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Gastro-intestinal *in vitro* digestions of protein emulsions monitored by pH-stat: influence of structural properties and interplay between proteolysis and lipolysis

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Abstract

This study describes an experimental design, based on pH-stat, to rapidly screen and assess food formulation effects on the degrees of hydrolysis (DH) of both proteins and lipids throughout *in vitro* gastro-intestinal digestions. This approach was used to quantitatively compare and hierarchize key structure parameters of protein emulsions. Six matrices (15 wt% whey proteins, 0 or 10 wt% oil), each differing by at least one structure characteristic, were studied. The physical state of the bulk and the oil droplet size were the major structural levers to modulate the hydrolysis of proteins (final DH between 51.7 and 58.3%) and lipids (final DH between 46.9 and 72.7%), with non-trivial interplays between proteolysis and lipolysis. Additionally, pH-stat measurements in presence of a pancreatic lipase inhibitor proved to be an efficient way to widen the scope of the proposed experimental approach to foods that are intrinsically made of both proteins and lipids.

Keywords

Food; Structure; Emulsion gel; Chemical-physics; Hydrolysis; Orlistat

Abbreviations

LCP: Liquid Continuous Phase; GCP: Gelled Continuous Phase; LFE: Liquid Fine Emulsion; GCE: Gelled Coarse Emulsion; GCEi: Gelled Coarse Emulsion with a modified o/w interface; GFE: Gelled Fine Emulsion

1. Introduction

Food structure is characterized by different spatial scales, from nano to macroscopic levels. Soluble molecules are typically in the nanometer scale and are generally embedded in structures organized at larger scales: biopolymer networks (with subunit-structure of about 10-100 nm), droplets (0.1-5 µm, typically), etc. To better understand how these structures break down during digestion and assess their impact on the release of nutrients, both *in vivo* and *in vitro* experiments can be undertaken. While *in vivo* studies are mandatory to unambiguously demonstrate that food structure can modulate the fate of nutrients in the host, *in vitro* experiments can be used to predict outcomes of *in vivo* digestions (Bohn et al., 2018) and more thoroughly investigate the underlying mechanisms.

When considering lipids, which are dispersed as oil droplets in most foods, both *in vivo* and *in vitro* studies have shown that smaller droplet size leads to faster lipolysis kinetics (Armand et al., 1999; McClements & Li, 2010), since they develop a larger interfacial area for lipase adsorption. The type of emulsifiers at the interface is another key factor to consider as it may control not only the ability for lipases to access their substrate (Mun, Decker, & McClements, 2007), but also the emulsion stability. In the changing conditions of the digestive tract, the composition of the interface may change and droplets interact with each other. This can lead to droplet flocculation and/or coalescence during both the gastric (Golding et al., 2011; Sarkar, Goh, Singh, & Singh, 2009) and intestinal (Giang et al., 2015, 2016; Li, Ye, Lee, & Singh, 2013; Sarkar, Horne, & Singh, 2010) phases of digestion, and lower the rate of lipid absorption as estimated from the postprandial blood triglyceride concentrations (Golding et al., 2011; Keogh et al., 2011).

The kinetics of protein digestion also depends on food structure. The macrostructure is of course a key parameter, with a slower rate of proteolysis generally observed for solid foods.

For instance, dairy proteins have been reported to be much more rapidly metabolized in mini-pigs when eaten in the liquid state compared to rennet gels of identical composition (Barbé et al., 2013). Nanostructure and microstructure effects are also important to consider. For example, when used as emulsifiers, dairy whey proteins show an enhanced susceptibility to hydrolysis by pepsin because of their modified conformation upon adsorption at the oil interface (Macierzanka, Sancho, Mills, Rigby, & Mackie, 2009). Similarly, thermal denaturation and/or aggregation of β-lactoglobulin, the main protein of dairy whey, can lead to an improved hydrolysis by both pepsin (Guo, Fox, Flynn, & Kindstedt, 1995; Singh, Øiseth, Lundin, & Day, 2014) and intestinal proteases (Stănciuc, van der Plancken, Rotaru, & Hendrickx, 2008). Differently structured whey protein gels can therefore lead to highly contrasting digestion profiles (Macierzanka et al., 2012).

Few studies have also focused on food emulsions rich in proteins and on the interplay between lipolysis and proteolysis. Gelatin gels have been shown to slow down the rate of pancreatic lipolysis of embedded oil droplets because of a reduced lipase diffusion into the protein network (Sarkar et al., 2015). Others have investigated the influence of the structure of whey protein emulsion gels on lipid digestion, with interesting findings on the relations between their mechanical properties and their rate of disintegration upon simulated gastric contractions (Guo, Ye, Bellissimo, Singh, & Rousseau, 2017). Beyond confirming a slower lipolysis in presence of a surrounding protein network, it has been shown that the release of oil droplets from whey protein emulsion gels is delayed for hard gels compared to soft ones (Guo, Ye, Lad, Dalgleish, & Singh, 2014b), leading to a reduced rate of lipid hydrolysis (Guo, Bellissimo, & Rousseau, 2017; Guo, Ye, Lad, Dalgleish, & Singh, 2016). For emulsion gels of identical composition (10% whey proteins, 20% oil), the same team also observed that the size of the dispersed oil droplets can modify the structural and mechanical properties of the

gels, and hence their digestion kinetics, with less coalescence and phase separation when embedded droplets were small (1 µm *vs* 6 and 12 µm) (Guo, Ye, Lad, Dalgleish, & Singh, 2014a).

This literature on the digestion of emulsion gels remain quite recent and is still scarce, but nicely illustrates how protein digestion can govern the way lipids entrapped in a protein network are released. Such findings are interesting to gain a better view of the fate of complex foods in the gastro-intestinal tract. Nonetheless, most of these studies have been conduct from a lipid digestion perspective, with no concomitant measurements of the protein digestion extent to more thoroughly quantify the interplays between protein and lipid hydrolysis. Previous studies of our group have shown that pH-stat can be used to monitor the degrees of hydrolysis of proteins during the gastric phase of *in vitro* digestions (Mat, Cattenoz, Souchon, Michon, & Le Feunteun, 2018), and of both proteins and lipids during the intestinal phase (Mat, Le Feunteun, Michon, & Souchon, 2016). Building upon these previous developments, our present work intends to show how pH-stat can be used, as a rather simple and high throughput approach, to quantitatively assess food formulation effects on the gastro-intestinal *in vitro* digestion of both their protein and lipid contents. More specifically, this study aimed at applying such approach to quantitatively compare the effects of three key structure parameters of protein emulsions: the state of proteins in the continuous phase (liquid and gelled state), the thickness and degree of denaturation of the protein layer at the oil/water interface, and the lipid droplet sizes (1 *vs* 20 µm). Additionally, because pH-stat is sensitive to both proteolysis and lipolysis in intestinal conditions, this method is generally considered inappropriate to the study of complex foods that are intrinsically made of both proteins and lipids. To overcome this issue, we also present a

means, based on the use of a lipase inhibitor during pH-stat intestinal monitoring, that appears suitable to evaluate the contribution of each reaction with such complex foods.

2. Material and methods

2.1. Material

Whey protein isolate powder (Prolacta 95, 95 wt% of proteins on dry powder) was obtained from Lactalis, France. Rapeseed oil (Fleur de colza, Lesieur, France) was purchased at a local supermarket. Pepsin (P6887), pancreatin (P7545), pancreatic lipase (L3126) and bile extract (B8631), all of porcine origin, were obtained from Sigma-Aldrich, France, as well as the Orlistat lipase inhibitor (O4139). Enzyme activities and bile salts concentrations were determined according to the protocols described in (Brodkorb et al., 2019; Minekus et al., 2014). Water was Milli-Q water and all other materials were of standard analytical grade.

2.2. Designed matrices

In total, 6 different matrices where designed for the purpose of this study: 4 protein emulsions and 2 protein-only matrices, the latter corresponding to the continuous phases of the emulsions. Their compositions are presented in Table 1, as well as their schematic structure and size characteristics.

A gelled coarse emulsion (GCE) was produced based on to the protocol previously described in (Mat et al., 2016). In short, an emulsion was prepared with 0.3 wt% of whey proteins and 30 wt% of oil using a rotor-stator homogenizer (Polytron PT3100D, Kinematica AG, Switzerland) fitted with a PTDA32/2-B250 for 5 min at 10,000 rpm. It was then heated for 5 min at 70 °C. This preliminary step was performed to ensure a certain amount of denaturation of the adsorbed proteins, and to test if their partial cross-linking at the

130 interface could influence lipolysis. The warm emulsion was mixed with a solution of 22.3
131 wt% of proteins prepared beforehand to achieve the final composition of 15 wt% of proteins
132 and 10 wt% of oil. The preparation was then heated for 30 min at 80 °C in a water bath to
133 perform gelation.
134 An equally-composed liquid fine emulsion (LFE) was prepared as described in (Mat et al.,
135 2016). In short, it consisted in emulsification by the same rotor-stator treatment, followed
136 by sonication at 20 kHz and 130 W using a 13 mm probe (VCX 130, Sonic & Materials, UK) for
137 10 min (effective time, with on/off cycles of 10 s) in order to further reduce the sizes of the
138 oil droplets. The mean temperature in the emulsion was maintained below 30 °C with an ice
139 bath.
140 The corresponding lipid-free matrices consisted in a 15 wt% of whey protein solution for the
141 liquid one (liquid continuous phase, LCP), while its gelled counterpart (gelled continuous
142 phase, GCP) was obtained after a heat treatment (80 °C, 10 min) of the same solution.
143 The other two emulsions were prepared in similar ways as GCE with slight variations. On the
144 one hand, a gelled coarse emulsion with a modified oil/water interface (GCEi) was made
145 with omission of the first heat treatment (at 70 °C), meaning that whey proteins were not
146 heat-denatured before the dispersion of the droplets in the protein solution. On the other
147 hand, a gelled fine emulsion (GFE), in which the pre-emulsion was produced with a higher
148 quantity of whey proteins (6.0 wt% instead of 0.3 wt%) and a prolonged sonication step of
149 15 min to produce oil droplets similar in size to those in LFE. These two additional emulsions
150 were designed to change only one parameter at a time (interface structure and droplet size,
151 respectively) when compared to GCE, and more properly assess the effect of the physical
152 state of the continuous phase by comparing LFE with GFE.
153

2.3. Matrix characterization

The oil droplet size distributions in the emulsions were controlled with a laser light scattering particle size analyzer (Mastersizer 2000, Malvern, France), using refractive index of 1.47 and 1.33 for oil and water (dispersant), respectively. A value of 0.001 was set as the absorption of the emulsion. Droplet sizes (Table 1) are given as volume-weighted mean diameters, calculated using $d_{4,3} = \sum n_i d_i^4 / \sum n_i d_i^3$. Similar $d_{4,3}$ values were obtained for LFE and GFE: 1.22 ± 0.06 µm and 1.37 ± 0.01 µm, respectively; and for GCEi and GCE: 19.13 ± 0.03 and 18.83 ± 0.64 µm, respectively.

The evolution of the rheological properties of the protein emulsions upon heating up to 80 °C was also measured in triplicate according to the protocol used in (Mat et al., 2016). Final storage modulus values (G') are reported in Table 1. G' was similar for protein emulsions with large oil droplets (GCE and GCEi) and for their lipid-free counterpart (GCP), whereas it tended to be slightly higher for the protein emulsion containing small oil droplets (GFE) because of the increased interactions between dispersed and continuous phases.

2.4. Gastro-intestinal *in vitro* digestions monitored by pH-stat

The digestion experiments were all performed in triplicate, and consisted in a 3-phase digestion based on the recommendations of the Infogest consortium (Brodkorb et al., 2019; Minekus et al., 2014), where details can be found on the composition of digestive fluids and enzyme activities. The only noticeable difference is that $NaHCO_3$ salts were replaced by NaCl at the same molar ratio in all electrolyte solutions to avoid unwanted pH drift (Mat et al., 2016), meanwhile maintaining the same ionic strength. Gastro-intestinal digestions were carried out in a 200 mL jacketed beaker maintained at 37 °C by water circulation using a

177 constant magnetic stirring (250 rpm). This set-up was mounted onto an automatic titration
178 unit (Titroline7000, VWR, France).
179
180 *Oral phase*: Solid matrices were demolded and grinded with a domestic kitchen food
181 chopper (Braun Turbo, 600W, type 4191, Spain) for 3 s at maximum power to produce
182 submillimeter particles. 7.5 g of the grinded matrix were then mixed with 7.5 mL of
183 simulated salivary fluid in the jacketed beaker and let to reach temperature equilibrium. This
184 oral phase was only carried out for electrolyte concentration considerations, with no added
185 enzymes at this stage.
186
187 *Gastric phase and pH-stat measurements:* The pH probe and the titration cone of the
188 titration unit were put in place. 13.5 mL of gastric electrolytes were added and the pH was
189 adjusted to 3.0 using HCl 1 N. Once pH and temperature equilibria were achieved, 1.5 mL of
190 a pepsin solution (to reach 2,000 U/mL in the final mixture) were added and titration was
191 immediately turned on in a pH-stat mode. It was programmed to maintain a constant pH
192 value of 3.0 for 2 h using HCl 0.3 N as a titrant.
193
194 *Intestinal phase and pH-stat measurements:* At the end of the gastric phase, the beaker was
195 reconnected to another titration unit (same reference). The volume of the chyme was
196 completed with water up to 33 mL in order to always start the intestinal phase with the
197 same volume, regardless of the volume of titrant added previously. 25.5 mL of intestinal
198 electrolytes containing a pre-established amount of NaOH 1 N, to bring the pH to 7.0, were
199 first added. 2.5 mL of bile solution (prepared beforehand by melting bile extract in a 55 °C
200 water bath) were then added to the mix. Pre-established amounts of pancreatin and

pancreatic lipase powders (to achieve a trypsin activity of 100 U/mL and a lipase activity of 2,000 U/mL in the final mixture), conserved at -20 °C in a tube, were rapidly rehydrated with an intestinal electrolyte solution containing a pre-established amount of NaOH 1 N to bring the pH of the solution to 7.0. The tube was left in the water bath to reach 37 °C, and after 5 min, the pH was checked, and adjusted to 7.0 if needed. 5 mL of the enzymes solution were then added into the jacketed beaker to complete the intestinal fluid (33 mL). The titration program was immediately turned on for 2 h in a pH-stat mode to maintain a pH value of 7.0 using NaOH 0.2 N as a titrant.

Three blank intestinal digestions, with no food, were also conducted as it appeared that the mixing of the intestinal solutions induced a small pH-stat signal, possibly induced by interactions between bile extract constituents and pancreatic enzymes. This contribution was subtracted from all the titration curves obtained during the intestinal digestion of the studied matrices.

Intestinal phase in presence of Orlistat: In order to test whether protein hydrolysis could be solely monitored during the intestinal digestion of GCE and LFE matrices (*i.e.* despite their high lipid content and the lipolytic activity of pancreatin), the action of a lipase inhibitor (tetrahydrolipstatin, branded as Orlistat) was tested during additional digestions experiments. For these experiments, the gastric phase was carried out as described above. However, 660 µL of the intestinal electrolyte solution were substituted by 660 µL of an Orlistat solution (4 mg/mL in DMSO) in order to achieve a final concentration of 40 µg/mL. Moreover, pancreatic lipase was not added in complement to pancreatin in order to limit the lipolytic activity of the intestinal secretions, while maintaining the same proteolytic activity.

225

226 2.5. Determination of the degree of hydrolysis

227 *Gastric proteolysis:* The degree of hydrolysis of proteins during the gastric phase (DH_{prot_G})
228 was estimated according to the relation previously described in (Mat et al., 2018):

$$DH_{prot\_G} = 100 \times \frac{V \times N}{m \times h_{tot}} \times \frac{1}{1 - \alpha_{COOH}} \quad (1)$$

229 where *V* is the volume of added HCl (mL), N is the normality of the acid, m_{prot} is the mass
230 of proteins (g), h_{tot} = 8.8 meqv/g is the total number of peptide bonds in whey proteins
231 (Spellman, McEvoy, O'Cuinn, & FitzGerald, 2003), and α_{COOH} = 0.080 is the mean degree of
232 dissociation of the peptide carboxylic groups at pH = 3.0 and 37 °C (Mat et al., 2018).

233

234 *Intestinal proteolysis:* The degree of hydrolysis of proteins during the intestinal phase
235 (DH_{prot_I}) was estimated for the lipid-free matrices (LCP and GCP) and for LFE and GCE in
236 presence of Orlistat, according to the following relation:

$$DH_{prot\_I} = 100 \times \frac{V \times N}{m_{prot} \times h_{tot}} \times \frac{1}{\alpha_{NH_2}} + DH_{prot\_G}(end) \quad (2)$$

237 where *V* is the volume of added NaOH (mL), $DH_{prot\_G}(end)$ is the degree of hydrolysis at
238 the end of the gastric phase, and α_{NH_2} is the mean degree of dissociation of the α-amino
239 groups. The value of α_{NH_2} was estimated to be 0.1118 and 0.1111 from the results obtained
240 with LCP and GCP, respectively, using the same procedure as in (Mat et al., 2016) that relies
241 on independent determinations of the degree of hydrolysis of end samples, *i.e.* collected at
242 the end of the experiments, using the OPA (ortho-phthalaldehyde) method. A value of α_{NH_2}
243 = 0.1114 was therefore used in Eq. (2).

244

Intestinal lipolysis: The degree of hydrolysis of lipids during the intestinal phase (DH_{lip}) was estimated according to the following relation:

$$DH_{lip} = 100 \times \frac{\Delta V \times N \times M_{lip}}{m_{lip} \times 2} \times \frac{1}{\alpha_{COOH}} \quad (3)$$

where ΔV (mL) is the difference between the volumes of NaOH added for the studied emulsion and its lipid-free counterpart (LCP or GCP), m_{lip} is the oil mass (g), M_{lip} is the molar weight of the triglycerides in the oil (calculated as 930 g/mol), and α_{COOH} is the mean degree of dissociation of the free fatty acids' carboxylic group. α_{COOH} was estimated to be 0.77 according to complementary 12h long intestinal digestions, performed on reduced quantities (1.5 g) of a liquid emulsion made with 10 wt% of rapeseed oil and 0.1 wt% of whey proteins in order to reach 100% release of fatty acids (controlled by the appearance of a sustained plateau during pH-stat measurements).

2.6. Statistical analysis

One way ANOVA was used to compare the degree of hydrolysis between two matrices, or two categories of matrices (*e.g.* liquid *vs* gelled). The initial reaction rates and the final extent of hydrolysis were compared 3 min and 120 min after the start of the gastric and intestinal phases, respectively. Statistically significant effects were accepted at the 95% level. All statistical analyses were conducted using the statistics toolbox of Excel™.

3. Results & Discussion

3.1. Effects of structural properties on gastric proteolysis

Fig. 1 shows the kinetics of protein hydrolysis by pepsin at pH = 3.0 for the 6 studied matrices measured by pH-stat during gastric digestion (Mat et al., 2018). Results are spitted into Fig. 1A and 1B for legibility, and those obtained for the gelled coarse emulsion (GCE) are duplicated in both subfigures for comparison purposes. The degree of hydrolysis (DH) of proteins measured after 3 and 120 min of reaction are also reported in Table 2.

A high initial reaction rate followed by a progressive slowdown was observed for all foods. Results also shows that the beginning of the reaction was slightly slower ($P < 0.001$ at t = 3 min, Table 2) for 4 gelled matrices (GCP, GCE, GCEi, GFE) than for the 2 liquid ones (LCP and LFE). This can be directly related to the physical state of the protein phase since the substrate is readily accessible to pepsin in solutions, whereas its accessibility is initially limited to the external surface of gel fragments for solid matrices. It also appears that the slowdown was more pronounced for liquid matrices than for the solid ones, leading to final DH values (Table 2) significantly higher ($P < 10^{-6}$) for solid matrices (5.5-6.0%) than for LCP and LFE (3.4-3.8%). This can be explained by an enhanced susceptibility of denatured proteins to peptic hydrolysis. Indeed, β-lactoglobulin, the major constituent of whey proteins, has been reported to be rather resistant to pepsin in its native form (Astwood, Leach, & Fuchs, 1996), and more sensitive to pepsinolysis after denaturation by heat treatments above 70 °C (Reddy, Kella, & Kinsella, 1988). Overall, the degrees of hydrolysis we measured are in line with the values found in the literature for pepsin digestion of whey proteins: 1.7% after 4 h with native proteins (Asselin, Hébert, & Amiot, 1989), between 3 to 10% after 2 h with heat-treated proteins (Kim et al., 2007), or 7.9% after 3 h with whey protein gels (Luo, Boom, & Janssen, 2015).

Fig. 1A and 1B also show that the trends observed for protein emulsions closely followed those observed for their lipid-free counterparts (LCP and GCP), with undistinguishable kinetics and final degree of hydrolysis ($P > 0.7$) for the 4 solid matrices (Table 2). This suggests that, whatever their size or interface (Table 1 and Fig. 1B), embedded oil droplets had a negligible influence on the peptic digestion of our emulsion gels. The final DH was slightly (by 0.4% on average) but statistically ($P = 0.045$) higher for LCP than for LFE, however. This difference, which appeared in the early stages of the reaction (Fig. 1A), could reflect a decreased pepsin-substrate meeting probability in oil-droplet containing solutions, but additional data would be needed to confirm such an effect.

In summary, these results confirm the significant effect of whey protein denaturation on their hydrolysis by pepsin, and show a lesser influence of embedded oil droplets, whatever their size or interface, on our protein-rich emulsions (15 wt% proteins and 10 wt% oil).

3.2. Effects of structural properties on intestinal proteolysis as inferred from lipid-free matrices

The cumulative volume of NaOH recovered by pH-stat during the subsequent intestinal digestion are presented in Fig. 2A for all matrices. However, in the neutral conditions of intestinal digestion, both proteolysis and lipolysis reactions contribute to the pH-stat signal. This is the reason why the volume of added titrant was much larger for lipid-containing matrices.

To address the impact of the protein structure on the proteolysis kinetics, one should therefore focus on the comparison between LCP and GCP. In a similar but more substantial way than during the gastric phase (Fig. 1A), the reaction was initially faster ($P = 10^{-5}$) for LCP than GCP (Fig. 2A), illustrating again the effect of substrate accessibility. By the end of the

311 experiments, a sustained plateau was clearly reached with both matrices, meaning that the
312 reaction was complete in each case. The final volumes of added titrant were similar, though
313 they tended (P = 0.08) to be higher for GCP (2.79 ± 0.05 mL) than for LCP (2.66 ± 0.03 mL).
314 The overall quantity of peptide bonds hydrolyzed during the intestinal phase were thus
315 about the same for denatured and native whey proteins. This small difference, if any, is
316 consistent with the previous report that heat denaturation of whey proteins has a much less
317 pronounced effect on chymotrypsin action (major constituent of pancreatic proteases) than
318 pepsin action (Reddy et al., 1988). It is noteworthy that we previously found a bigger
319 difference in a previous work on comparable matrices (Mat et al., 2016). We since figured
320 out that protons entrapped in too large gel fragments (e.g. > 1 mm) during the gastric phase
321 can slowly release during the intestinal phase, hence leading to an overtitration by pH-stat
322 and an overestimation of the proteolysis extent. Special care was therefore taken in the
323 present study to avoid such effects by grinding the solid matrices into submillimeter pieces
324 (averaged minimal Feret diameters of 0.5 mm, data not shown) and wait enough time for pH
325 adjustment at the transition between gastric and intestinal phases.
326 The evolution of the intestinal DH of proteins, which accounts for the hydrolysis achieved
327 during the gastric phase (*i.e.* DH of 5.7% for GCP and of 3.8% for LCP at t = 0 min of the
328 intestinal phase), are presented in Fig. 2B. The final DH obtained for LCP (51.7 $\pm$ 0.4%) was
329 smaller (P = 0.024) than the one measured for GCP (57.4 $\pm$ 2.7%), hence confirming the
330 higher overall susceptibility of gelled whey proteins (GCP) than native proteins (LCP) to
331 digestive proteases. We may highlight in here that LCP and CGP matrices have been shown
332 to induce contrasted impacts on both the gastrointestinal physiology and the intestinal
333 microbiota in rats, associated to a higher protein content reaching the caecum for LCP

(Beaumont et al., 2017; Oberli et al., 2018). A higher resistance of this native protein solution to protease action therefore appears consistent with these *in vivo* findings.

From Fig. 2A, we may finally note that the titration curves for GCE (pre-heated initial emulsion) and GCEi (unheated initial emulsion) were indistinguishable, hence suggesting that the higher quantity of proteins and cross-links at the oil droplet interface of GCE was of negligible influence on the overall digestion kinetics. We may indeed expect both liquid matrices, on the one hand, and the 4 solid matrices, on the other hand, to behave similarly from a pancreatic proteolysis point of view, as demonstrated during the gastric phase. However, it is not possible to ensure this statement from the analyses of the results of Fig. 2A, nor to investigate the possible effects of oil droplets on the intestinal protein hydrolysis kinetics, because of the contribution of lipid hydrolysis.

3.3. pH-stat monitoring of intestinal digestion in the presence of a lipase inhibitor

Most foods are intrinsically made of both proteins and lipids, with no simple way of manufacturing a lipid-free equivalent, as performed in this study, to evaluate the contributions of proteolysis and lipolysis to the pH-stat signal in intestinal conditions. To overcome this limitation, we investigated another experimental strategy that relies on the use of a lipase inhibitor, tetrahydrolipstatin, branded as Orlistat. This molecule has been studied both *in vitro* and *in vivo* and proved very efficient in inhibiting the human pancreatic lipase (Carrière et al., 2001; Tiss, Lengsfeld, Carrière, & Verger, 2009; Wilcox, Brownlee, Richardson, Dettmar, & Pearson, 2014). It prevents the hydrolysis of triglycerides by covalently reacting with the active site of the lipase. According to the specificity of its mechanism of action, Orlistat is also assumed to solely inhibit lipases and leave protease action unhindered.

Fig. 3A and 3B present the titration kinetics obtained when Orlistat was added into the reaction mixture during intestinal digestions of LFE and GCE, respectively. Results were very close to the ones obtained with the lipid-free matrices, LCP and GCP, hence confirming that Orlistat is a very good inhibitor of intestinal lipolysis, with no, or very little, effects on proteases. This strategy therefore constitutes a very interesting alternative, which should be suitable to all types of food, to unveil the contributions of intestinal proteolysis and lipolysis when using pH-stat. However, the lipid hydrolysis reaction cannot be perfectly prevented, as evidenced by the slow but progressive increase towards the end of the experiments. Nonetheless, present results clearly show that this approach enables to reach the same general conclusions as those obtained from the lipid-free matrices strategy. The present approach can moreover provide a means to estimate the effects of lipids on the hydrolysis of proteins, as illustrated by the slower initial rate of proteolysis for GCE than for GCP ($P < 0.001$ at t = 3 min) during the first hour (Fig. 3B).

3.3. Effects of structural properties on intestinal lipolysis

The lipolysis contribution to the intestinal titration was determined by subtracting the volumes of titrant added for the lipid-free matrices. The resulting curves were then converted into DH values, as presented in Fig. 4 and Table 2.

The intestinal titration curves were the same for GCE and GCEi (Fig. 2A), leading to similar final DH ($46.9 \pm 8.8\%$ and $48.1 \pm 2.2\%$, respectively). Alongside to our conclusion on protein hydrolysis, the higher quantity proteins and of cross-links at the oil droplet interface of GCE also appeared to be of negligible influence on the lipolysis kinetics. This finding is also in excellent agreement with the previously reported limited effect of interfacial β-lactoglobulin

cross-linking on the *in vitro* intestinal digestion of liquid emulsions (Sandra, Decker, & McClements, 2008).

Compared to GCE and GCEi, both LFE and GFE showed marked enhanced lipolysis profiles (Fig. 4, $P < 0.001$ at 120 min). The mean diameter of oil droplets in GFE and LFE was almost 10 times smaller than in GCE and GCEi (Table 1), hence demonstrating the major influence of the interfacial surface on the lipid hydrolysis rate even when oil droplets are embedded within a protein gel. The most rapid initial reaction rate was obtained with LFE ($P < 0.05$ from 3 to 9 min) most certainly because of the higher accessibility of oil droplets in this liquid matrix when compared with GFE. However, the subsequent slowdown of lipid hydrolysis was less pronounced for GFE than for LFE, hence leading to statistically comparable final DH ($P = 0.19$). This suggests that the physical state of the continuous phase also has an influence on lipid hydrolysis. The entrapment of the oil droplets in the protein gel may help stabilizing them throughout the intestinal reactions, whereas droplets in LFE can more easily enter into contact, coalesce and cream, thereby almost disappearing from a reaction point of view (Giang et al., 2015). This was visually witnessed as no oil layer was formed at all with GFE, which contrast with what was observed with LFE, GCE, and SECc. The solid fine emulsion thus appeared to enable an initially more gradual, but overall comparable, release of lipolysis products than its liquid counterpart. Such finding might be of interest to better understand nutrient interactions within the gut, or in the formulation of lipid-based delivery systems.

Conclusions

In this study an integrated method of *in vitro* static digestion, respecting the Infogest recommendations, was presented. It relies on the use of the pH-stat titration during both

405 the gastric and intestinal phases of digestion. The method proved efficient at determining
406 the kinetics of gastric proteolysis, intestinal lipolysis and intestinal proteolysis on both liquid
407 and solid matrices, which may be considered as good models of complex foods rich in both
408 proteins and lipids. The physical state of the proteins (particularly in the bulk) and the size of
409 the oil droplet were identified as major structural parameters to modulate protein and lipid
410 hydrolysis. Overall, pH-stat proved to be a simple, rapid, and very efficient method to
411 quantitatively monitor gastro-intestinal proteolysis and lipolysis of food products. The use of
412 Orlistat as an efficient inhibitor of lipolysis makes it possible to study the proteolysis of food
413 matrices in which lipids are naturally present.
414
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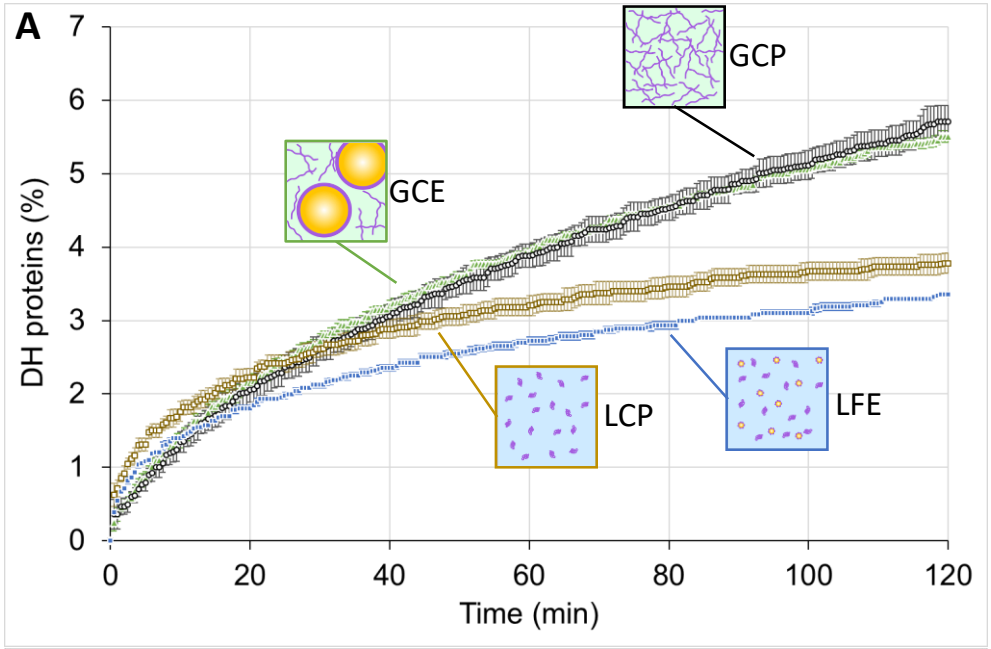


Fig. 1 Evolution of the degree of hydrolysis (DH) of proteins measured by pH-stat during the course of *in vitro* gastric digestion (pH = 3.0). LCP stands for Liquid Continuous Phase (squares), GCP for Gelled Continuous Phase (circles), LFE for Liquid Fine Emulsion (squares), GCE for Gelled Coarse Emulsion (triangles, duplicated in A and B), GFE for Gelled Fine Emulsion (diamonds), and GCEi for Gelled Coarse Emulsion with a modified o/w interface (stars). Data represent means ± SEM over at least 3 replicates.

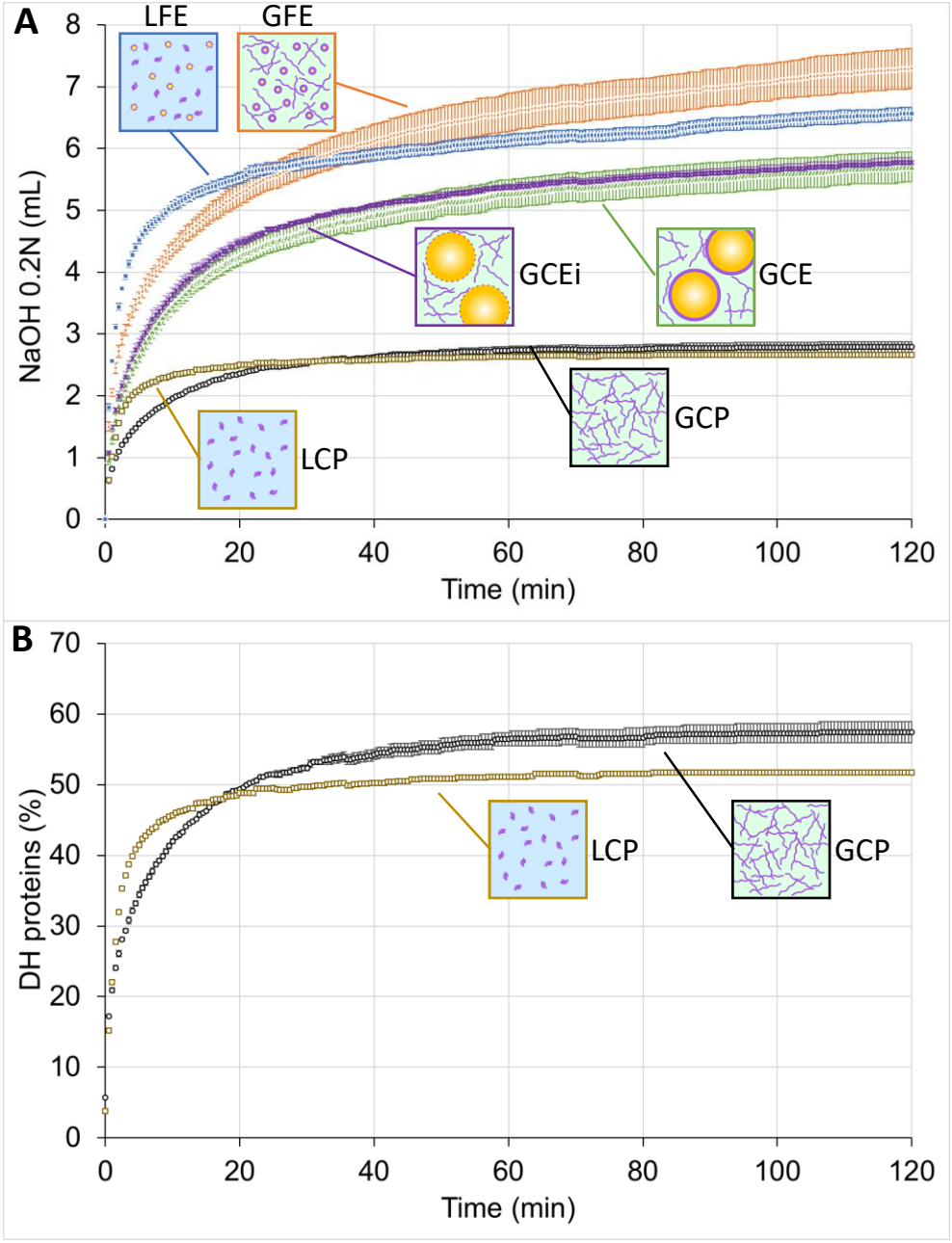


Fig. 2 Results of the pH-stat titration during the course of *in vitro* intestinal digestion (pH = 7.0): (A) Titration curves; and (B) Evolution of the degree of hydrolysis (DH) of proteins for lipid-free matrices that accounts for the preceding gastric phase. LCP stands for Liquid Continuous Phase (squares), GCP for Gelled Continuous Phase (circles), LFE for Liquid Fine Emulsion (squares), GCE for Gelled Coarse Emulsion (triangles), GFE for Gelled Fine Emulsion (diamonds), and GCEi for Gelled Coarse Emulsion with a modified o/w interface (stars). Data represent means ± SEM over at least 3 replicates.

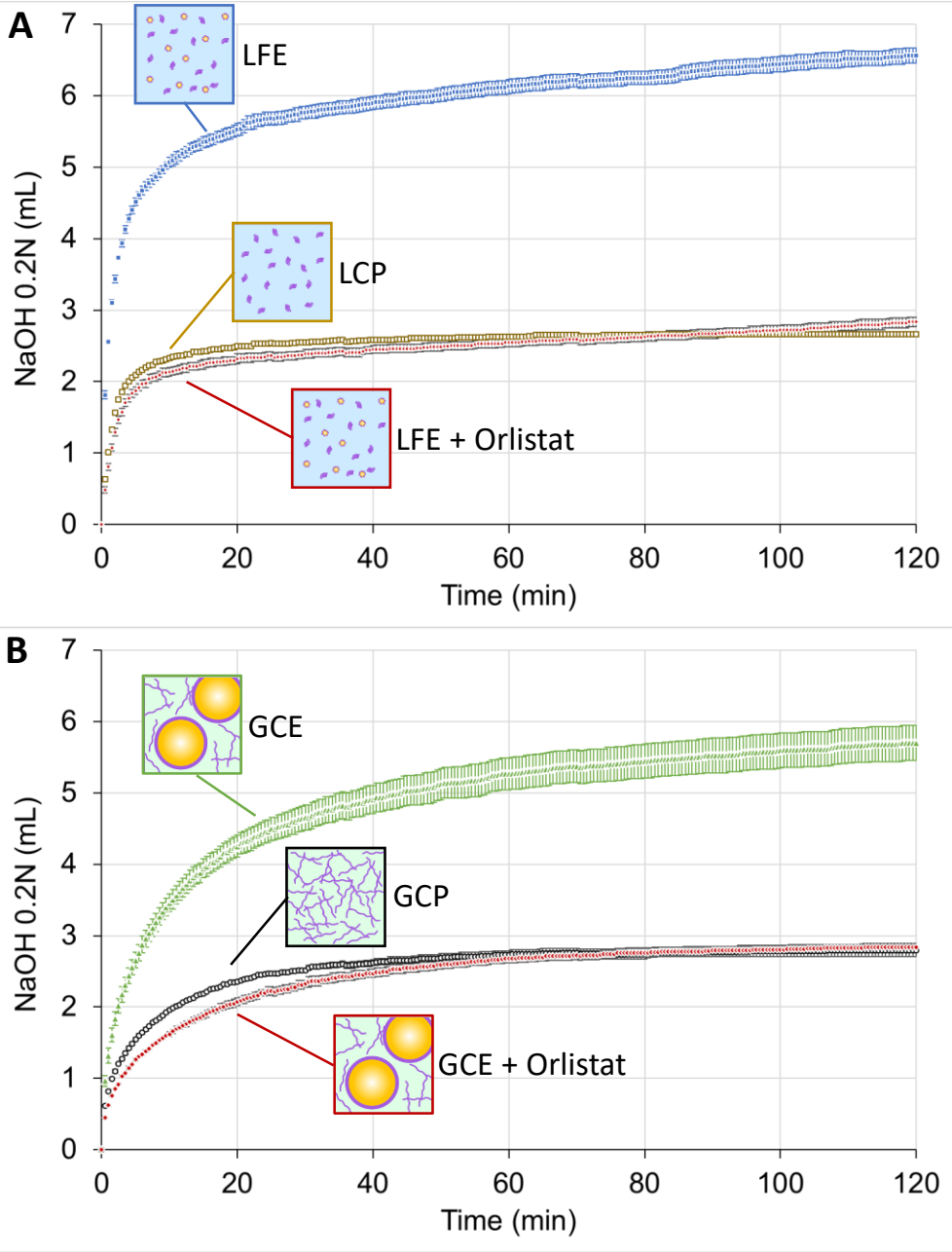


Fig. 3 pH-stat titration curves during the course of *in vitro* intestinal digestion (pH = 7.0) for (A) liquid matrices: Liquid Fine Emulsion (LFE) in absence (squares) or presence (diamonds) of Orlistat to be compared with its lipid-free counterpart (LCP, squares); and (B) gelled matrices: Gelled Coarse Emulsion (SCE) in absence (triangles) or presence (diamonds) of Orlistat to be compared with its lipid-free counterpart (GCP, circles). Data represent means ± SEM over at least 3 replicates.

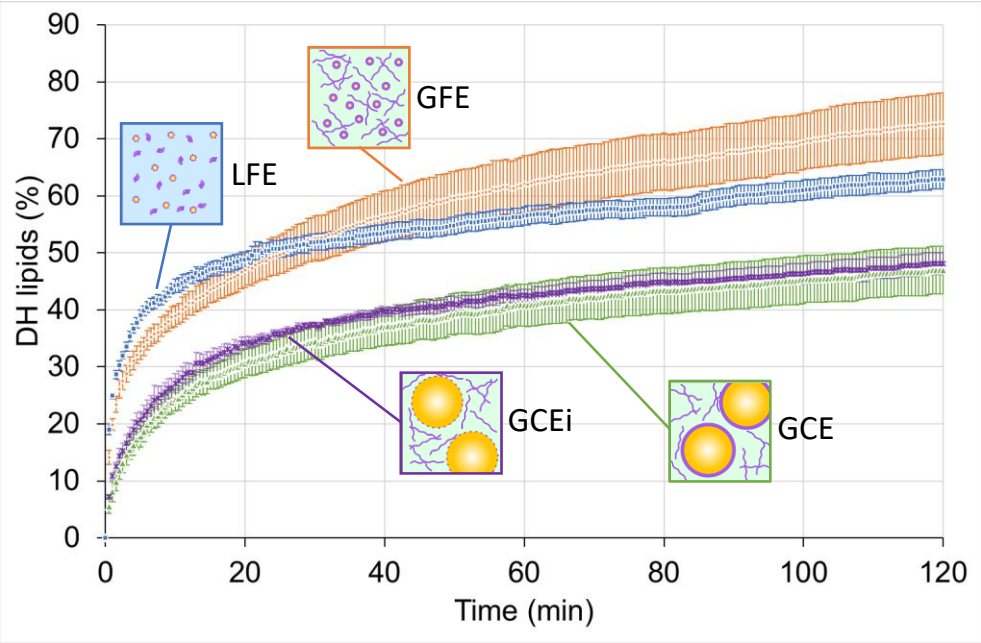


Fig. 4 Evolution of the degree of hydrolysis (DH) of lipids measured by pH-stat during the course of *in vitro* intestinal digestion (pH = 7.0). LFE stands for Liquid Fine Emulsion (squares), GCE for Gelled Coarse Emulsion (triangles), GFE for Gelled Fine Emulsion (diamonds), and GCEi for Gelled Coarse Emulsion with a modified o/w interface (stars). Data represent means ± SEM over at least 3 replicates.

Table 1: Overview of the designed matrices. All matrices contain 15 wt% whey proteins. When lipids are present, they represent 10 wt% of the emulsion. Data represent Mean ± SD over at least 3 replicates.

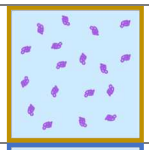
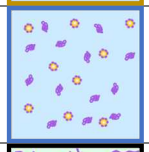
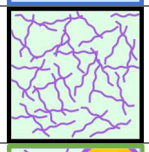
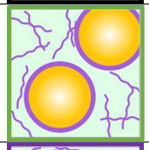
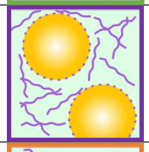
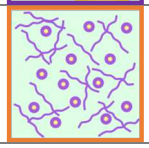
Matrix	Continuous phase	Protein state / heat treatment	G' for solid matrices (kPa)	$d_{4,3}$ (µm) of oil droplet	Schematic representation
LCP	Liquid	Native	–	–	
LFE	Liquid	Native	–	1.22 ± 0.06	
GCP	Gel	Gelation at 80 °C	39.0 ± 1.3	–	
GCE	Gel	Pre-emulsion at 70°C Gelation at 80 °C	46.9 ± 4.4	18.83 ± 0.64	
GCEi	Gel	Gelation at 80 °C	41.3 ± 0.5	19.13 ± 0.03	
GFE	Gel	Pre-emulsion at 70°C Gelation at 80 °C	68.8 ± 3.6	1.37 ± 0.01	

Table 2: Degrees of hydrolysis (DH) of proteins and lipids after 3 min and 120 min of gastric and intestinal digestion. Data represent Mean ± SD over at least 3 replicates.

	DH proteins (%) Gastric phase		**DH proteins (%) Intestinal phase**		**DH lipids (%) Intestinal phase**	
	3 min	**120 min**	**3 min**	**120 min**	**3 min**	**120 min**
LCP	1.1 ± 0.2	3.8 ± 0.2	37.1 ± 0.3	51.7 ± 0.4 [1]	–	–
LFE	0.9 ± 0.1	3.4 ± 0.1	32.1 ± 1.7 [2]	54.8 ± 2.2 [2]	33.6 ± 1.3	62.9 ± 4.0
GCP	0.6 ± 0.1	5.7 ± 0.4	29.4 ± 0.6	57.4 ± 2.7 [1]	–	–
GCE	0.6 ± 0.2	5.5 ± 0.2	24.8 ± 0.4 [2]	58.3 ± 1.7 [2]	14.6 ± 3.9	46.9 ± 8.8
GCEi	0.8 ± 0.1	6.0 ± 1.1			16.6 ± 2.4	48.1 ± 2.2
GFE	0.7 ± 0.1	5.7 ± 0.1			28.8 ± 3.0	72.7 ± 10.0

[1] DH of proteins determined using the OPA method, and used to estimate the value of α_{NH_2} in Eq. (2).

[2] DH of proteins determined by pH-stat in presence of Orlistat assuming a total lipase inhibition.