In vitro gastric lipid digestion of emulsions with mixed emulsifiers: correlation between lipolysis kinetics and interfacial characteristics

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Abstract

Emulsions stabilized by a mixture of emulsifiers may show a tailored functionality depending on the compatibility of the emulsifiers employed. This was proposed in a previous work dealing with distinct lipolysis kinetics of citrus pectin (CP) and Tween 80 (TW80) based emulsions. CP provided a fast a high extent of gastric lipid hydrolysis, TW80 presented negligible digestion and emulsions stabilized with mixtures presented intermediate lipolysis kinetics. The current research work aimed to investigate the interfacial mechanisms underlying gastric lipolysis to further understand the encountered behavior on a fundamental basis. This was achieved by monitoring the interfacial tension kinetics and measuring the interfacial rheology of interfacial layers during in vitro digestion using a modified pendant drop technique. This allowed the correlation of emulsions *in vitro* lipolysis kinetics and interfacial phenomena during digestion. Two extremely different interfacial tension kinetics were observed when single layers of CP versus TW80 and intermediate kinetics were observed for the binary interfacial layers, importantly correlating with results obtained with emulsions. These findings are discussed in detail and confirm that competitive adsorption between emulsifiers and lipase (e.g. orogenic displacement) is a key factor determining the lipolysis kinetics of emulsions stabilized by mixed emulsifiers.

Keywords

Gastric lipase; lipid digestion, orogenic displacement; interfacial tension; interfacial rheology, OCTOPUS.

1. Introduction

Oil-in-water (o/w) emulsions are widely spread among commercial food products such as milk, dressings, soups, sauces, but also among a range of nutraceutical and pharmaceutical products (Bouyer et al., 2012; McClements, 2018). Rational emulsion property design to obtain a particular functionality has been a recent trend in the field. Nutritionally speaking, the emulsion lipid phase contributes with the energy and essential fatty acids provision for our body. Additionally, emulsions allow the encapsulation, protection and transport of lipophilic compounds, tailoring the delivery inside the gastrointestinal tract (Corstens et al., 2017). In this aspect, interfacial engineering of o/w emulsions gained increasing interest in recent years in the food science and technology field. Their interfacial initial properties often determine their physicochemical stability and/or susceptibility to enzymatic degradation in the gastrointestinal tract (Troise et al., 2020).

The emulsion interface needs to be thermodynamically stabilized, otherwise the dispersed oil droplets undergo different instability phenomena (e.g. flocculation, coalescence, Ostwald ripening). For this reason, emulsifiers are essential emulsion ingredients normally added to stabilize the oil-water interface. These emulsifiers can be broadly classified as low-molecular-weight surfactants (e.g. Tweens, phospholipids, monoglycerides, and others) and amphiphilic biopolymers (e.g. β-lactoglobulin, pectin, and others) (Berton-Carabin & Schroën, 2019). O/w emulsions can be stabilized by a single type of emulsifier. Nevertheless, the physicochemical stability and digestive functionality of emulsions can be fine-tuned by combining emulsifiers, instead of using individual ones (Dickinson, 2011; McClements & Jafari, 2018). Moreover, mixes of emulsifiers are normally employed in more complex emulsified food products.

The emulsion interfacial properties govern the adsorption of lipases to the oil-water interface, and therefore, determine the lipid digestion kinetics (Berton-Carabin & Schroën, 2019). Three main phenomena are impacted by the nature of the surface-active compounds adsorbed to the interface: the emulsion physical stability, competitive adsorption between emulsifiers and lipases, as well as the potential digestion phenomena taking place at the interface (Singh & Ye, 2013). The first phenomenon is intrinsically related to the interface. Depending on the nature of the emulsifier stabilizing the interface, this emulsifier may

undergo physicochemical changes due to digestive conditions (e.g. acidity, ionic strength) leading to its desorption from the interface and further emulsion destabilization (Verkempinck, Salvia-Trujillo, Moens, et al., 2018). This emulsion instability induces changes in the available surface for lipase adsorption which impacts the kinetics of lipid digestion. The second phenomenon linked to the emulsion interface is the competitive adsorption between emulsifiers and lipases. If the emulsifiers are strongly adsorbed to the interface, the access of lipases to the interface can be hindered or even completely inhibited (Scheuble et al., 2016). In this aspect, there is limited information regarding the impact of emulsions stabilized by a mix of emulsifiers on their behavior along the gastrointestinal tract. Only few studies assessed the lipid digestion kinetics of emulsions stabilized by mixed emulsifiers and none of them in the gastric compartment (Dickinson, 2011; Klinkesorn & McClements, 2009; Li & McClements, 2014; Wulff-Pérez et al., 2010). A third phenomenon linked to the interfacial composition is the potential hydrolysis of the interfacial layer by digestion enzymes (Singh et al., 2009). This may occur to surface-active compounds such as proteins, digestible carbohydrates (e.g. modified starches), and/or digestible lipids (e.g. lecithin, mono- and diglycerides), but not to other compounds such as tweens or fibers like pectin. The cleavage of these compounds at the interface mainly affects the emulsion physical stability.

In this aspect, there is a research opportunity to describe lipolysis in the gastric phase. Diverse *in vivo* implications may be triggered by gastric lipid digestion**.** Gastric lipolysis, although limited, has been suggested to stimulate pancreatic lipid digestion by causing droplet disruption, increasing the solubilization of digestion products, promoting hormone release and augmenting colipase (coenzyme) binding capacity of pancreatic lipase (McClements, Decker, and Park 2008). Lipid digestion in the stomach is also highly relevant for people suffering of exocrine pancreatic insufficiency (low pancreatic enzymes production) (Layer & Keller, 2005). Moreover, gastric lipase is an essential enzyme for infant lipid digestion since their pancreas is not yet fully developed at birth, resulting in a relatively low secretion of pancreatic lipase, while on the contrary, their HGL expression is already fully mature (Bourlieu et al., 2014; Lindquist & Hernell, 2010).

Lipid digestion is considered an interfacial phenomena as lipases have to adsorb at the oil-water interface in competition with the emulsifiers in order to access the oil and hydrolyze triacyclglycerols into fatty acids and monoglycerides. Thus, interfacial changes in emulsion along the gastrointestinal tract play a very important role during lipid digestion (Maldonado-Valderrama, 2019). Two important interfacial properties that can be monitored during *in vitro* digestion are the interfacial tension and interfacial rheology. These properties are indicators of the interactions between the initially adsorbed emulsifiers, bile salts and lipases. One important interaction among these elements is the displacement of emulsifiers by bile salts and/or lipases. Most lipid digestion studies have characterized interfacial layer changes during the small intestinal phase since it is the compartment where lipids are majorly digested (Aguilera-Garrido et al., 2021; Corstens et al., 2017; Macierzanka et al., 2009; Maldonado-Valderrama et al., 2013; Wei et al., 2021). This work shows for the first time, mixed interfacial layers composed of a small surfactant and biopolymer subjected to *in vitro* gastric digestion in presence of a relevant substitute of gastric lipase. In the gastric phase, only one study have monitored the interfacial tension kinetics during *in vitro* gastric lipid digestion (Scheuble et al., 2016). Therefore, there are research opportunities to obtain insights on the interactions between emulsifiers and gastric lipase.

In our previous work, diverse emulsions stabilized by a single emulsifier were subjected to *in vitro* gastric digestion (Infantes-Garcia, Verkempinck, Gonzalez-Fuentes, et al., 2021). From these experiments, citrus pectin (CP)- and Tween 80 (TW80)-based emulsions showed distinct physical stability and lipolysis kinetics under simulated gastric conditions. Substantial amount of research has demonstrated the interfacial activity (capacity to adsorb onto oil-water interfaces) of certain types of pectin (Humerez-Flores et al., 2021, 2022; Neckebroeck et al., 2020; Verkempinck, Kyomugasho, et al., 2018; Verkempinck, Salvia-Trujillo, Denis, et al., 2018; Wei et al., 2021). More specifically, the CP-based emulsion exhibited a moderate physical stability and high lipolysis extent, while the TW80-based emulsion a high stability but negligible lipolysis extent. For that reason, we mixed these two stabilizing agents with the objective of forming emulsions with both of them adsorbed to the interface exhibiting a complementary functionality.

In our most recent work, the *in vitro* gastric lipolysis behavior was modulated by emulsions with mixed interfaces (Infantes-Garcia et al., 2022). A competitive adsorption phenomenon was hypothesized to explain the detected lipolysis kinetics modulation. Accordingly, the objective of this research was to specifically design and *in vitro* digest interfaces stabilized by single versus mixed interfaces, and correlate their interfacial tension behavior with the lipid digestion trends observed in our previous studies. Section 3.1 of the results and discussion part compiles and merges the most interesting findings of our two previous studies regarding some emulsion physical stability indicators and *in vitro* gastric lipolysis behavior. Section 3.2 discusses the interfacial characterization of single and mixed interfacial layers during *in vitro* gastric digestion in which a correlation with the lipolysis kinetics is also included. These interfacial layers were characterized in terms of interfacial tension kinetics and interfacial dilatational rheology, which allowed us to obtain insight into the competitive adsorption phenomenon. These interfacial layers were characterized in terms of interfacial tension kinetics and interfacial dilatational rheology, which allowed us to obtain new insight into the composition of the interface and the interfacial interactions.

2. Experimental methods

2.1 Production of o/w emulsions

Triolein (> 99 %, Acros Organics, Geel, Belgium) was utilized as oil phase since different commercial plant-based oils such as olive, canola or sunflower oil are rich in this triglyceride (Karupaiah & Sundram, 2007). Citrus pectin (CP, degree of methylesterification ≥ 85 %, Sigma-Aldrich, Diegem, Belgium) and Tween 80 (TW80, Sigma-Aldrich, Diegem, Belgium) were utilized as emulsifying agents. Since one of the objectives in this research was to form emulsions with mixed interfaces, the difference in surface activity of CP and TW80 should be considered as interesting rationale for their combined selection. For instance, it is well known that large molecules, like CP, adsorb at a slower rate than small surfactants such as TW80 (McClements, 2016). For this reason, it was decided to follow a post-homogenization procedure to prepare the emulsions with mixed interfaces. Firstly, an emulsion stabilized by CP was prepared, and secondly, this

emulsion was mixed with a TW80 solution. This strategy permitted the formation of mixed interfacial layers without the total desorption of CP (McClements & Jafari, 2018).

The coarse CP emulsions were produced with a high-shear mixer (Ultra-Turrax T25, IKA, Staufen, Germany) at 13500 rpm for 5 min, to obtain emulsions consisting of triolein (10 % w/w), CP (2 % w/w) and ultrapure water (88 % w/w). Afterwards, the coarse emulsions were homogenized in a high-pressure homogenizer (Stansted Fluid Power, Pressure cell homogenizer, U.K.) at 100 MPa (one cycle). Next to the homogenization step, each CP emulsion was mixed with one of the two TW80 solutions in a ratio 1:1 resulting in contents of 5% of triolein, 1% of CP, and TW80 concentrations of 0.00625 or 0.1 %. The level of 1% CP was chosen because it generated a stable emulsion without significantly increasing the bulk viscosity (Verkempinck, Salvia-Trujillo, Denis, et al., 2018). These mixtures were gently stirred for 1 h. For the emulsions stabilized by a single emulsifier (CP or TW80), the same mixing and homogenization conditions explained above were applied. The pH of all emulsions containing CP was around 2.7, while the TW80 emulsion exhibited a pH around 6.8. On the one hand, the low pH of the emulsions containing CP was due to the acid extraction process of pectin. On the other hand, TW80 production process does not require strong acids or alkalis, and thus the pH of the emulsion was close to neutrality. Even if these values are largely different from each other, during *in vitro* gastric digestion, the pH is adjusted to 3. At that pH, CP emulsions present a relatively good physical stability, while TW80 is unaffected by acid pH values.

2.2 *In vitro* gastric digestion of the generated emulsions

The static *in vitro* INFOGEST protocol was employed to analyze the kinetics of gastric lipolysis of the generated emulsions (Brodkorb et al., 2019). In this case, a scaled-down version of the INFOGEST protocol was employed exactly as described in our previous work (Infantes-Garcia, Verkempinck, Gonzalez-Fuentes, et al., 2021). In this aspect, a gastric lipase activity of 60 U/mL (tributyrin-based) was set in the gastric phase performed at pH 3 (Grundy et al., 2021). The gastric lipid digestion kinetics were evaluated by digesting independent samples (tubes) as a function of time (5; 10; 15; 30; 45; 60; 90; 120 min after

156 enzyme addition). Lipolysis was stopped through chemical inhibition (10 μL of Orlistat 100 mM in
157 ethanol).

158 2.3 Monitoring of oil droplet characteristics during *in vitro* gastric digestion

159 Additional chyme samples were generated at 4 different gastric digestion moments (after 15; 30; 60 and
160 120 min in the gastric phase) in an independent experiment and their oil droplet characteristics were
161 determined (i.e. particle charge, microstructure, and particle size).

162 **Microstructure:** Microstructural visualization of oil droplets was carried out using an optical microscope
163 (Olympus BX-41) fitted with an Olympus XC-50 digital camera (Olympus, Opticel Co. Ltd., Tokyo, Japan)
164 at 40x magnification (Infantes-Garcia et al., 2020).

165 **Particle size:** The particle size of all samples was determined employing a laser diffraction equipment
166 (Beckman Coulter Inc., LS 13 320, FL, USA). All equipment parameters were set as explained in our
167 previous work (Infantes-Garcia, Verkempinck, Gonzalez-Fuentes, et al., 2021).

168 **Particle charge:** The ζ-potential of oil droplets was measured using a dynamic light scattering
169 electrophoresis equipment (Zetasizer NanoZS, Malvern Instruments, Worcestershire, UK). Emulsions or
170 chyme samples were diluted in a ratio 1:10 with pure ultrapure water or ultrapure water adjusted to pH 3,
171 respectively (Infantes-Garcia, Verkempinck, Gonzalez-Fuentes, et al., 2021).

172 2.4 Analysis of lipid digestion products

173 An HPLC-charged aerosol detector method was employed to quantify triolein (TAG) and its hydrolysis
174 products diolein (DAG), monoolein (MAG) and oleic acid (FFA). Lipids were extracted and quantified
175 according to our previous work which is briefly described below (Infantes-Garcia, Verkempinck, Guevara-
176 Zambrano, et al., 2021).

177 2.4.1 Extraction of lipids

In sum, 1 mL of sample was placed in a glass tube together with 0.2 mL sulphuric acid (2.5 M), 2 mL ethanol, and 3 mL diethylether:heptane (DEE:H=1:1). The tube was vortexed for 2 min, followed by an end-over-end rotation at 15 rpm for 30 min. Afterwards, the upper non-polar layer was collected in a 5 mL volumetric flask. A second extraction was done by addition of 1 mL of DEE:H into the previous tube, vortexed for 2 min, and mixed in an end-over-end rotator at 15 rpm for 15 min. The upper layer was collected in the same volumetric flask, which was brought to 5 mL by adding DEE:H. The lipid extract was filtered (Chromafil PET filters, 0.20 µL pore size, 25 mm diameter) and stored at −80 °C until analysis.

2.4.2 Chromatographic lipid quantification

The extracted lipids were injected in an HPLC system (Agilent Technologies 1200 Series, Diegem, Belgium) fitted with a silica column (Chromolith Performance Si, 100–4.6 mm, Merck, Darmstadt, Germany) preserved with a guard column (Merck, Darmstadt, Germany). An external oven (Chromaster 5310, VWR, Hitachi Ltd., Tokyo, Japan) was used to keep the column temperature at 40 °C. Lipids species were separated by a quaternary gradient: isooctane (solvent A), acetone:ethyl acetate (2:1 v/v) containing 0.02% (v/v) of acetic acid (solvent B), isopropanol:water (85:15 v/v) containing 0.5% (v/v) of acetic acid and triethylamine (solvent C), and isopropanol (solvent D). Detection was performed in a charged aerosol detector (Corona Veo, Thermo Fisher Scientific, Geel, Belgium), operated with a gas pressure of 5.5 bar and evaporator temperature of 35 °C. Calibration curves were constructed using lipid standards and employed to quantify lipolysis products.

Glycerol (GLY) and residual triolein were calculated per digestion time as indicated by (Infantes-Garcia et al., 2020). In sum, a molar balance based on the sequential lipolysis reactions sequential starting from triolein was performed to calculate the residual triolein and glycerol.

2.5 Interfacial characterization

In a separate experiment, the characterization and *in vitro* digestion of single versus mixed interfaces composed of CP and/or TW80 was performed using the OCTOPUS: a pendant drop film balance equipped

with a multisubphase exchange system which is described in detail elsewhere (JMaldonado-Valderrama et al., 2013; Maldonado-Valderrama, 2019). In this equipment, a single aqueous solution droplet was formed at the tip of a double capillary directly immersed in the oil phase, kept in a heated (37 °C) cuvette (Hellma®). The OCTOPUS allows the online measurement of the interfacial tension kinetics and the interfacial dilatational rheology as surface-active compounds adsorb onto the oil-water interface. The software packages DINATEN© and CONTACTO© were employed to measure the interfacial tension and interfacial dilatational rheology data, respectively. Images of the drop were taken as function of time by a charge-coupled camera (Pixelink®)Experimetal drop profiles were extracted and fitted to the Young-Laplace equation to determine the volume, surface tension, and the interfacial area of the pendant drop through Axisymmetric Drop Shape Analysis (ADSA). The equipment uses a fuzzy logic area control to maintain the interfacial area constant during the experiment. After stabilizing, or *in vitro* digesting, the interfacial layer at constant interfacial area (20 mm^2), volume oscillations of the drop were applied to evaluate the dilatational viscoelasticity of the interfacial layer. The dilatational modulus (*E*) of the interfacial layer is defined by a real and an imaginary component:

$$E = E' + iE'' = \varepsilon + i\eta\nu \quad (1)$$

In equation (1), the term *E'* is the storage modulus that accounts for the interfacial elasticity (ε), while *E''* is the loss modulus accounting for the interfacial viscosity (η) of the adsorbed layer and ν is the angular frequency of the oscillation. The oscillation amplitude was maintained below 5% variation to assure linear regime and the oscillation frequency was set to 0.1 Hz. At this relatively high frequency (0.1 Hz), the dilatational rheology will be mainly elastic and the dilatational modulus can be approximated by the storage modulus or interfacial elasticity as the viscosity or loss modulus can be neglected (results not shown) (Maldonado-Valderrama et al., 2005). The dilatational elasticity contains information about the inter- and intramolecular associations at the interfacial layer and provide information on the composition of the interfacial layer (Aguilera-Garrido et al., 2021). Also, at 0.1 Hz, the dilatational response highlights differences between proteins, who develop a cohesive layer with high elasticity and surfactants, which

develop a more mobile and fluid interface with lower *E*, also allowing comparison with other experimental works (Maldonado-Valderrama & Patino, 2010).

In the OCTOPUS, the normal capillary is substituted by a double capillary which allows simultaneous injection and extraction of liquid from the droplet (Cabrerizo-Vílchez et al., 1999). This allows a subphase exchange of the bulk and simulating the digestive process in a single droplet, as explained in detail elsewhere (Maldonado-Valderrama et al., 2013; Maldonado-Valderrama, 2019). Single and mixed interfacial layers were formed and subjected to *in vitro* gastric conditions through subphase exchange of the droplet bulk solution with gastric media.

The oil phase was high-oleic sunflower oil (~70% oleic acid), kindly donated by Vandemoortele (Ghent, Belgium). It was employed in this independent experiment due to technical reasons. However, this oil presents a very high triolein content which makes it suitable as a replacement of pure triolein. This oil was previously purified with Florisil resins (oil:Florisil 2:1) reaching an interfacial tension of ~30 mN/m in ultrapure water (Maldonado-Valderrama et al., 2013). In case of the single interfacial layers, either 1% CP or TW80 solutions were utilized to form drops and allow the adsorption of the surface-active compounds. For the mixed interfacial layers, a two-step interfacial layer formation was followed by first forming a drop containing 1% CP, permitting the biopolymer to adsorb. Afterwards, solutions containing 0.00625 or 0.1% TW80 were pumped and replaced the droplet bulk solution allowing the formation of a mixed interfacial layer. After stabilization of these single or mixed interfacial layer, they were subjected to *in vitro* gastric digestion by replacing again the bulk solution with simulated gastric media by means of subphase exchange. We intended to simulate the digestive conditions as close as possible as in the *in vitro* digestion protocol for o/w emulsions previously described. For this purpose, a simulated gastric fluid (SGF) adjusted to pH 3 containing electrolytes and a rabbit gastric extract (60 U/mL tributyrin-based) was utilized as recommended by the international network INFOGEST (Brodkorb et al., 2019). The *in vitro* gastric digestion of the interfacial layers was carried out for 2 hours at constant interfacial area of 20 mm^2 (by slightly varying the droplet volume). As a final step, the droplet bulk solution is exchanged with water to force desorption of

emulsifiers and soluble products of digestion. This exchange depletes the bulk solution of material leaving a mixture of lipophilic digestive products at the interface and undigested emulsifier. In the case of TW80, the SGF contained also enough concentration of TW80 to avoid spontaneous desorption owing to depletion of TW80 in the bulk solution. A final desorption step consisting of subphase exchange of droplet bulk solution by a simulated gastric fluid, which depletes the bulk of soluble products of lipolysis and allows evaluating the presence of lipolytic digestive products at the interface (Maldonado-Valderrama, 2019).The interfacial tension was simultaneously monitored throughout the whole simulated digestion and the interfacial rheology was measured at the end of each adsorption/desorption step.

2.7 Statistical analysis and modeling

2.6.1 One-way ANOVA and comparison test

The oil droplet size, ζ-potential during *in vitro* gastric digestion and interfacial properties were statistically compared with one-way ANOVA and Tukey HSD comparison analyses ($P < 0.05$) using the statistical software JMP (JMP pro14, SAS Institute Inc., Cary, NC, USA).

2.6.2 Single-response kinetic modeling

Single-response modeling was employed to compare the lipid hydrolysis behavior of emulsions stabilized by single versus mixed interfaces. The selected response for this analysis was the lipid hydrolysis percentage (%HYD), which refers to the number of cleaved ester bonds with respect to the total number of ester bonds available. This response is described in equation (2):

$$\%\mathrm{HYD}(t) = \frac{n_{FFA(t)}}{(n_{TAG(t)} \times 3 + n_{DAG(t)} \times 2 + n_{MAG(t)} + n_{FFA(t)})} \quad (2)$$

%HYD(t) is the hydrolysis percentage as a function of gastric digestion time t; $n_{FFA(t)}$, $n_{TAG(t)}$, $n_{DAG(t)}$, and $n_{MAG(t)}$ are the number of moles of oleic acid, residual triolein; diolein, and monoolein, respectively.

Data sets were analyzed by means of the software JMP (JMP pro14, SAS Institute Inc., Cary, NC, USA). After a model discrimination process, the best fit was obtained for the modified Gompertz equation (Zwietering et al., 1990), which is indicated below in equation (3).

$$\%\mathrm{HYD}(t) = \mathrm{HYD}_f \exp\left\{-\exp\left[\frac{k\exp(1)}{H_f}\left(t_{lag} - t\right) + 1\right]\right\} \quad (3)$$

The parameter %HYD(t) is the percentage hydrolyzed lipids at a time t (min); HYD_f (%) is the extent of the hydrolyzed lipids ($t = \infty$); k (min^{-1}) is the reaction rate constant; and t_{lag} (min) is the lag time prior lipid hydrolysis.

3. Results and discussion

3.1 Oil droplet properties and *in vitro* gastric lipid digestion of emulsions formed with CP and/or TW80

All prepared emulsions were characterized in terms of oil droplet properties (particle charge, microstructure, and particle size) and lipolysis product content.

3.1.1. Oil droplet properties

The CP-based emulsion (pH ~2.7) presented an initial negative ζ-potential (-20.1 mV). This negative magnitude is possibly linked to the presence of ionized carboxylic groups in the CP molecule (Ngouémazong et al., 2015). The ζ-potential of CP-based emulsion changed to slightly positive values at the end of gastric digestion. This is possibly due to charge neutralization of pectin and the presence of ionized fatty acids generated as a result of lipolysis. The CP-based emulsion exhibited an increase in average particle size from 1.1 to around 4 μm (Figure 1). The micrographs in Figure 1 revealed that a certain extent of flocculation and coalescence led to this increase in particle size. In case of the TW80-based emulsion, a neutral particle charge was expected due to the non-ionic nature of TW80. However, it presented a slightly negative particle charge of -1.4 mV. This magnitude possibly reflected a certain extent

of contamination by fatty acids impurities (Chang & McClements, 2016). When the TW80-based emulsion was subjected to *in vitro* gastric digestion, the ζ-potential did not significantly change which may be an indication of a limited production of fatty acids. The physical stability of the TW80-based emulsion did not change during *in vitro* digestion (Figure 1).

In case of the emulsions stabilized by mixed interfaces, the ζ-potential values laid in between the ones of emulsions stabilized by CP or TW80 (Table 1). In this aspect, an inverse relation can be observed between the TW80 concentration and the particle charge. This inverse relation between particle charge and TW80 concentration might be an indication of the increased presence of TW80 at the interface due to CP displacement. Therefore, interfaces containing both CP and TW80 at different proportions were possibly created. This latter hypothesis will be further discussed in Section 3.2 (interfacial study). The magnitude of the ζ-potential for the emulsion containing 0.00625% TW80 retained a more negative value after gastric lipolysis compared to the one of 0.1% TW80. This could be due to the formation a mixed layer composed of TW80 compressing the interfacial network formed by CP which remained to a large extent at the interfacial layer. Conversely, the magnitude of the ζ-potential for the emulsion containing 0.1% TW80 diminished to a similar value to the one obtained after gastric lipolysis of the emulsion stabilized by TW80 only (Table 1). This is indicative for the predominant presence of TW80 at the interface even when mixed with CP. In this latter case, CP seemed to be largely displaced from the emulsion interface. Emulsions stabilized by mixed interfaces showed an improved physical stability compared to a CP-based emulsion (Figure 1). This phenomenon most likely occurred because TW80 is a non-ionic surfactant. Therefore, TW80 molecules did not present electrostatic interactions with the ionic CP molecules allowing compatibility between both surface-active compounds (Guzmán et al., 2016).

3.1.2. *In vitro* gastric lipolysis behavior

The percentage of lipid hydrolysis was utilized to compare the *in vitro* digestion behavior of the generated emulsions by means of single-response modeling (modified Gompertz equation). The representation of the single-response modeling is given in Figure 2, while the estimated parameters are presented in Table 2.

Single-response modeling was not applied to the TW80 data set because it showed a static trend and very low extent of lipid hydrolysis.

The lag time t_{lag} is defined as the period before the response starts to significantly increase in magnitude. For the emulsions stabilized by only CP, and by CP and a 0.00625% TW80; this lag time was limited (Figure 2) as values between 7.7 and 8.2 min were estimated by single-response modeling (Table 2). This suggests that the interfaces stabilized by the previous single or combined compounds did not represent a barrier for the (rapid) adsorption of gastric lipase allowing lipid cleavage from the first minutes of digestion. In Figure 2, it can be observed that the emulsion containing 1% CP and 0.1% TW80 presented a considerable lag phase with an estimated duration of 16.8±2.1 min. This finding may be related to the higher interfacial concentration of TW80. TW80 is known to be a surfactant with a high surface activity, thus gastric lipase probably competed for the interface with CP molecules. Therefore, we hypothesize that the lag phase might be correlated to the competitive adsorption phenomenon between lipase and CP molecules since TW80 molecules cannot be desorbed by gastric lipase.

The reaction rate constant *k* indicates the maximum rate with which the final extent of digestion is obtained. In this aspect, there was a clear effect on this kinetic parameter if a high TW80 concentration (0.1%) is used during emulsion preparation. As observed in Table 2, the *k* magnitude for this latter emulsion (0.19 min^{-1}) was significantly lower compared to the other two emulsions (0.86 min^{-1}). The rate constant is highly influenced by amount of gastric lipase adsorbed to the interface (McClements, 2018). Therefore, the emulsion containing 0.1% TW80 probably permitted a lower interfacial concentration of gastric lipase as compared to the other emulsions. As explained above, a higher concentration of TW80 at the emulsion interface most probably caused a stronger competitive adsorption phenomenon between gastric lipase and the initial emulsifiers. As a consequence, a lower lipase interfacial concentration was most likely reached for this emulsion.

Regarding the extent of lipid hydrolysis HYD_f, this kinetic parameter was also affected by the TW80 concentration in the initial emulsion. A high concentration of this non-ionic surfactant provoked a lower

extent of gastric lipid hydrolysis. During gastric digestion, a product inhibition phenomenon can limit the lipase action through the entrapment of the enzyme by lipolysis products (Pafumi et al., 2002). When a certain critical concentration of lipolysis products is reached, these products surround gastric lipase and restrict the access to the triglyceride core. Since the emulsion interface formed with 0.1% TW80 possibly permitted a low degree of gastric lipase adsorption, gastric lipase was entrapped faster because a lower concentration of lipolysis products was needed. Therefore, the lipid hydrolysis extent for this emulsion was much lower compared to the emulsions with no or low amounts of TW80. In case of the TW80-based emulsion, a negligible extent of digestion was obtained probably because this surfactant formed a compact interfacial layer which restricted gastric lipase adsorption as also observed by other researchers (Couëdelo et al., 2015; Infantes-Garcia, Verkempinck, Gonzalez-Fuentes, et al., 2021).

Since particle size was similar for all emulsions containing CP during *in vitro* gastric digestion, then emulsion physical stability was not the predominant factor impacting the lipolysis kinetics. Small differences in initial particle size are there but that these level of differences have not been linked to different digestion kinetics before (Infantes-Garcia et al., 2022). At this point, we can hypothesize that competitive adsorption between gastric lipase and the surface-active compounds at the interface possibly played a crucial role in the modulation of the lipolysis kinetics. Therefore, it is necessary to study the lipid digestion from an interfacial perspective. This interfacial approach is explored in the following section 3.2 to prove the intermediate hypotheses established in this section.

3.2 Interfacial study of single versus mixed interfaces during *in vitro* gastric digestion

As previously discussed, the lipolysis kinetics during *in vitro* gastric digestion could be modulated by designing emulsions stabilized by CP and TW80 at different ratios. This second part aims to obtain insights into the interfacial phenomena taking place during *in vitro* gastric digestion. In this aspect, a mixed interface composed of CP and TW80 has been created and subjected to *in vitro* gastric digestion. The OCTOPUS equipment allowed the evaluation of the adsorption kinetics and interfacial rheology by means of a modified

pendant drop technique. In this equipment, a water drop containing the compound(s) of interest is formed inside the oil phase. First, interfaces were formed using a similar approach as for the emulsions previously evaluated: Single interfaces of CP or TW80 were formed in one step. Mixed interfaces in two sequential steps by saturating the interface with CP and then allowing TW80 adsorption. Second, the formed interfaces were exposed to *in vitro* gastric conditions including a relevant gastric lipase source (i.e. rabbit gastric extract).

3.2.1 Formation of interfaces

This sub-section describes the process of single and mixed interfacial layers formation and its characterization in terms of interfacial tension and interfacial rheology.

Single interfaces: Figure 3A-B shows the adsorption kinetics of CP (blue experimental points) and TW80 (orange experimental points), respectively. Both compounds were able to decrease the interfacial tension of the oil-water interface (initial value of 30 mN/m) as depicted in Figure 4A. However, it can be observed in Figure 3 that the biopolymer CP has a slower adsorption rate compared to the surfactant TW80. In addition, TW80 reduced the interfacial tension to lower values (6 mN/m) than CP (15 mN/m). These findings are coherent since the CP (60 kDa) is a larger molecule compared to TW80 (1.2-1.3 kDa) (Gomes et al., 2018; Ngouémazong et al., 2015). This result can be interpreted as a higher surface activity of the TW80 molecules as compared to CP, which can be related to the adsorption mechanism of these compounds. On the one hand, TW80 molecules contain a carbon chain of oleic acid, which may facilitate the anchoring of this surfactant to the oil phase (Gomes et al., 2018). On the other hand, CP molecules contain hydrophobic groups, such as protein moieties, which serve as anchor sites to the oil phase, while the largest part corresponds to the hydrophilic polysaccharide units which orient towards the aqueous phase (Ngouémazong et al., 2015). A small fraction of proteins chemically bound (~2 %) to pectin is normally co-extracted as previously quantified in our research unit (Kyomugasho et al., 2017). Other studies showed comparable interfacial tension kinetics for CP or TW80 at the same concentrations (Gomes et al., 2018; Verkempinck, Kyomugasho, et al., 2018). The interfacial activity of CP has also been proven in our

previous work, in which the interfacial composition of emulsions prepared with 1% CP was determined. This was possible through the use of a centrifugation step followed by the quantification of CP via a specific spectrophotometric method (Infantes-Garcia et al., 2022). We proved that pectin was adsorbed to the oil-water interface of emulsions.

An interesting result to highlight is the significantly different viscoelastic behavior of the CP and TW80 interfacial layers as observed in Figure 4B. The interfacial layer composed of CP presented an elastic modulus of 82 mN/m, while the one stabilized by TW80 a value of 6 mM/m at 0.1 Hz. At this relatively high frequency the viscous modulus is negligible (E''=0) and the interfacial layers are mainly elastic where the dilatational modulus coincides with the elastic modulus (E=E') (results not shown). This result reflects the formation of a compact layer of CP, with highly interconnected molecules. This interfacial network is composed of irreversibly adsorbed CP (data not shown), displaying similar values to globular protein interfacial layers measured at similar frequency (Maldonado-Valderrama et al., 2013). Adsorption/desorption steps of interfaces formed by CP without including a digestion step confirmed that this biopolymer was irreversibly adsorbed (data not shown). The elastic behavior of CP at the interface can be related to the number of hydrogen bonds that are formed among the CP molecules. Other authors found a dilatational elastic modulus of 60 mN/m for a CP layer adsorbed to a rapeseed oil-water interface (Schmidt et al., 2017). This value is slightly lower than obtained in the present study, most probably this is related to the differences in structural characteristics of CP among the two studies. In case of TW80, the low dilatational elasticity, obtained even at this high frequency, indicates the formation of a fluent, mobile interfacial film and is consistent with values of soluble surfactants which reversibly adsorb at the interface (Mackie et al., 2001). Consequently, CP showing a high viscoelasticity stabilizes emulsions by the formation of a cohesive viscoelastic network at the interface which resists deformation. The surfactant TW80 stabilizes emulsions by the Gibbs-Marangoni mechanism based on the formation of fluid and mobile films with high surface activity. Measurements of dilatational rheology at this frequency of oscillation allow differentiating between the two types of interfacial layers formed by CP and TW80 and was therefore used

hereafter to evaluate changes in the interfacial composition of the mixtures (Maldonado-Valderrama & Patino, 2010).

Mixed interfaces: Figures 3C-D illustrates the interfacial tension kinetics during the formation of mixed interfaces containing both CP and TW80. This strategy consisted in a sequential adsorption of these two surface-active agents. As observed in Figures 3C and D, an interfacial layer saturated by CP was first formed followed by the adsorption of TW80 which was tested at two concentrations (0.00625 versus 0.1%). Both concentrations were above the critical micellar concentration of TW80 (~0.003%) (Bąk & Podgórska, 2016). However, distinct adsorption kinetics were observed after TW80 was injected into the system at the concentrations tested. In case of the lower concentration (0.00625%), a gradual adsorption of TW80 molecules was observed leading to a slight decrease in interfacial tension from around 16 to 14 mN/m as depicted in Figure 4A. The higher TW80 concentration (0.1%) resulted in a faster and significant lower interfacial tension value (decrease from 16 to 9 mN/m). These different behaviors can be attributed to the phenomenon known as orogenic displacement, discussed in the following paragraph.

The orogenic displacement mechanism was proposed after studying oil-water mixed interfaces composed of milk proteins (biopolymer) and Tween 20 (surfactant) by means of atomic force micrographs and surface tension measurements (Mackie et al., 2000). As explained by Mackie et al. (1999), this phenomenon consists of three main steps after the formation of a biopolymer interfacial layer. *(i)* First, minor domains of surfactant are formed within the interface due to the presence of small defects in the biopolymer film. This does not affect the original interfacial layer formed by the polymer. *(ii)* Second, when the surfactant concentration increases, the domains containing the surfactant grow. This causes a disturbance of the biopolymer interfacial layer leading to a partial displacement from the interface. *(iii)* Third, as the surfactant concentration keeps on increasing, the biopolymer layer collapses and is completely removed from the interface (Alan R. Mackie et al., 1999). Therefore, the orogenic displacement mechanism suggests that the disturbance and potential displacement of the biopolymer film by the surfactant depends on the surfactant concentration.

445 Based on our findings, an orogenic displacement phenomenon can also explain the behavior of the mixed
446 interfaces formed by CP and TW80. For instance, in case of the mixed interfacial layer formed after the
447 addition of TW80 in low concentrations (0.00625%), TW80 possibly adsorbed onto small defects at the CP
448 interfacial layer. The presence of low concentrations of the highly surface active TW80 at the interfacial
449 layer slightly reduces the final interfacial tension (Figure 4A), but drastically affects the dilatational
450 elasticity as TW80 molecules disrupted the interfacial network and interconnection between adsorbed CP
451 molecules (Figure 4B). Possibly, the interfacial layer is composed of small spots of TW80 compressing the
452 interfacial network of CP, which probably remains at a relatively high extent at the interface. As a
453 consequence, no or limited displacement took place and the interfacial tension decreased only to a slight
454 extent. The ζ-potential measurement of the emulsion in Table 1 also indicated a similar behavior (no or
455 limited CP displacement). However, the presence of TW80 at the interfacial layer caused a significant
456 decrement in the interfacial elastic modulus reaching a magnitude of 37 mN/m (Figure 4B). This means
457 that the elasticity of the initial CP film (90 mN/m) was diminished as TW80 molecules disturbed the
458 intermolecular CP interactions, more specifically the hydrogen bonds among CP molecules. It can be
459 hypothesized that in case of the addition of 0.00625% TW80, only the first phase of orogenic displacement
460 occurs. As the TW80 concentration increased to 0.1%, the adsorbed TW80 regions probably grew and
461 provoked displacement of CP from the interface. In this case, the interfacial elastic modulus decreased from
462 87 to 11 mN/m (Figure 4B), proving that the elastic network of CP at the interface was seriously damaged
463 and the interfacial layer contained large regions of TW80. It must be noted that a complete displacement of
464 CP molecules most likely did not occur since the interfacial layer presented a slightly higher elastic behavior
465 (11 mN/m) as compared to the single TW80 film (6 mN/m). Hence revealing the anchoring of some CP
466 molecules to the interfacial layer, although incapable of creating an interconnected network. Thus, the
467 interfacial characterization indicated that mixed interfacial layers were successfully created.

468 In our previous work, the interfacial composition of emulsions prepared with 1% CP, and 0.1 or 0.00625%
469 TW80 was determined using a centrifugation step followed by the quantification of CP and TW80 (Infantes-

Garcia et al., 2022). We proved that a mixed interfacial layer could be formed in the emulsion. In addition, it was observed that higher TW80 concentrations used to prepare the emulsions led to a higher degree of CP desorption from the interface. In the current research, the formation of these mixed layers using the pendant drop technique showed that as the concentration of TW80 increased, the interfacial layer of CP was compressed and/or desorb from the interface. This finding can also be linked to the interfacial composition determination in our previous work (Infantes-Garcia et al., 2022). The interfacial layer evolved from a CP dominated cohesive interconnected network to a TW80 dominated fluid film, stabilizing emulsions by the Gibbs–Marangoni mechanism.

3.2.2 Behavior of interfaces under *in vitro* gastric digestion

Once the single and mixed interfacial layers formation was achieved, these were subjected to *in vitro* gastric lipolysis using the OCTOPUS system and according to the INFOGEST protocol (Brodkorb et al., 2019). These conditions included an acidic pH (3), the presence of electrolytes, and the inclusion of a relevant gastric lipase with an activity of 60 U/mL (tributyrin based).

Single interfaces: In Figure 3A-B, the kinetics of interfacial tension are shown after CP and TW80 single interfaces were subjected to *in vitro* gastric conditions (yellow triangles). Specifically for the CP interfacial layer (Figure 3A), a very fast decrease was observed in the first minutes of gastric digestion which can be linked to the adsorption of gastric lipase at the interface. Gastric lipase quickly adsorbed into the defects present in the CP interfacial film, compressing the interfacial layer. This was also observed for other biopolymers, such as milk proteins (Mackie et al., 2001; Maldonado-Valderrama et al., 2013). Additionally, a competitive adsorption phenomenon could have occurred between CP and gastric lipase resulting in the desorption of the biopolymer from the interface. Afterwards, a more gradual reduction in interfacial tension was observed, reflecting the production of surface-active lipolysis products (e.g. FFAs and MAGs). Regarding the interfacial elastic modulus, this property decreased in magnitude from 82 to around 29 mN/m indicating a loss of the elasticity (Figure 4B). If this latter value was compared with the elastic modulus after digesting an oil-water interface without emulsifiers (29 mN/m) (Figure 5), then it can be deduced that

the interfacial layer at the end of the gastric digestion was mainly composed of lipolysis products. This means that CP was possibly totally displaced from the interface after 2 hours of *in vitro* gastric digestion. The CP removal most likely followed the orogenic displacement mechanism, but in this case, the surfactants (e.g. FFAs and MAGs) were endogenously produced through the hydrolysis of the oil phase. Gastric lipase and lipolysis products are more surface-active than CP molecules, and possibly caused their removal from the interface. The desorption step, showed no changes in interfacial tension and interfacial elastic moduli. This could be explained by the water insoluble character of the lipolysis products of high-oleic sunflower oil, which accumulate at the interface (Pafumi et al., 2002). In absence of bile salts, these lipolysis products cannot be removed from the interface and thus are not solubilized in the form of mixed micelles (Macierzanka et al., 2019; Maldonado-Valderrama et al., 2011). The digestive behavior of this interface was also similar as compared to the fast and extensive lipolysis behavior observed for the CP-based emulsion in Section 3.1.2.

A completely different scenario occurred for the TW80 interfacial layer under *in vitro* gastric digestion conditions. As observed in Figure 3B, the TW80 film blocked gastric lipase adsorption, and consequently, a negligible extent of lipid digestion took place. This behavior was also observed after comparing the final interfacial tension values of the TW80 interfacial layer before and after *in vitro* gastric digestion (Figure 4A). Moreover, the change in interfacial elastic modulus of the initial TW80 film was very similar to the one after the digestion step which confirmed that the fluid nature of the TW80 film was maintained. Therefore, the more surface active TW80 and highly mobile TW80 film restricted the access of enzymes possibly by Gibbs-Marangoni effects upon deformation of interfacial films (Mackie et al., 2001). In other words, gastric lipase was not capable to compete for the interface with the more surface active TW80 molecules which rapidly occupied the available interfacial area. This is the reason why very limited lipid hydrolysis was observed when *in vitro* digesting TW80-based emulsions as discussed in Section 3.1.2. The desorption phase is difficult to interpret as the TW80 should desorb as the interface is depleted. Conversely, the interfacial tension remains unchanged after subphase exchange with water. This suggests the presence

of irreversibly adsorbed compounds at the interfacial layer. The composition of this final interface is difficult to interpret as many phenomena might be overlapping; the forced dilution of bulk solution along with desorption of TW80, solubilization, residual gastric digestion as gastric lipase occupies available space at the interface, and others.

Mixed interfaces: Two distinct interfacial tension kinetics and interfacial rheology behaviors during *in vitro* gastric digestion were observed for the single interfacial layers of CP or TW80. In case of mixed interfacial layers, their kinetics of interfacial tension followed an intermediate behavior compared to the ones of CP and TW80 single interfacial layers. These kinetics highly depended on the TW80 concentration employed to form the mixed interfaces as observed in Figure 3C-D. In case of the low TW80 concentration (0.00625%), the interfacial tension kinetics were similar to the kinetics of a single CP film. This finding is more clear when comparing the decrease in interfacial tension magnitude before and after *in vitro* digestion between a single CP film (difference of around 5 mN/m) versus the mixed interface formed with the low TW80 concentration (difference of around 5 mN/m) (Figure 4A). This differential decrease in interfacial tension indicates that a similar extent of lipid hydrolysis was reached. However, the more progressive decrease of interfacial tension during the first minutes of *in vitro* digestion for the mixed interface compared to the CP single interface was an interesting difference observed from Figures 3A and 3C. For the mixed interface, the presence of adsorbed TW80 domains at the CP interface modulated the adsorption of gastric lipase. In this aspect, there was probably a competitive adsorption phenomenon between gastric lipase and CP molecules taking place. These latter findings may explain the lag-phase encountered during the *in vitro* gastric digestion of the emulsion stabilized by 1% CP and 0.00625% TW80 (Section 3.1.2). Regarding the interfacial elastic modulus (Figure 4B), this mixed interface became less elastic as the interconnected interfacial CP film is partially disrupted by TW80 and/or lipolytic products, presenting a lower magnitude after *in vitro* digestion (24 mN/m). If the elastic modulus of the single CP and the mixed interface with a low TW80 concentration after digestion are compared, they were rather similar, yet the one of the mixed interface was slightly lower. This is possibly because the interface also contained TW80 regions which

disrupted the elastic network and hence, decreased the interfacial elastic modulus. Moreover, the formation of these rather complex interfaces could be responsible for the complex trends observed during the ζ-potential measurements for this mixed system (Table 1). The desorption phase reflected the desorption of TW80 as the interfacial tension slightly increased. The parallel increase in dilatational elasticity revealed the presence of lipolysis products anchored at the interface already.

The digestion of the mixed interfaced formed using the high TW80 concentration (0.1%) presented a behavior closer to the one of a single TW80 interface digestion. This is also consistent with findings regarding the evolution of the ζ-potential (Table 1). As observed in Figure 3D, there was a limited adsorption of gastric lipase, and consequently, a slow decrease in the interfacial tension which can be interpreted as a low lipid hydrolysis extent. As mentioned in Section 3.3.1, the mixed interfacial layer formed with the 0.1% TW80 solution presented large regions of adsorbed surfactant while possibly only few CP molecules remained anchored at the interface. During *in vitro* digestion, gastric lipase probably had limited access to the interface since the defects in the CP film were already largely occupied by TW80 molecules. Thus, the enzyme most likely competed for the interface with the small fraction of remaining CP. Under these conditions, a small amount of gastric lipase could adsorb onto the interface, eventually resulting in a low lipolysis extent. This low extent was also evidenced in Figure 4A, where the differential interfacial tension before and after *in vitro* digestion was lower than 2 mN/m. With respect to the interfacial dilatational rheology, Figure 4B shows an elastic modulus of around 15 mN/m, which is somewhat higher than the one of the interface before *in vitro* digestion (11 mN/m). This possibly means that the interface was composed of a significant fraction of TW80 molecules as well as some lipolysis products. In another interesting interfacial study, an interfacial layer covered by TW80 completely inhibited lipid digestion by HGL. Only in presence of bile salts that removed TW80 from the interface, HGL could initiate the hydrolysis process (Gargouri et al., 1986). The desorption phase could also reflect the desorption of TW80 as the interfacial tension slightly raised for the mixed systems. This was accompanied by an increase of the dilatational elasticity that relates to the presence of lipolysis products anchored at the interface. The values

reached at this final desorption again serve as prove of the formation of a mixed interfacial layer that allowed with a fine-tune lipid digestibility.

4. Conclusions

This study presented new insight into emulsions stabilized by binary mixed interfaces and their lipid digestion kinetics evaluation under *in vitro* gastric conditions. All emulsions showed a relatively good to excellent physical stability during *in vitro* gastric digestion. Therefore, it was hypothesized that lipid digestion mainly depended on the interfacial composition. The addition of TW80 to CP-based emulsions permitted the modulation of the lipolysis kinetics as a function of TW80 concentration. A marked lag-phase was present in the emulsions stabilized by CP and 0.1% TW80. Both the rate and extent of gastric lipid hydrolysis was decreased by incrementing the TW80 concentration during emulsion preparation. A competitive adsorption phenomenon was possibly the main driving force for the modulation of lipid digestion kinetics. Next to this, hypotheses linked to the lipolysis behavior of these emulsions were tested in a separate experiment consisting of the formation and *in vitro* gastric digestion of interfacial layers. Overall, it can be claimed that the *in vitro* gastric digestion behaviors of single versus mixed interfacial layers were analogous to the trends observed for the digestions of their respective o/w emulsions. Two very distinct interfacial tension kinetics during digestion were observed when single layers of CP or TW80 were exposed to *in vitro* gastric conditions. When both CP and TW80 were incorporated to form mixed interfacial layers, intermediate kinetics of gastric lipase adsorption and lipolysis were observed as function of TW80 concentration. This interfacial study confirmed that the competitive adsorption phenomenon (e.g. orogenic displacement) was the determining factor for lipid hydrolysis of emulsions stabilized by CP and/or TW80, proving that interfacial design can allow steering of lipolysis kinetics of emulsions. Emulsions stabilized by mixed interfaces showed a promising tailored lipid digestibility in the gastric phase, but more research should be conducted to also modulate lipid digestion in the small intestinal phase.

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Declaration of interests

The authors of this work declare no conflict of interests.

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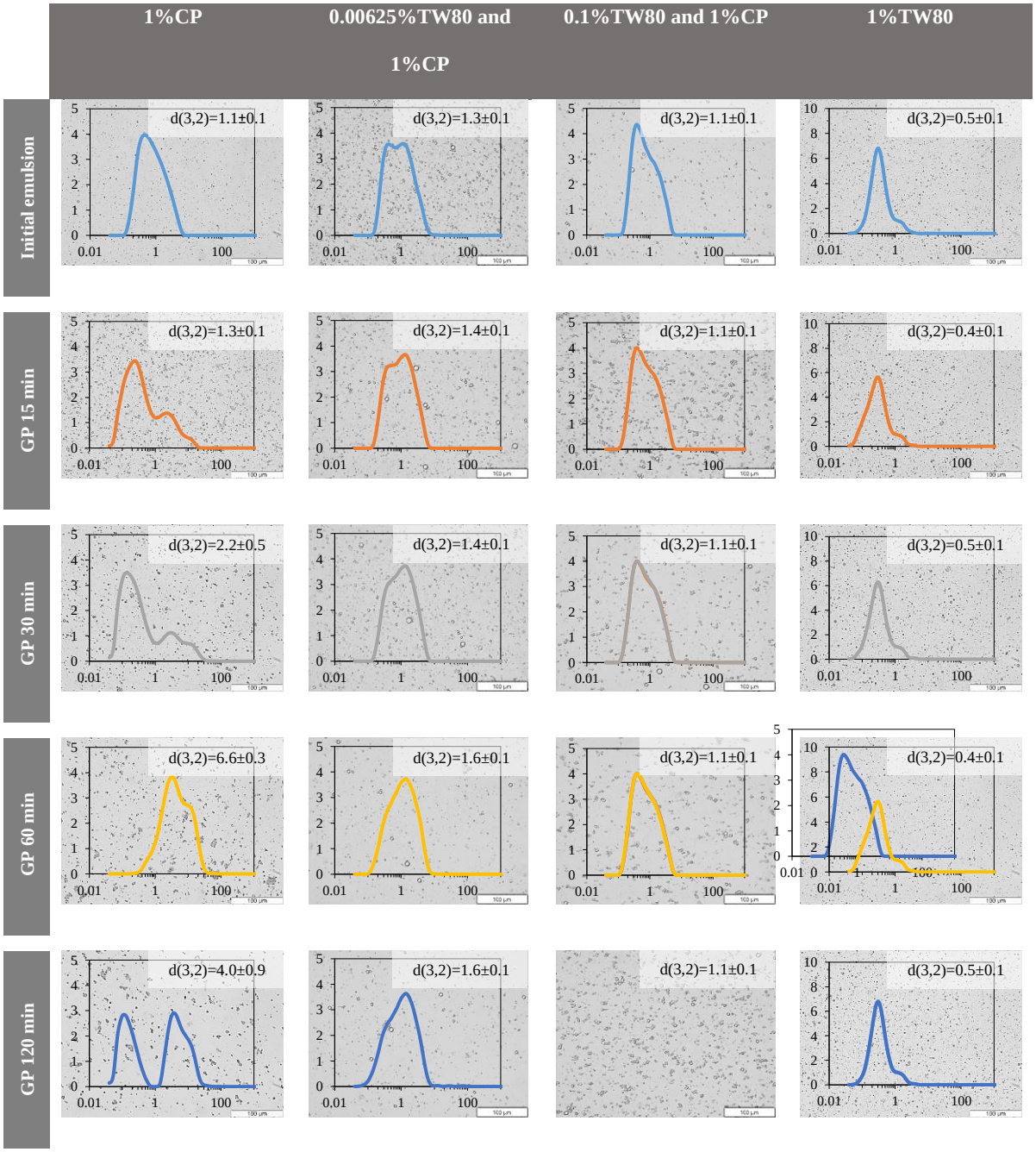


Figure 1. Effect of *in vitro* gastric conditions (GP) on the particle size distribution (PSD), average surface-based particle size d(3,2) and microstructure of o/w emulsions stabilized by mixed interfaces composed of citrus pectin (CP) and/or Tween 80 (TW80). Scale bars in the micrographs represent 100 μm. X-axis and Y-axis of PSD graphs indicate the oil droplet diameter (μm) and surface fraction (%), respectively.

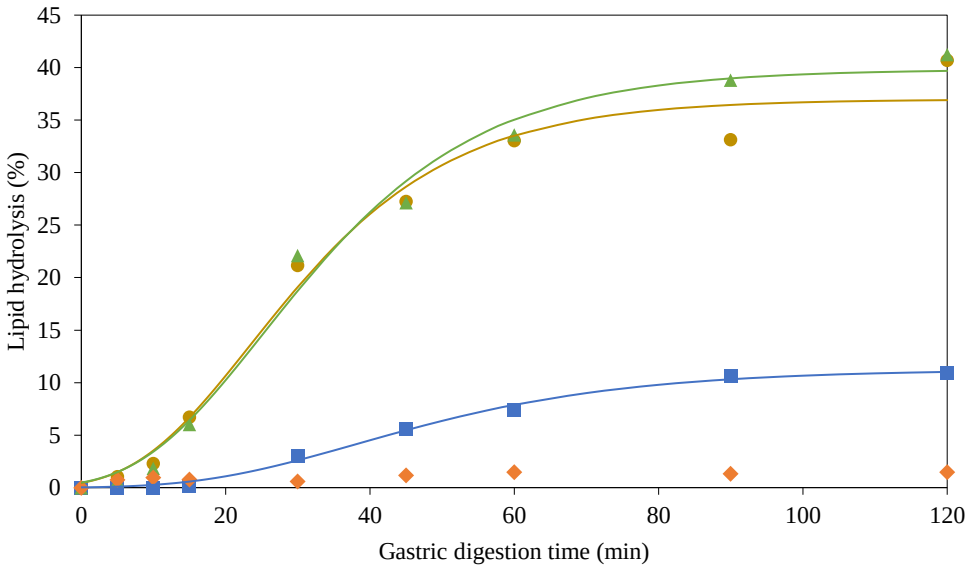
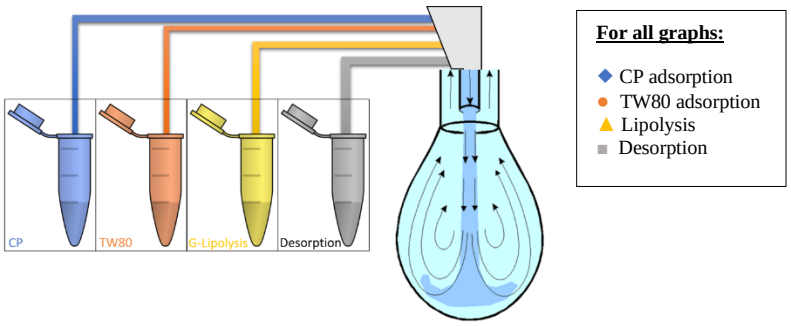
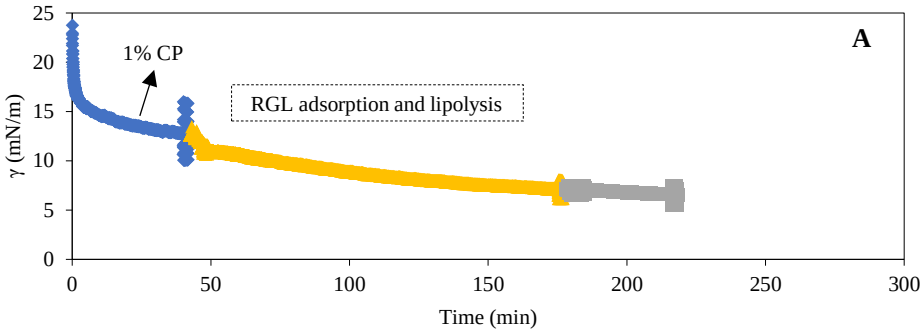
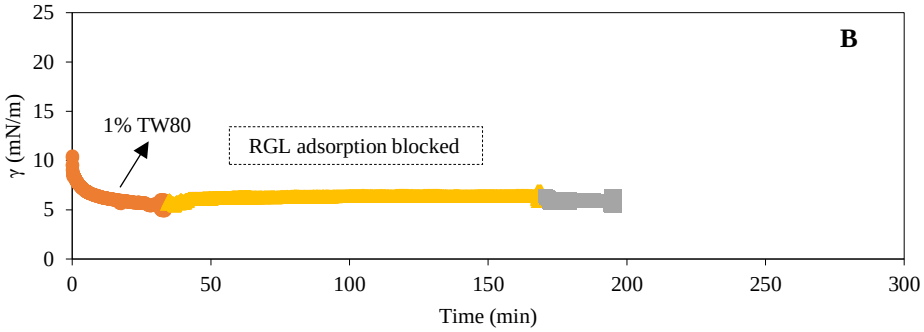
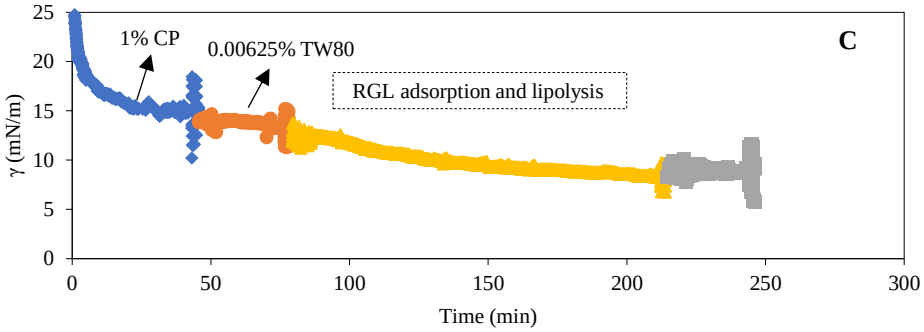


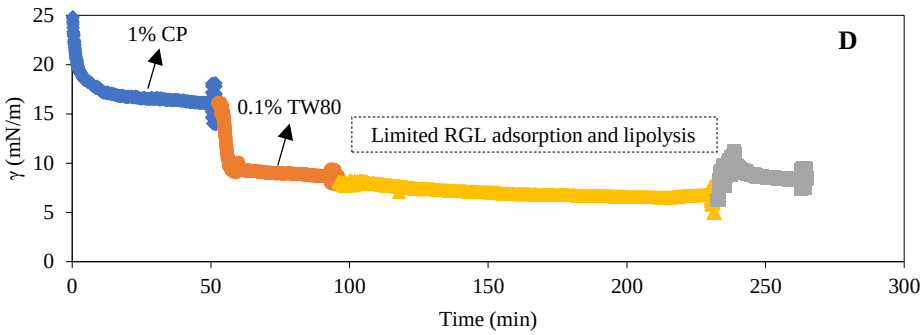
Figure 2. Modulation of the lipid hydrolysis percentage of emulsions stabilized by single and mixed interfaces containing citrus pectin (CP) and/or Tween 80 (TW80) and subjected to *in vitro* gastric digestion: (▲) 1% CP, (●) 1% CP and 0.00625% TW80, (■) 1% CP and 0.1% TW80, and (◆) 1% TW80. Symbols represent the experimental analyte concentrations, while solid lines represent model curves fitted with the modified Gompertz equation.


CP
TW80
G-Lipolysis
Desorption
For all graphs:
CP adsorption
TW80 adsorption
Lipolysis
Desorption


A
1% CP
RGL adsorption and lipolysis
γ (mN/m)
Time (min)


B
1% TW80
RGL adsorption blocked
γ (mN/m)
Time (min)


C
1% CP
0.00625% TW80
RGL adsorption and lipolysis
γ (mN/m)
Time (min)



804

805 **Fig. 3.** Interfacial tension (γ) kinetics of immersed aqueous phase droplets in high-oleic sunflower oil measured with
806 the OCTOPUS system (modified pendant drop technique) for single interfaces formed after placing solutions of (A)
807 1% citrus pectin (CP), (B) 1% Tween 80 (TW80), and mixed interfaces formed after sequentially injecting solutions
808 of (C) 1% CP and then 0.00625% TW80, and (D) 1% CP and then 0.1% TW80. The formed interfaces were subjected
809 to *in vitro* gastric digestion (pH 3, rabbit gastric lipase activity (RGL) of 60 U/mL) followed by a desorption step
810 (injection of ultrapure water). Oscillatory values (vertical lines) of the interfacial tension at the end of each stage (e.g.
811 interface formation or digestion) correspond to the interfacial rheology measurement.

812

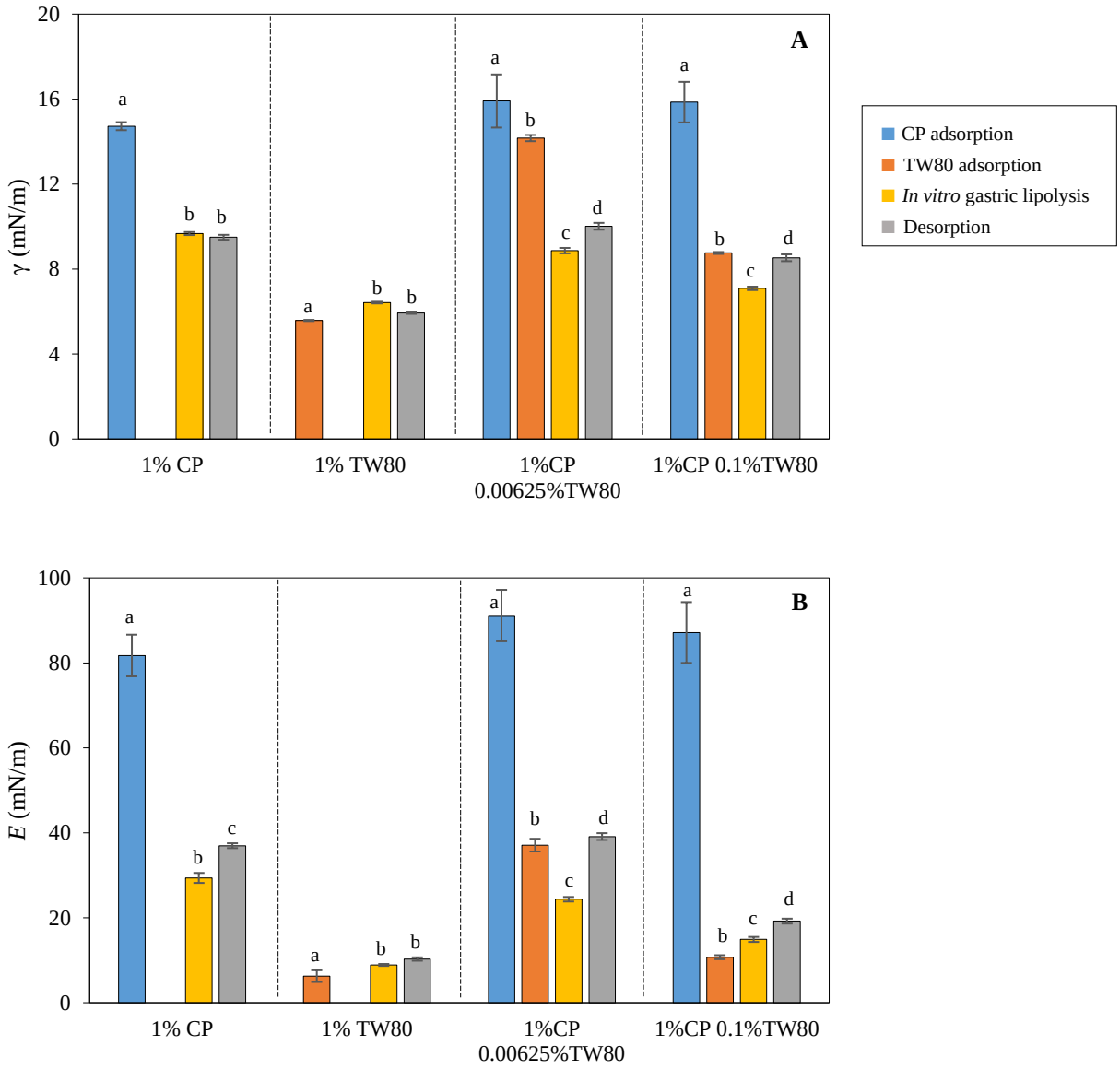


Figure 4. (A) Interfacial tension (γ) and (B) interfacial dilatational elastic moduli (*E*) of immersed aqueous phase droplets in high-oleic sunflower oil at equilibrium measured with the OCTOPUS system (modified pendant drop technique) for single interfaces formed after injecting solutions of 1% citrus pectin (CP), 1% Tween 80 (TW80), and mixed interfaces formed after subsequently injecting solutions of 1% CP and 0.00625% TW80, and 1% CP and 0.1% TW80. The formed interfaces were subjected to *in vitro* gastric digestion (rabbit gastric lipase activity 60 U/mL) followed by a desorption step. The oscillation amplitude to determine *E* was maintained below 5% variation and the oscillation frequency was set to 0.1 Hz. In graph B, the values represent measurements after each

adsorption/digestion/desorption step (vertical lines in Figure 3). Different lower case letters indicate significant differences among each adsorption/digestion/desorption step per interface type using Tukey HSD comparison tests.

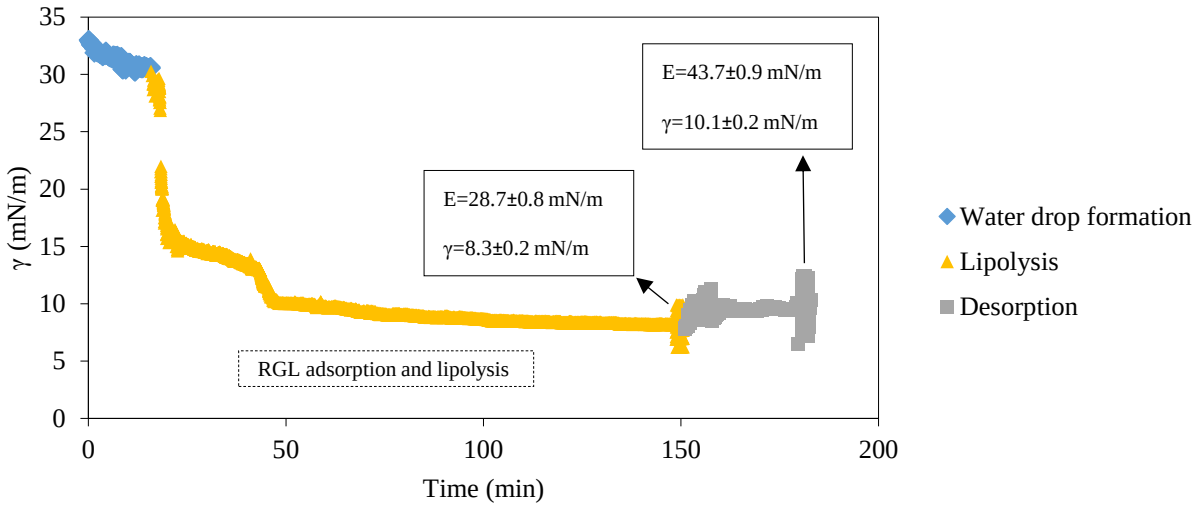


Fig. 5. Interfacial tension (γ) kinetics and interfacial dilatational elastic moduli (E) of an immersed water drop in high-oleic sunflower oil subjected to *in vitro* gastric digestion (pH 3, rabbit gastric lipase activity (RGL) of 60 U/mL) followed by a desorption step, and measured in the OCTOPUS system (modified pendant drop technique).

Table 1. ζ-potential of o/w emulsions stabilized by single and mixed interfaces during *in vitro* gastric digestion (GP). Different lower case letters indicate significant differences ($P < 0.05$) between different digestion times from the same emulsion.

Emulsifier concentration in emulsion	Initial emulsion	GP 15 min	GP 30 min	GP 60 min	GP 120 min
1%CP	-20.1 ± 0.8^{a}	-0.5 ± 0.1^{b}	1.9 ± 0.2^{c}	1.7 ± 0.2^{c}	1.5 ± 0.2^{c}
1%CP and 0.00625%TW80	-14.1 ± 0.1^{a}	-6.7 ± 0.2^{b}	-6.7 ± 1.9^{b}	-7.0 ± 0.4^{b}	-6.8 ± 0.3^{b}
1%CP and 0.1 %TW80	-6.8 ± 0.3^{a}	-0.8 ± 0.5^{b}	-0.4 ± 0.3^{b}	-0.8 ± 0.1^{c}	-2.0 ± 0.1^{d}
1%TW80	-1.4 ± 0.1^{a}	-2.0 ± 0.2^{b}	-1.8 ± 0.2^{b}	-1.9 ± 0.2^{b}	-1.8 ± 0.6^{b}

Table 2. Estimated parameters of the percentage of lipid hydrolysis using the modified Gompertz equation of emulsions stabilized by citrus pectin (CP) and/or Tween 80 (TW80) subjected to *in vitro* gastric digestion. The parameter t_{lag} is the estimated lag time (min), k is the estimated hydrolysis rate constant (min^{-1}), and H_f is the estimated final hydrolysis extent (%) as indicated in equation 3. Different lower case letters indicate significant differences among each parameter estimate according to their confidence intervals (95%).

	Estimated lipid hydrolysis parameters		
	t_{lag} (min)	k (min^{-1})	H_f (%)
1%CP	8.2 ± 2.3^{a}	0.86 ± 0.09^{a}	39.8 ± 1.5^{a}
1%CP and 0.00625%TW80	7.7 ± 2.8^{a}	0.86 ± 0.12^{a}	36.9 ± 1.7^{a}
1%CP and 0.1 %TW80	16.8 ± 2.1^{b}	0.19 ± 0.02^{b}	11.3 ± 0.4^{b}