface. This adsorption is facilitated by the presence of BS, released from the gall bladder through the bile duct, and colipase secreted by the pancreas (Fillery-Travis, Foster, & Robins, 1995; Mun et al., 2006). The adsorption of BS facilitates the emulsification of the lipids, as they affect the interfacial layer of emulsion droplets and prepare them for enzymatic hydrolysis (Bauer et al., 2005; Bellesi et al., 2014; Torcello-Gómez, Maldonado-Valderrama, Jódar-Reyes, Cabrerizo-Vílchez, & Martín-Rodríguez, 2014). Therefore, the nature of the interfacial layer surrounding the lipid droplets should have an important role in the lipid digestion. Previous works have shown that the characteristic of interfacial films could affect the BS adsorption under simulated intestinal conditions, which could alter the lipid digestion (Bellesi et al., 2014; Maldonado-Valderrama et al., 2008; Mun, Decker, & McClements, 2007; Singh & Sarkar, 2011; Singh et al., 2009; Torcello-Gómez & Foster, 2014). When the lipase is adsorbed at the o/w interface, the triglycerides are hydrolyzed into free fatty acid (FFA), monoglycerides and diglycerides. These products are then incorporated into the BS micelles and transported to be absorbed by the epithelium layer. Moreover, the intestinal proteases (trypsin and chymotrypsin) carry out the hydrolysis of the protein interfacial films (Di Maio & Carrier, 2011; Martigne, Julien, & Sarda, 1987).

It has been recently described the behavior of lipid emulsions under gastrointestinal conditions focusing in different aspects (Golding et al., 2011; Singh & Ye, 2013; Wooster et al., 2014; Ye et al., 2013). Malaki Nik et al. (2011) have reported the effect of the emulsifier type in the physicochemical behavior of o/w emulsions during the in vitro digestion. They observed that soy protein isolate (SPI) emulsions were more digested than whey protein isolate (WPI) emulsions. Torcello-Gómez, A., et al. (2014) have analyzed the behavior of different non-ionic surfactants (pluronics) under simulated duodenal conditions from an interfacial point of view using olive oil as the oil phase. These authors concluded that the surfactants could resist the interfacial displacement of BS, retarding or limiting the lipase activity. Ye et al. (2013) have focused on the effect of calcium in the kinetics of FFA release in emulsions stabilized by WPI, Tween 20, sodium caseinate and lecithin under duodenal conditions and they showed that the addition of calcium promote the rate and extent of FFA release (through the removal of FFA from the surface area). Moreover they concluded that the increase of FFA was dependent on the emulsifying agent. The proteins, capable to interact with calcium, reduced the availability of calcium and consequently decreased the lipase activity more than low molecular weight emulsifiers (lowest capacity to interact with calcium). It has also been demonstrated that emulsions stabilized whit gum arabic were more digested than emulsions stabilized by proteins (WPI), as the former showed the higher FFA release. The protein molecules adsorbed at the interface could hinder the arrival of the lipase molecules to the interface (Helbig, Silletti, Timmerman, Hamer, & Gruppen, 2012).

In a previous work (Bellesi et al., 2014) it was shown from the analysis of the competitive and sequential adsorption of three proteins and BS that soy protein film was particularly more resistant to BS displacement than β-lg or egg white film, which could impact in further action of lipase and thus on lipid digestion. Therefore, the objective of the present work was to study the behavior of soy protein stabilized o/w emulsions under simulated gastrointestinal digestion in comparison with β-lg, which has been extensively used to stabilized o/w emulsions (Sarkar, Horne, & Singh, 2010a, 2010b; Singh et al., 2009), and a non-ionic polysaccharide (hydroxypropylmethylcellulose (HPMC)), that has been selected because of its known interfacial activity (Camino, Sanchez, Rodríguez Patino, & Pilosof, 2012) and resistance to enzymes action. Furthermore, the rate of lipid digestion was determined and related to the performance of emulsions upon in vitro digestion.

2. Materials and methods

2.1. Materials

BioPURE β-lactoglobulin (β-lg) was obtained from DAVISCO Foods International, Inc. (Le Sueur, Minnesota) with a protein composition (dry basis) of 97.8%, being β-lactoglobulin 93.6% of total proteins. Denatured soy protein isolate (thermally procedure), being 98% water soluble, was obtained from defatted soybean flour (Sambra S.A., Brazil) as indicated by Carp, Bartholomai, and Pilosof (1997).

Methocell (food grade) HPMC from the Dow Chemical company was kindly supplied by Colorcon (Argentina) and used without purification. The characteristics of this HPMC was indicated previously by Camino and Pilosof (2011).

2.2. Methods

2.2.1. Emulsion preparation

Oil–Water emulsions were prepared by mixing a commercial sunflower oil and emulsifiers solutions (2% w/w) at a 10:90 ratio using an ultrasonic processor Vibra Cell, model VCX 750 (Sonics & Materials, Inc., Newton, CT, USA) at a frequency of 20 kHz and an amplitude of 20% for 15 min. The glass tube with the sample was introduced in a glycerine-jacketed at 0.5 °C to dissipate the heat produced during the sonication keeping the sample temperature below 25 °C (Camino & Pilosof, 2011; McClements, 2004).

2.2.2. In vitro digestion model

15 ml of o/w emulsion were previously incubated at 37 °C. The in vitro digestion begins with the addition of 15 ml of a simulated gastric fluid (SGF) (pH 2.5, 100 mM NaCl, 3 mM $CaCl_2$, 5 mM NaH_2PO_4, and 22 mM KCl) under continuous and moderate agitation. Pepsin from porcine gastric mucosa (P700, powder ≥ 250 units/mg solid) and L-α-phosphatidylcholine from egg yolk (type XVI-E, P3556) both purchased from Sigma–Aldrich were dissolved in SGF. The different aliquots were withdrawn at different times of the gastric stage (t_g): 0 (t_{g0}), 10 (t_{g10}), 30 (t_{g30}) and 60 (t_{g60}) min. The aliquot t_{g0} (at the beginning of the gastric stage) was immediately taken after the incorporation of pepsin and PC and it is associated with the initial effect of this biomolecules. Finally, the proteolysis was stopped by increasing the pH to 7 (1 M $NaHCO_3$).

Thereafter, the digestion continued by incorporating 7.5 ml of simulated intestinal fluid (SIF) (pH 7.0, 39 mM K_2HPO_4, 150 mM NaCl and 30 mM $CaCl_2$), prepared as indicated by Sarkar, Horne, and Singh (2010b) containing bovine bile extract (B3883), lipase from porcine pancreas (L3126, type II, 100–400 units/mg protein using olive oil – 30 min incubation), and the proteases: trypsin (type I, T8003) and L-α-chymotrypsin (type II, C4129), both from bovine pancreas. All the biochemical agents were purchased from Sigma–Aldrich. This mixture was stirred for 1 h at 37 °C and in the meanwhile the pH was continuously monitored and controlled to maintain the pH at 7 by neutralizing the FFA released by lipase activity using NaOH 0.5 M. Aliquots were taken at different time during duodenal stage (t_d): 10 (t_{d10}) and 60 (t_{d60}) min and the enzymes were inhibited using suitable concentrations of Orlistat (inhibitor of lipase activity) and trypsin and chymotripsin inhibitor, both purchased from Sigma–Aldrich (Jiménez-Saiz, Ruiz-Henestrosa, López-Fandiño, & Molina, 2012; McClements & Li, 2010; Miller, Schricker, Rasmussen, & Van Campen, 1981; Sarkar, Goh, Singh, & Singh, 2009; Sarkar et al., 2010a, 2010b; Singh & Sarkar, 2011).

2.2.3. Particle size distribution

The droplet size distributions of emulsions were determined, after the emulsion preparation and during the simulated gastro-duodenal process, by static light scattering using a Mastersizer 2000 with a Hydro 2000MU as dispersion unit (Malvern Instruments Ltd, UK.) The pump speed was set at 1800 rpm. The refractive index (RI) of the dispersed phase (1.47) and its absorption parameter (0.001) were used. Droplet size is reported as the volume–surface mean diameter or Sauter diameter (D_{32}) (Eq. (1)) and the equivalent volume–mean diameter or De Broucker diameter (D_{43}) (Eq. (2)).

$$D_{32} = \sum n_i * d_i^3 \Big/ \sum n_i * d_i^2 \quad (1)$$

$$D_{43} = \sum n_i * d_i^4 \Big/ \sum n_i * d_i^3 \quad (2)$$

where n_i is the number of droplets of diameter d_i. D_{32} provides a measure of the mean diameter while D_{43} is related with changes in particle size involving destabilization processes (Arzeni et al., 2012; Camino et al., 2012; Galazka, Dickinson, & Ledward, 1996; Gu, Decker, & McClements, 2005; Guzey, Kim, & McClements, 2004; Huang, Kakuda, & Cui, 2001). It is also reported the values corresponding to the specific surface area (SSA) that indicate the surface area per unit mass of the dispersed phase.

The results are reported as the average and standard deviation of ten readings made on a sample.

2.2.4. Optical microscopy

The microstructure of the o/w emulsions (fresh and digested emulsions) were analyzed by optical microscopy (OLYMPUS BX43). Each sample was agitated before analysis and a drop of emulsion was placed on the slide and covered with a cover slip, and observed with an objective magnification of 400×. Two replications were done for each sample.

2.2.5. Free fatty acid release

The extent of the lipolysis was analyzed from the amount and rate of the FFA released during the duodenal stage. The lipolysis was monitored, by a titration method, as described by Li and McClements (2010). The lipase activity could be analyzed as percent of free fatty acid released (% FFA) in the digestion time as follows (Eq. (3)):

$$\%\,FFA = ((V_{NaOH}(t) * M_{NaOH} * MW_{TG})/m_{TG} * 2) * 100 \quad (3)$$

where V_{NaOH} (l) is the volume of NaOH solution to maintain the pH at 7 in the duodenal stage (after lipase addition), M_{NaOH} is the molarity of the NaOH solution used to titrate the sample. MW_{TG} is the molecular weight of the triacylglycerol oil, and m_{TG} is the total mass of triacylglycerol oil present at the beginning of the intestinal stage (g). MW_{TG} was calculated as a mean of typical composition of commercial sunflower oil (Chowdhury, Banu, Khan, & Latif, 2007; Rosa et al., 2009).

3. Results

3.1. Droplet size distribution and microstructure

3.1.1. Fresh emulsions

The droplet size distributions of fresh emulsions were measured during 24 h to corroborate the stability with time before the digestion process (data not shown). It can be deduced from Fig. 1 the ability of the emulsifiers to produce small oil droplets. It is related to the high interfacial activity of these emulsifiers and the use of the ultrasonic technique (Bellesi et al., 2014; Camino, Pérez, Sanchez, Rodriguez Patino, & Pilosof, 2009; McClements, 2004; Torcello-Gomez, Maldonado-Valderrama, Martin-Rodriguez, & McClements, 2011).

It is shown in Fig. 1 (A and B) that the particle size appears to be smaller in the original emulsions containing protein-coated droplets (fine and monomodal droplet size distribution, with a peak at 0.3 μm) than those containing HPMC (Fig. 1C). This suggests that the proteins were more effective than HPMC at producing smaller droplets during the emulsification and to prevent their subsequent aggregation (Malaki Nik et al., 2011; Sarkar et al., 2010b; Singh & Sarkar, 2011). These results are closely related to the better interfacial properties observed for the proteins (Bellesi et al., 2014) in comparison with the HPMC (Camino et al., 2009).

The emulsion formed with HPMC showed a droplet size distribution similar to those reported by Camino and Pilosof (2011), presenting a population with a shoulder between 0.15 and 0.5 μm and a mean peak at 1 μm (Fig. 1C).

The values of the mean droplet sizes of the fresh emulsions (t_0) in Table 1 show that both protein stabilized emulsions exhibited the same values of D_{32}, being the values of D_{43} for soy protein stabilized emulsions slightly higher than that for β-lg emulsions, which would indicate that the former was more flocculated. The microstructures obtained by optical microscopy (Fig. 2), corroborated that the emulsions have a uniform distribution of the droplets, which is in accordance with the droplet size distributions obtained by light scattering and the low D_{43} values observed in Table 1.

The initial values of the SSA of the protein emulsions were much higher than that of HPMC emulsions (Table 1), which is in agreement with their lower droplet sizes. Nevertheless, as the emulsions pass along the gastrointestinal tract (GIT), there are changes in the emulsion properties due to flocculation and/or coalescence that could affect the final interfacial area that is exposed to the lipase adsorption when arriving to the small intestine. These changes are illustrated below.

3.1.2. Emulsions under in vitro gastric conditions

Prior to the gastric digestion process, the fresh emulsions were mixed with the SGF (without pepsin and PC) for 1 h at 37 °C, to analyze the effect of the pH and the ionic strength. The emulsions underwent a slight change but the droplet size distributions remained almost unchanged (data not shown). This result indicates that the absence of pepsin and PC resulted in no significant effect on

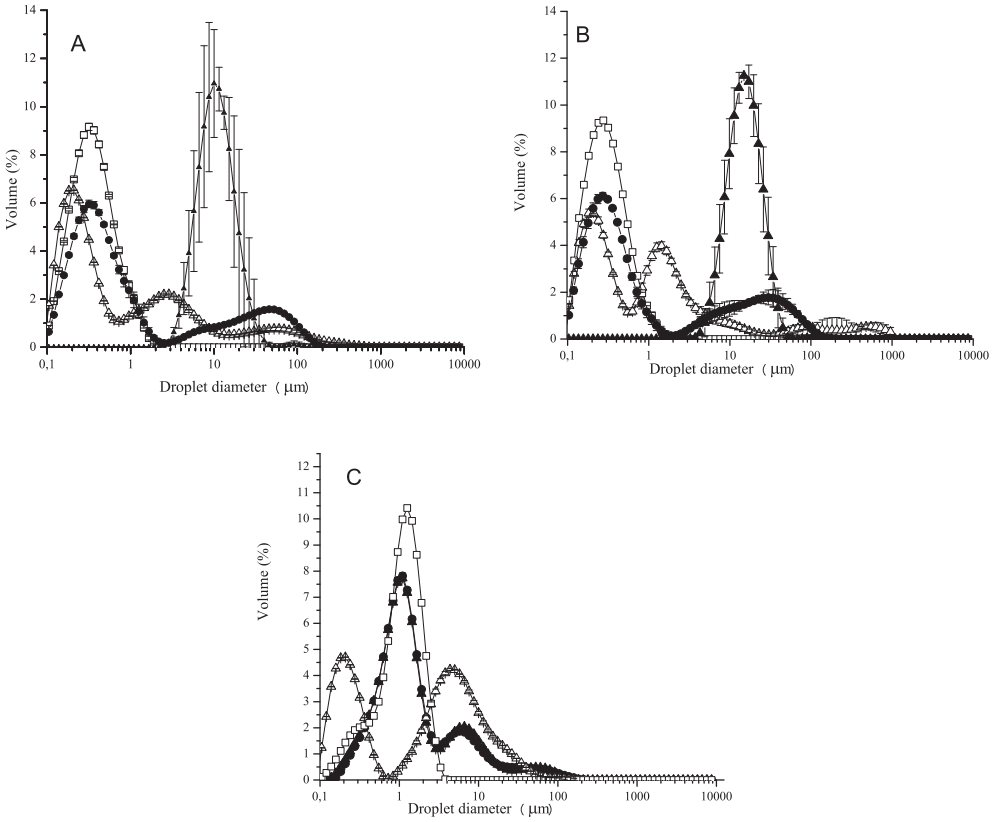

Fig. 1. Volume particle size distribution of oil in water emulsions stabilized by soy protein isolate (A), β-lactoglobulin (B) and HPMC (C), during the simulated gastrointestinal digestion. In all cases: Original emulsion (□), at the beginning of gastric stage (●), at the end of gastric stage ((▲), 60 min later) and at the end of intestinal stage ((Δ), 120 min later).

Table 1
Specific surface area (SSA) and mean droplet size (D_{32} and D_{43}) obtained for fresh emulsions (t_0), at the end of the gastric stage (t_{g60}), at 10 min of the duodenal stage (t_{d10}) and at the end of the duodenal stage (t_{d60}).

	Soy			β-lg			HPMC		
	SSA (m^2/g)	D_{32} (μm)	D_{43} (μm)	SSA (m^2/g)	D_{32} (μm)	D_{43} (μm)	SSA (m^2/g)	D_{32} (μm)	D_{43} (μm)
$\mathbf{t_0}$	17.5 ± 0.6	0.278 ± 0.001	0.531 ± 0.002	22.15 ± 0.07	0.277 ± 0.001	0.3895 ± 0.0003	7.56 ± 0.01	0.73 ± 0.08	1.19 ± 0.07
$\mathbf{t_{g60}}$	0.581 ± 0.008	7.4 ± 0.1	12.9 ± 2.9	0.41 ± 0.04	14.6 ± 0.1	18.3 ± 3.0	5.4 ± 0.6	1.01 ± 0.05	7.2 ± 0.9
$\mathbf{t_{d10}}$	9.97 ± 1.30	0.61 ± 0.08	11.7 ± 4.1	10.25 ± 0.63	0.57 ± 0.02	6.7 ± 0.9	7.07 ± 1.46	0.9 ± 0.1	13.3 ± 2.4
$\mathbf{t_{d60}}$	14.2 ± 2.6	0.43 ± 0.08	9.7 ± 0.8	12.9 ± 0.9	0.46 ± 0.03	34.6 ± 0.4	11.3 ± 0.1	0.53 ± 0.01	6.1 ± 0.3

the droplet size distributions. Therefore, the change of pH and ionic strength appeared not to affect the interfacial film of the emulsifiers. Similar effect was also observed by Malaki Nik et al. (2011) in o/w emulsions prepared with WPI and SPI, where they concluded that in the absence of enzymes and biosurfactants the changes in the emulsions were not significant. However, it has been reported, at an interfacial level, a significant effect in the interfacial parameters of β-lg film (o/w interface) due to the modifications in the ionic strength (Maldonado-Valderrama, Miller, Fainerman, Wilde, & Morris, 2010; Maldonado-Valderrama, Wilde, Mulholland, & Morris, 2012).

When the protein emulsions were mixed with SGF containing pepsin and PC, they were destabilized with time (Fig. 1 (A and B)) showing a bimodal droplet size distribution with a second peak ranging from 2 to 100 μm. Similar effects have been reported by Singh and Sarkar (2011) using β-lg as emulsifier. It was demonstrated that although β-lg solutions can resist the proteolysis (carried out by pepsin), the protein was susceptible when unfolded at the o/w interface (Mackie & Macierzanka, 2010; Singh & Sarkar, 2011). Fig. 3 shows the mean droplet size increment with the digestion time for protein emulsions. Nevertheless, in spite of having similar initial droplets sizes, the β-lg emulsions underwent a stronger coalescence/flocculation under the gastric digestion stage, reaching at the end a D_{32} value twice than that of soy protein emulsions (Table 1). There is an association between the emulsion stability upon gastric conditions and gastric emptying, with the fine emulsion having a slower rate of emptying relative to a coarse emulsion that may impact on the satiety (Golding & Wooster, 2010).

At the end of the gastric digestion (t_{g60}) monomodal particle size distributions were observed (Fig. 1 (A and B)) and the soy protein emulsion exhibited a D_{43} value almost twice the D_{32} value, which indicates a high degree of flocculation (Table 1). Optical microscopy showed that the oil droplets appeared to flocculate during the gastric stage even at t_{g0}, being the soy protein emulsion more flocculated (Fig. 2 (A and B)) (Kenmogne-Domguia, Meynier, Viau, Llamas, & Genot, 2012; Li, J. et al., 2012; Malaki Nik et al., 2011; Sandra, Decker, & McClements, 2008).

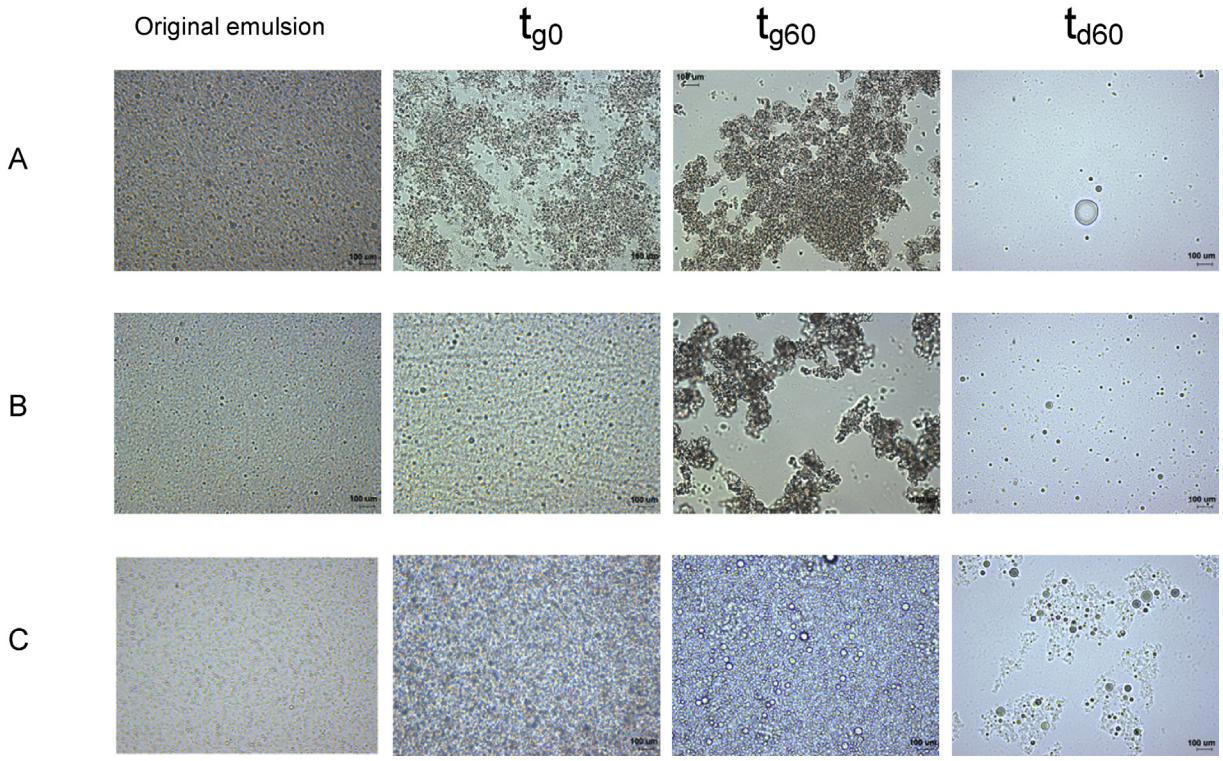

Fig. 2. Optical microscopy images before and during the digestion process (objective lens 400×) of emulsions stabilized by soy protein isolate (A), β-lactoglobulin (B) and HPMC (C).

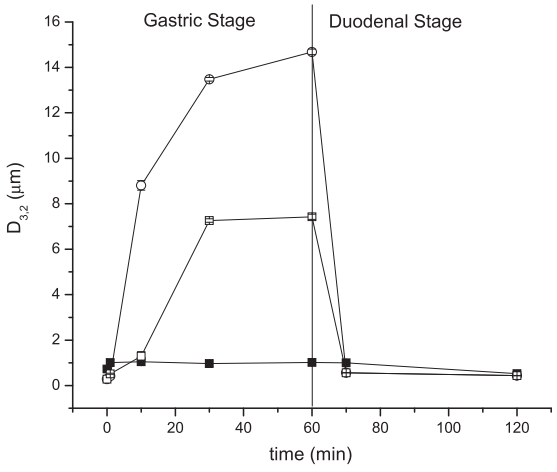

Fig. 3. Change in the average particle size (D_{32}) during the gastrointestinal process of emulsions stabilized by soy protein isolate (□), β-lactoglobulin (○) and HPMC (■).

These results agree with previous works which have reported that the size distribution of oil droplets shifted toward larger sizes during the gastric digestion due to the faster digestion of smaller oil droplets compared to larger droplets (Gallier, Tate, & Singh, 2013). This fact could indicate that the proteolysis of the interfacial layer promotes the formation of aggregates of oil droplets as it causes a gradual loss in the superficial charge of the oil droplets and reduces the thickness of the interfacial layer. Therefore the reduction of the electrostatic repulsion between oil droplets and the thickness of the interfacial layer promote the aggregation process (Li, Ye, Lee, & Singh, 2012; Malaki Nik et al., 2011; Sarkar et al., 2009; Singh & Sarkar, 2011; Tikekar, Pan, & Nitin, 2013).

The interfacial area of both protein emulsions at the end of the gastric stage was comparable, being that for soy protein emulsion slightly higher (Table 1).

Contrarily to the behavior of the protein emulsions, after few minutes of gastric digestion the HPMC-coated droplets showed a slight change in the particle size distribution which remained almost constant for the rest of the gastric digestion (Fig. 1C). Because of the lower number of ionizable groups reported for this HPMC (Camino & Pilosof, 2011) compared to these proteins and the fact that the pepsin has not effect on fats and carbohydrates (Gallaher, Hassel, Lee, & Gallaher, 1993; Mudgil & Barak, 2013; Slavin, 2003, 2005), it could be associated the slight changes observed in Fig. 1 with the interfacial activity of PC. The corresponding microstructures showed a small flocculation of the oil droplets (Fig. 2C) that is reflected in a small increase of the mean droplet size diameters (Fig. 3) at the end of the gastric stage. This result corroborates the fact that HPMC emulsion is more resistant under gastric conditions than protein emulsions. This lower degree of destabilization under the gastric condition presents a special interest, since it has been associated with the rate of gastric emptying delay, alterations in the release of the hormones involved in human digestion and consequently with the satiating effect (Malaki Nik, Wright, & Corredig, 2010; Marciani et al., 2009; Marciani et al., 2007).

The values of D_{32} during the gastric stage (Table 1) only increased from 0.73 to 1.01 μm, but the D_{43} value at the end of this stage (7.2 μm) indicated that the emulsion was flocculated as it can be seen in Fig. 2C. However, the extent of flocculation of HPMC emulsions was much lower than that for protein emulsions (Fig. 2 (A and B)). As a result of the small decrease of the droplets size for HPMC emulsions, a slight decrease of the SSA occurred from 7.56 m^2/g to 5.4 m^2/g, at the end of the gastric digestion (Table 1).

3.1.3. Emulsions under in vitro duodenal conditions

Large changes in the mean oil droplets size occurred when exposing the emulsions from the simulated gastric conditions to

the in vitro intestinal conditions. It can be observed in Fig. 1 that there were several changes in the oil droplets size diameter due to the environmental change: pH, ionic strength and the presence of biological components with interfacial activity (bile salts, enzymes, etc.) (Sarkar et al., 2009; Singh & Sarkar, 2011). It has been reported that the major changes during the duodenal stage could be attributed to the displacement of the original emulsifiers by the surface active substances, such as bile salts together with the interfacial proteolysis caused by the intestinal proteases. Fig. 1 shows that, at the end of intestinal stage, the particle size distributions of the protein emulsions became multimodal and there existed predominant peaks in the region of the smaller diameters (0.1–0.7 μm), which possibly occurred due to the formation of micelles and vesicles containing lipolytic products as monoglycerides and fatty acids that form micelles with bile salts. D_{32} values at the end of the duodenal digestion (Table 1) were similar for the emulsions (0.43–0.53 μm), as well as their final SSA values. Optical microscopy images emulsions (Fig. 2) showed that at the end of the duodenal stage some flocs still occurred. The reduced number of droplets could be attributed to the dilution that occurs when the SIF is incorporated (Hur, Decker, & McClements, 2009; Torcello-Gómez et al., 2011).

The presence of particles in the region of 10–100 μm is associated with the displacement or digestion of the emulsifiers, that were originally stabilizing the oil droplets, that promotes the coalescence or flocculation of the oil droplets (Gallier et al., 2013; Hur et al., 2009; Sandra et al., 2008). Furthermore, as mentioned in the literature, the increase in the pH from 2.5 to 7 causes several changes in the charge of the protein-coated droplet while passing through the isoelectric point, that promotes the flocculation process (Singh & Ye, 2013).

At the end of the digestion, the protein stabilized emulsions showed a higher degree of polydispersity (multimodal distributions) than the emulsion stabilized by HPMC (Fig. 1). It has been shown in recent studies that emulsion droplets undergo flocculation, dissociation and coalescence during the digestion with pancreatic lipase under the duodenal conditions (Singh & Sarkar, 2011; Ye, Cui, & Singh, 2010). The droplet aggregation may have occurred due to bridging flocculation caused by some component in the lipase extract that adsorbed to the surfaces of more than one lipid droplet. The optical images (Fig. 2) showed that some coalescence appeared to occur, which is probably due to the effect of the BS and the high ionic strength. The BS appeared to displace the original emulsifier (partially or fully) from the lipid droplets; however, the negative charge they impart to the oil droplet surface could be neutralized by the presence of cations from the intestinal solution (Ca^{+2}, Na^{+1}) that promotes the droplet flocculation and thereby the consequent coalescence (Bellesi et al., 2014; McClements, 2004). Torcello-Gómez et al. (2011) have reported that the presence of BS promoted droplet coalescence when using non-ionic surfactants in olive o/w emulsions.

3.2. Kinetics of FFA release

The influence of the emulsifier on the digestibility of lipid droplets was determined, as it was explained previously, by the neutralization of the free fatty acids (FFA) released during the simulated duodenal digestion because of the pancreatic lipase action. The consumed amount of NaOH during the digestion is related to the total amount of FFA released as described previously (Abrahamse et al., 2012; Li & McClements, 2010; Zhu, Ye, Verrier, & Singh, 2013).

Fig. 4 shows the profile of the released FFA with time for the different emulsions. There was a rapid release of FFA during the first 10 min and after this period, an almost constant value was reached.

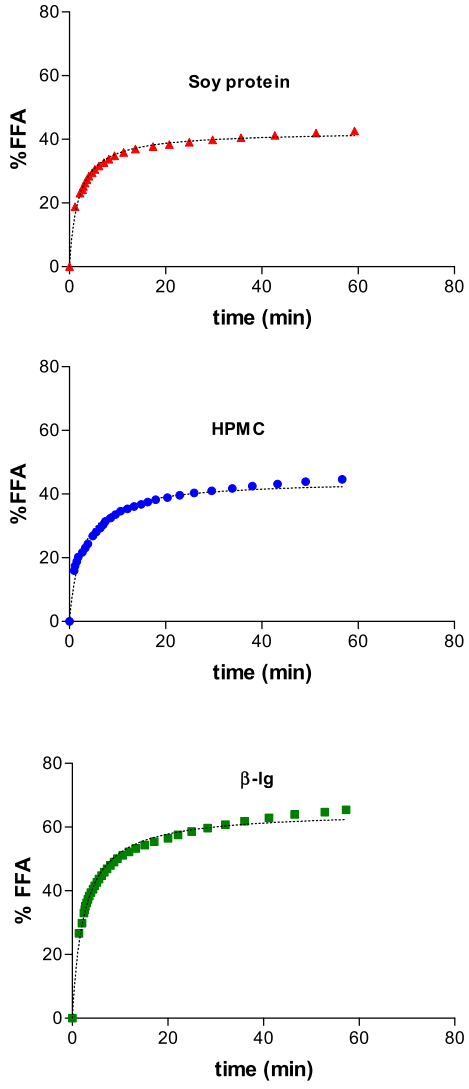

Fig. 4. Free fatty acid release (FFA) during the duodenal stage from emulsions stabilized by soy protein isolate (▲), β-lactoglobulin (■) and HPMC (●). Dot lines indicate the fitting curves.

As reported in the literature, when working at a high lipase concentration, the adsorption of lipase at the o/w interface occurs rapidly and consequently, the digestion process begins almost immediately after the lipase incorporation to the reaction vessel (McClements & Li, 2010; Mun et al., 2007). The slower rate of FFA release after 15 min of the digestion process could be associated with the accumulation of the lipolysis products at the droplets surface which could reduce the lipase activity. It has been demonstrated that the FFA molecules released have interfacial activity so they could compete with the lipase molecules in order to get adsorbed at the interface (Reis, Holmberg, Watzke, Leser, & Miller, 2009; Troncoso, Aguilera, & McClements, 2012).

The amount of FFA released during the digestion process over time (Fig. 4) was fitted with the following empirical model (Eq. (4)) in order to describe the kinetics of the FFA release:

$$\%\,\mathrm{FFA}(t) = \left[(\%\,\mathrm{FFA})_{max} * t\right] / (B + t) \quad (4)$$

where % FFA and $(\%\,\mathrm{FFA})_{max}$ are the % FFA released at the time t and at the "pseudo-equilibrium", respectively, and B is the time (min) needed to reach half the maximum % FFA [$(\%\,\mathrm{FFA})_{max}/2$], that is the

Table 2
Kinetic parameters describing the release of FFA for each emulsion with time using the empirical model developed by Pilosof et al. (1985).

Emulsifier	R^2	FFA_{max} (%)	B (min)	K_0^{FFA} (1/min)	$K^{FFA}*10^3$ (1/min)
β-lg	0.9890	65.14 ± 0.55	2.55 ± 0.09	25.6 ± 1.1	6.03 ± 0.22
HPMC	0.9742	44.36 ± 0.68	2.76 ± 0.19	16.4 ± 1.4	8.3 ± 0.6
Soy	0.9922	42.53 ± 0.36	1.97 ± 0.08	21.58 ± 1.05	11.9 ± 0.5

lipolysis half time. Equation (4) was able to fit the experimental data very well (Fig. 4), as it can be deduced from the R^2 values in Table 2, where the values of $(\%\ FFA)_{max}$ and B obtained are also included. The maximum FFA released ($(\%\ FFA)_{max}$) was similar for soy protein and HPMC emulsions, but the release of FFA for the β-lg emulsions was 50% higher (Table 2).

According to Pilosof, Boquet, and Bartholomai (1985), the initial rate of FFA release (K_0) and the overall digestion rate constant (K) can be calculated from the model by means of Eqs. (5) and (6):

$$K_0^{FFA} = (\%\ FFA)_{max}/B \tag{5}$$

$$K^{FFA} = \left((\%\ FFA)_{max}{*}B\right)^{-1} \tag{6}$$

The soy protein emulsion showed the highest overall rate of lipolysis K^{FFA} and the β-lg emulsion the lowest one (Table 2). A different information arose from the initial rate of FFA release (K_0) that increased as follows, β-lg > soy > HPMC. The β-lg emulsion that was initially hydrolyzed at the highest rate also produced a greater amount of FFA at the end of the duodenal digestion (FFA_{max}). Li et al. (2011), also found a positive correlation between the initial rate of lipid digestion and the amount of FFA released from a corn oil-in-water emulsion stabilized by β-lg. Nevertheless, soy and HPMC emulsions exhibited similar FFA_{max} despite having different initial rates of digestion (Table 2). This indicates that besides the influence of initial rates of lipid digestion, there are other factors that can affect the release of FFA, as follows.

3.3. Effect of the particle size/surface area

As the lipolysis is an interfacial reaction (pancreatic lipase has to bind to the o/w interface via complexation with BS and/or colipase) (Jódar-Reyes, Torcello-Gómez, Wulff-Pérez, Gálvez-Ruiz, & Martín-Rodríguez, 2010; McClements & Li, 2010), the size of the oil droplets within the small intestine should impact in their digestion rate, being more efficient the lipid hydrolysis when in presence of smaller oil droplets (higher surface area) (Armand et al., 1992; Bauer et al., 2005; Helbig et al., 2012; Li et al., 2011; McClements & Li, 2010; Singh & Ye, 2013).

It is important to point out that besides the D_{32} value of an emulsion is generally used to analyze the impact of the droplets size/area, the interfacial area is modulated by the possible flocculation of droplets, which is described by D_{43} values. Thus, soy and β-lg emulsions, which had the same initial D_{32} values, had different surface areas (Table 1) available for the lipase adsorption.

Therefore the surface areas (Table 1) were related to the rate constants describing the kinetics of FFA release (Table 2). It was found a correlation between the initial rate of FFA release (K_0^{FFA}) and the initial values of the SSA (Fig. 5). As it is shown in Table 1, the droplet sizes of protein emulsions and their SSA underwent many changes as they passed the gastric stage due to changes in pH, ionic environment and by the action of pepsin, which resulted in a strong increase of the droplet mean size and a concomitant decrease of the SSA. Despite this increase in the droplet size in the gastric stage mostly due to flocculation, the emulsions retained their smaller

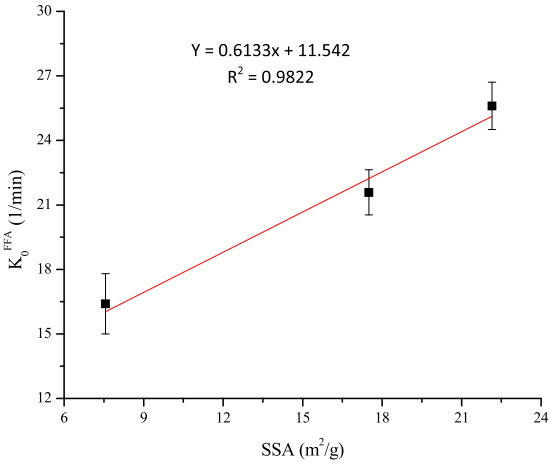

Fig. 5. Correlation between initial rate of lipolysis, K_0^{FFA}, and initial specific surface area, SSA, of fresh emulsions.

size under duodenal environment. Most of the aggregated emulsions in the stomach are re-dispersed when they pass through the antrum/pylorus to the duodenum (Golding & Wooster, 2010). Contrarily, HPMC stabilized droplets underwent small changes because of their non-ionic nature and undigestibility.

This result points out that irrespective of the emulsifier used, the initial interfacial area of the emulsions has a significant impact in the initial rate of lipolysis. This result is consistent with other in vitro digestion studies. Li and McClements (2010) reported an increase in the rate of lipid digestion with an increase of the droplets size of β-lg emulsions. Interestingly, this rate constant was expressed per unit time as the initial rate K_0^{FFA} in our model. Armand et al. (1999) investigated the effect of the fat globule size of emulsions on fat digestion in humans, concluding that a lower initial droplet size enhances lipolysis.

Regarding the maximum % FFA released from each emulsion, it was not correlated nor to the initial SSA of the emulsions, nor to the values of the SSA at the end of the gastric digestion (Table 1), thus pointing out that the structural characteristics of the interfacial films could be the key factor to explain the differences in the FFA release.

3.4. Effect of the composition/structure of the interfacial films

One of the key interfacial processes that regulate fat digestion in pancreatic lipolysis is the surfactant action of the BS (Golding & Wooster, 2010). One of the roles of the BS is to prepare the interface of the fat to improve the access of lipolytic enzymes to the lipid substrates. BS adsorbs and penetrate into the interfacial films to allow the colipase/lipase to adsorb at the o/w interface and to hydrolyze the lipids. Thus, the way an interfacial film reaching the small intestine is penetrated by the BS should be a key property as some emulsifiers could make more difficult the BS penetration and even their displacement by the BS from the interface, thus hindering the enzymes to adsorb at the interface and to access the lipid core in order to initiate the hydrolysis. Accordingly, the interfacial composition of the lipid droplets could ultimately determine the accessibility of fat mediated by the BS (Maldonado-Valderrama, Wilde, Macierzanka, & Mackie, 2011). Several studies have been made in the last years on the in vitro digestion of interfacial layers to get a better understanding of the BS action on protein or lipid stabilized interfaces. It has been proved that the interfacial structures formed by native β-Lg cannot resist the displacement by the BS. Also, it was shown that the mechanism of displacement was

orogenic, involving the growth of the BS domains (Maldonado-Valderrama et al., 2008).

The adsorption of BS to interfaces occupied by lipids appears to be strongly dependent on the lipid head group, how it interacts and packs at the interface and the degree of interaction with the BS. This fact determines how effectively BS can adsorb into lipid films and hence prepare the interface for lipolysis (Maldonado-Valderrama et al., 2011). AFM imaging studies (Chu et al., 2010; Maldonado-Valderrama et al., 2008) have shown that the addition of BS to phospholipid monolayers does indeed disrupt the structural organization at the interface, which may allow a better access for the lipolytic enzymes.

Li et al. (2012) suggested that the initial surface layer of sodium caseinate emulsions had only limited impact on the lipid digestion (as measured by FFA release) in the presence of BS, probably because BS, with their strong surface-active properties, displace the interfacial material from the droplets surface that are stabilized by either the initial protein or the products of its proteolysis. The rate of the lipids digestion was also decreased for chitosan-coated droplets (Mun et al., 2006). It was thought that the lecithin–chitosan interfacial complex provided a physical barrier that prevented lipase/BS from accessing the interface.

Recently, Bellesi et al. (2014) have observed in dynamic co-adsorption and sequential experiments that soy protein was more resistant than β-lg to BS interfacial displacement at the o/w interface and it could then compete with the BS for the interface. These facts could indicate a lower perturbation of the interfacial structure formed by soy proteins, which could difficult the ability of pancreatic lipase to access to the lipid surface and consequently it can explain the lowest number of FFA released from soy protein emulsions as compared to β-lg emulsions (Fig. 4).

Mun et al. (2007) have also shown that the digestion rate of emulsified lipids depend on the interfacial composition. The extent of the lipolysis was much greater for the protein stabilized emulsions than those stabilized by lecithin or Tween 20.

Another key role of the BS on the duodenal digestion is the removal of the lipolysis products from the interface. As the lipolysis progress there is an increase of fatty acids and monoglycerides at the emulsion interface. These products have high surface activity and tend to displace both gastric lipase and pancreatic lipase from the interface. Reis et al. (2008) found that 2-monoglycerides were the most surface active product of lipolysis followed by the fatty acids and diglycerides. Moreover, 2-monoglycerides were found to dominate the interface when present in high concentrations, meaning that they displace many surface active molecules from the interface of lipid emulsions, including the lipase molecules. The 2-monoglyceride appears to push the lipase into a sub-layer in the aqueous phase just beneath the monoglycerides covered interface. Lipolysis is therefore inhibited because the enzyme is no longer in contact with the lipid. BS and to a lesser extent phospholipids, remove these digestion products from the interface by solubilizing them in mixed micelles in the bulk aqueous phase. This facilitates further lipid digestion by removing these inhibitory surfactants and by driving the reaction equilibrium towards continued lipolysis (Golding & Wooster, 2010). The solubilization of the monoglycerides and fatty acids in micelles for their transport involves the desorption of these surface active molecules from the interface, which can be affected by the structure and composition of the interface. We can anticipate that an interface extensively disrupted by the adsorption of the BS should be prone to the interaction between monoglycerides or fatty acid and BS to form micelles detaching from the interface. In this scenario, the soy protein interface, that is more resistant to BS penetration (Bellesi et al., 2014) could be more rapidly saturated with the lipolysis products, thus decreasing the degree of FFA release in the in vitro duodenal digestion (Fig. 4). Contrarily, the β-lg interface that is totally disrupted by BS (Maldonado-Valderrama et al., 2008) will exhibit a delayed saturation, reaching a higher degree of lipolysis (Fig. 4).

Other factors that are related to the structure/composition of the protein stabilized interfaces can also influence the extent of lipolysis. One of them is how the interfacial proteolysis of soy and β-lg is modulated by the BS. It has been reported in previous works that the hydrolysis of some proteins, carried out by the pancreatic proteases, was enhanced by the presence of the BS. It has been suggested that BS destabilized the protein structure making it more susceptible to the proteolysis (Gass, Vora, Hofmann, Gray, & Khosla, 2007; Mackie, et al., 2010). Furthermore, the ability of the soy protein to interact with calcium (Canabady-Rochelle, Sanchez, Mellema, & Banon, 2009; Yuan et al., 2002) could further reduce the availability of calcium to remove FFA and consequently inhibit the lipase activity (Ye et al., 2013), thus yielding a lower extent of lipolysis than β-lg emulsion (Fig. 4).

Regarding the HPMC stabilized interfaces, it has been shown to be easily penetrated by the BS (data not shown), thus the lower extent of lipolysis observed (Fig. 4), similar to that for soy protein, should be attributed to its indigestible nature and non-ionic structure that results in small changes of the particle size and interfacial structure upon gastric digestion.

4. Conclusions

Fat digestion is controlled by the ability of lipase to bind to the emulsion interfaces which is controlled by the emulsion size or more precisely by the interfacial area and interfacial composition/structure (Golding & Wooster, 2010). Due to the complexity of the digestion events and the limited understanding of the behavior of oil–water interfaces upon the digestion conditions, as well as the desorption of the lipolysis products, it is difficult to explain the rate and extent of lipolysis by simple factors. It has been shown in this study that irrespective of the composition/structure of emulsions, the initial surface area is very important in determining the initial rate of lipolysis. However, as the lipolysis progress this factor is not able to explain the extent of lipolysis, because of important changes of the interfacial film as a result of the enzymes action and BS and phospholipids competition. The lower extent of lipid digestion of soy protein emulsion, as measured from the in vitro FFA release, could be related to the behavior of the soy protein interfaces that could resist the displacement carried out by the BS (Bellesi et al., 2014; Favé, Coste, & Armand, 2004). As a result, the adsorption of lipase would be less facilitated as well the desorption of the products of the lipolysis which is also mediated by the BS. Regarding HPMC emulsions, the lower extent of lipolysis seems to relay on its resistance to enzymatic hydrolysis and non ionic character. Ongoing studies will be presented to further support these findings.

Finally, the control of the structure of oil–water interfaces seems to be the key point to control the lipid digestion.

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