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In vitro digestion of foods using pH-stat and the INFOGEST protocol: Impact of matrix structure on digestion kinetics of macronutrients, proteins and lipids

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Abstract

It is currently admitted that food structure can facilitate or delay the release of nutrients during digestion and their absorption by the Human body. The aim of this study is to propose an *in vitro* method able to assess the behavior of lipo-proteinic matrices with different structures during digestion.Two model matrices of exactly the same compositions (10 % oil, 15 % whey proteins, w/w) were designed: i) A liquid emulsion (LE) made of small fat droplets (1 µm) dispersed in a liquid continuous phase containing native whey proteins, ii) A solid emulsion (SE) made of a continuous whey protein gel entrapping large oil droplets (20 µm). The two matrices were digested through an *in vitro* gastro-intestinal protocol based on the INFOGEST guidelines using pH-stat to monitor the enzymatic hydrolyses of both proteins and lipids. By further digesting lipid-free matrices in the same conditions, the contributions of the proteolytic and lipolytic reactions were evaluated. Significant differences were observed between matrices at both short and long digestion times. The initial rates of both proteolysis and lipolysis were slower for SE than LE because of the gel state of the continuous phase. At the end of the experiments, SE led to a smaller extent of lipolysis (DH_{lip_SE} = 51 % < DH_{lip_LE} = 81 %) but a greater extent of proteolysis (DH_{prot_SE} = 80 % > DH_{prot_LE} = 52 %) because of the higher sensitivity to digestion of denatured whey proteins. These results highlight the impact of matrix structure on enzyme accessibility, and show that the proposed method is suitable to monitor the digestion of complex food matrices.

Key words: Food structure; Gastro-intestinal digestion; Pancreatin; Lipolysis; Proteolysis; Emulsion gel.

Abbreviations

WPI: whey protein isolate; WP: whey proteins; FFA: free fatty acids; SSF: simulated salivary fluid; eSSF: electrolyte mix in the SSF; SGF: simulated gastric fluid; eSGF: electrolyte mix in the SGF; SIF: simulated intestinal fluid; eSIF: electrolyte mix in the SIF; LE: liquid proteinic emulsion-type matrix; LCP liquid proteinic continuous phase matrix; SE: solid proteinic emulsion-type matrix; SCP: solid proteinic continuous phase matrix; DH: degree of hydrolysis.

1 Introduction

It is now well-established that food structure can facilitate or limit the release and subsequent metabolization of nutrients. Understanding how food structure can modulate digestion kinetics and/or macronutrient digestibility has therefore become a main issue, which may lead to more-effective ways of maintaining a healthy diet. Knowledge on the food structure-physiology interactions may indeed be useful when considering the effects of particular diets and consumption habits, but also to design foods targeted for specific health functionalities (J. E. Norton, Wallis, Spyropoulos, Lillford, & Norton, 2014).

Food in the human digestive tract is subject to chemical-physics transformations while passing through the different compartments. In the stomach, gastric secretions contain hydrochloric acid decreasing the pH to the 1-5 range (Guerra et al., 2012), as well as gastric lipase and pepsin which initiate the lipolylitic and proteolytic reactions, respectively. In the case of solid food, the fragments are progressively grinded until their size is reduced enough to pass into the proximal part of the small intestine, the duodenum. In the small intestine, pH progressively rises to about 7, while the enzymes secreted in the pancreatic juice continue the hydrolysis of macronutrients. Pancreatic lipase, in association with its co-lipase, bile acids and other lipases perform lipolysis (H. Singh, Ye, & Horne, 2009), while proteases, such as trypsin and chymotrypsin, perform proteolysis (Mackie & Macierzanka, 2010; Turgeon & Rioux, 2011). The hydrolysis of lipids and of proteins is considered almost complete at the end of the small intestine.

In vitro gastrointestinal digestion experiments have proved to be a powerful means to gain knowledge on the nutritional value of foods as they allow condition controlling and easy sampling (Guerra et al., 2012). Although the capacity of *in vitro* approaches to mimic the physiological reality is never perfect, they avoid difficulties inherent to *in vivo* studies such as costs, ethical considerations, technical complexity and variability. *In vitro* systems are various, leading to a diversity of protocols and physiological relevance levels. Dynamic models consist of different compartments through which the

67 studied food passes while the digestive juices are dynamically added. In some cases, mechanical devices
68 are also incorporated in order to mimic the breakdown of foods. Even though these dynamic models are
69 based on *in vivo* digestion, their use is complex, mostly suitable for liquid foods, and involves
70 complicated engineering and high costs. On the other hand, cost effectiveness and good adaptability to
71 the needs of the studied foods are the main advantages of static *in vitro* systems. Their *in vivo* digestion
72 equivalence is nevertheless a drawback. Moreover, as they can be easily set up in most laboratories, a
73 great number of protocols have been proposed. This has raised the need for harmonization of the key
74 parameters (transit times, pH, enzymes used and their activities), a work that has been undertaken during
75 the INFOGEST cost action and which has recently led to the publication of an international consensus
76 (Minekus et al., 2014).

77 In association with static systems, the pH-stat titration method is a classical approach to monitor the
78 intestinal phase of *in vitro* digestions. It consists in maintaining constant the pH of a food in contact
79 with a digestive fluid mixture, whereas the enzyme-substrate reaction would normally modify it.
80 Therefore, the quantity of titrant added during the experiment versus time is directly related to the
81 production of hydrolyzed species. The method is easy to set up and directly provides the dynamics of
82 the reaction. pH-stat is most often used to measure the progress of the lipolytic reaction (Day et al.,
83 2014; Li & McClements, 2014), but can also be used to monitor the digestion of protein solutions or
84 gels. To our knowledge, the simultaneous study of lipolysis and proteolysis with pH-stat has not been
85 proposed yet.
86 Indeed, lipids and proteins are generally associated in real foods. Lipids are most often consumed in the
87 form of oil in water emulsions (milk, sauces…). In such systems, proteins can be found in the aqueous
88 continuous phase but also at the interface. The structural elements of an emulsion-type food matrix can
89 affect its digestion behavior. For instance, the size of the oil droplets is a major parameter governing the
90 kinetics of lipolysis (Armand et al., 1999; Giang et al., 2015). Lipolysis is an interfacial reaction.

Therefore, the rate of the reaction increases with the droplet surface area, i.e. smaller droplet sizes. Formulation and process conditions govern the droplet sizes in the emulsion, but transformations during digestion can also promote destabilization leading to a decrease in oil/water interface area (Clifton et al., 2011; Day et al., 2014). By controlling the emulsion stability against flocculation and coalescence, the nature of the interfacial layer is therefore also of importance for lipid digestion. Whey proteins can be used to achieve good stability of emulsions in gastric conditions (Clifton et al., 2011; Mantovani, Cavallieri, Netto, & Cunha, 2013). These proteins are reported to facilitate lipolysis compared to small surfactants, since they are subject to conformational changes through adsorption which can promote co-lipase anchorage and displacement action of bile salts (Mun, Decker, & McClements, 2007; Nik, Wright, & Corredig, 2010). Secondly, the physical state at a macroscopic scale of the continuous phase is an important parameter as liquid foods are prone to transiting through the stomach and intestine more rapidly than solid ones (J. E. Norton et al., 2014). Moreover, at a microscopic scale, whey proteins allow variations in the microstructure of the continuous phase. Their globular structure is sensible to heat and pH leading to various aggregated gel structures depending on gelation parameters. The sensitivity of whey proteins to hydrolysis is modulated by process conditions and is modified compared to native proteins (Macierzanka et al., 2012; T. K. Singh, Singh, Øiseth, Lundin, & Day, 2014).

The aim of this study was to develop a simple and robust method to assess the effects of the structures of complex food matrices on digestion. The association of a static *in vitro* digestion based on the INFOGEST protocol (Minekus et al., 2014) and a pH-stat method is proposed. The approach was used to investigate the digestion behaviors of two emulsion-type food matrices of identical compositions (10 wt% oil, 15 wt% whey proteins) but with different structures at the macroscopic and microscopic scales. Results are discussed in relation with the structural effects of the matrices on the kinetics and final extents (degrees of hydrolysis) of both lipolysis and proteolysis.

2 Materials and Method

2.1 Materials

Rapeseed oil (Fleur de Colza, Lessieur, France) was purchased from a local supermarket. Whey protein isolate (WPI) (Prolacta 80) was purchased from Lactalis, France. The protein content was 72.9 wt% on dry powder measured by the Kjeldahl method (Nx6.25) in triplicate. Pepsin (P6887), pancreatin (P1750), pancreatic lipase (L3126) and bile extract (B8631) were all of porcine origin and obtained from Sigma-Aldrich, France. The enzyme activities and bile salt concentrations in the bile extracts were measured according to the assays detailed in Minekus et al. (2014). All other materials were standard analytical grade. Water was Milli-Q water.

2.2 Preparation of food matrices

Liquid-emulsions (LE) were prepared as follows. 15 g of whey proteins (WP) were dispersed in 50 g of water and allowed to rehydrate under continuous stirring at room temperature for at least an hour. 10 g of rapeseed oil were then added with water to achieve a final weight of 100 g. A coarse emulsification was made using a rotor-stator homogenizer (Polytron PT3100D) for 5 min at 10,000 rpm. This coarse emulsion was then treated with ultra-sounds for 10 min (10 s on/off cycles) in order to further reduce the size of the oil droplets (Bioblock Ultrasonic Processor 20 kHz, 130 W). During the two emulsification processes, the temperature within the sample was maintained below 30°C using an ice bath. The final emulsion contained 15 wt% of whey proteins and 10 wt% of rapeseed oil.

Solid-emulsions (SE) were prepared as follows. 0.24 g of WP were dispersed in 60 g of water and allowed to rehydrate under continuous stirring at room temperature for at least an hour. 24 g of rapeseed oil were then added with water to achieve a final weight of 80 g and a coarse emulsion was made using the rotor-stator homogenizer for 5 min at 10,000 rpm. The emulsion was thereafter heated at 70°C for 5

136 min to allow a certain degree of denaturation of proteins adsorbed at the interface of oil droplets. It was
137 then mixed with a more-concentrated WP solution (22.33 wt%) in order to achieve final concentrations
138 of 15 wt% of whey protein and 10 wt% of rapeseed oil. The emulsion was finally poured into 40 mL
139 plastic cylindrical containers subsequently closed, immersed in a water bath, and heated for 30 min at
140 80°C to produce a hard gel.

141 In order to estimate the respective contributions of proteolysis and lipolysis from the pH-stat signal,
142 matrices only made of the continuous phases of the emulsions were also prepared and digested. The
143 liquid proteinic continuous phase matrix (LCP) therefore consisted in a 15 wt% native whey protein
144 solution while the solid proteinic continuous phase matrix (SCP) was made by heating LCP at 80°C for
145 30 min.

146 **2.3 Characterization of matrices**

147 *Granulometry:* the oil droplet size distribution of the liquid emulsion was measured using laser light
148 scattering (Mastersizer 2000, Malvern, France) with 1.47 as the refractive index of the oil and 1.33 as
149 the refractive index of the dispersant (water). An absorption value of 0.001 was chosen for the optical
150 properties of the emulsion. The volume-weighted mean diameter was calculated using $d_{4,3}$ ($d_{4,3} =$
151 $\sum n_i d_i^4 / \sum n_i d_i^3$).

152 *Confocal microscopy:* solid (SE) and liquid (LE) matrices were observed with the use of confocal laser
153 scanning microscopy (TCS SP2 AOBS by Leica, Solm, Germany) equipped with two He-Ne lasers with
154 excitation wavelengths of 543 nm and 633 nm. A labelling protocol (Huc et al., 2014) was performed
155 on lipids with the Bodipy 665/676 nm marker (Invitrogen, Carlbad, NM, USA) and on proteins with the
156 DyLight 488 nm marker (Thermo Fisher Scientific, Waltham, MA, USA). Images shown in this paper
157 correspond to superimpositions of images of the same area observed separately with the two markers.

158 *Gel rheology:* thermal gelation was also characterized using a rheometer (MCR 301, Anton Paar) fitted
159 with a Couette geometry ($R_{cup}/R_{cylinder}$ =1.085). The following sequences defined by the process used for
160 solid-emulsion were performed: a linear temperature ramp from 30 to 80 °C at 3°C/min, a stabilization
161 at 80°C for 30 min, a cooling back down to 30°C at 1°C/min, then a final stabilization of the temperature
162 at 30°C for 20 min. The shear storage modulus at 0.1 % deformation with 1 Hz frequency was measured
163 all along.

164 ## 2.4 *In vitro* digestion protocol

165 *In vitro* digestion experiments were carried out in a 50 mL bottle bathing in a jacketed beaker filled with
166 water that was maintained at 37°C by heat water circulation in the double wall. The whole set was
167 mounted on a magnetic stirrer (375 rpm).

168 *Electrolytes adjustment:* the digestion protocol used follows the recommendations given by Minekus et
169 al. (2014) but an adaptation in the composition of the electrolyte solutions was needed. Indeed,
170 experiments were first conducted by strictly following the protocol recommendations, but when trying
171 to perform pH-stat analyses during the intestinal phase the measured pH actually increased instead of
172 decreasing. As shown in Figure 1 (squares), the same phenomenon was observed when the simulated
173 intestinal fluid electrolyte solution alone (eSIF) was placed in an open beaker at the concentration used
174 during an intestinal digestion. The pH was continuously increasing above 7, while gas bubbles appeared
175 on the glass surfaces of the pH probe and the vessel. On the contrary, when replacing $NaHCO_3$ by NaCl
176 (circles in Figure 1**),** pH remained stable at 7. Following INFOGEST guidelines, $NaHCO_3$ is present at
177 85 mM, which corresponds to 7.14 g/L, and is the major contributor to hydrogenocarbonates in the final
178 solution. This concentration is much higher than the solubility limit of CO_2 (1.6 g/L at 20°C). Being in
179 contact with the ambient atmosphere with the pH-stat setting, the CO_2 concentration is therefore out of
180 equilibrium and is rejected in its gaseous form, leading to a basification of the milieu. In all prepared

181 solutions (oral, gastric and intestinal) of the present study, $NaHCO_3$ was therefore replaced by NaCl at
182 the same molar ratio in order to maintain the same ionic strength.

183 *Gastro-intestinal digestion:* apart from this salt replacement (NaCl instead of $NaHCO_3$) the protocol
184 used was the same as in Minekus et al. (2014). Briefly, 5 g of food matrix were mixed with 5 mL of
185 simulated salivary fluid (SSF) with no salivary α-amylase (no starch in the matrices). For solid
186 matrices, they were artificially grinded with a domestic kitchen food chopper (Braun Turbo 600 W type
187 4191) during 3 s to produce pieces of solid gel ranging from 3 to 5 mm beforehand. For liquid matrices,
188 the oral phase was only carried out for proper electrolyte concentrations and matrix dilution purposes.
189 A few minutes were taken to reach temperature equilibrium at 37°C before proceeding to the gastric
190 phase. To launch the gastric phase, 10 mL of simulated gastric fluid (SGF) were added to the mixture.
191 It consisted of SGF electrolyte solutions (eSGF), pepsin solution to achieve a 2,000 U/mL activity in
192 the final mixture, and HCl 1 N to lower pH to 3. The gastric phase was kept running for 2 h. Finally, 20
193 mL of simulated intestinal fluid (SIF) were added as the pH-stat titration was switched on. It consisted
194 of SIF electrolyte solutions (eSIF), bile extract solution (10 mM bile salts in the final mixture),
195 pancreatin added with pancreatic lipase (100 U/mL trypsin activity and 2,000 U/mL lipase activity in
196 the final mixture) and NaOH 1 N to adjust pH to 7. The intestinal phase was also 2 h long.

197 **2.5 pH-stat monitoring for the intestinal proteolysis and lipolysis**

198 As detailed in section 2.6 and 2.7, lipolysis and proteolysis performed by pancreatic enzymes lead to
199 the release of protons, and hence to a decrease in pH. To monitor the kinetics of the reactions, an
200 automatic titration unit (Titrino 7000, VWR, France) with a pH-stat programming to maintain the pH of
201 the mixture at 7 was used with NaOH 0.1 N as the titrant solution. All pH-stat titrations were carried
202 out in triplicates using a data acquisition frequency of 1 s.

Besides, 6 h long experiments were performed on the intestinal digestive mixture alone, without any added substrate, to determine whether the hydrolysis coming from the auto-digestion of the digestive fluids could be neglected. Results showed that this was not the case as there was a progressive addition of 0.420 mL of NaOH during the 2 h of intestinal phase (data not shown). As a conclusion, special care should be taken with such digestion protocol because auto-digestion of digestive fluids can contribute to the signal measured with pH-stat. In the present study, this contribution was taken into account for proper calculations of the degrees of hydrolysis.

2.6 Degree of Proteolysis

During the hydrolysis of a peptide bond, one carboxylic group is produced along with one α-amino group. According to their low pK values, carboxylic groups release their proton at pH 7, which are titrated by NaOH during the pH-stat experiment. However, the co-produced α-amino group show pK values of about 7.4 at 37°C (Adler-Nissen, 1986) and may therefore consume protons to form ammonium groups. In the latter case, the proton captured by the α-amino group therefore counteracts the one released by the carboxylic group and nothing is measured by titration (Nielsen, 1997). According to these phenomena, conversion from volumes of NaOH added into degree of hydrolysis ($DH_{protein}$, in %) can be calculated as follows (Asselin, Hébert, & Amiot, 1989; Spellman et al., 2003):

$$DH_{protein} = 100 \times \frac{V(NaOH) \times N(NaOH)}{\alpha(RNH_2) \times m(protein) \times h_{tot}} \quad (1)$$

where V(NaOH) is the volume (mL) of NaOH consumed during the titration, N(NaOH) is the NaOH normality (Eq/L), m(protein) is the protein mass (g), h_{tot} is the number of peptide bonds in the protein substrate (= 8.8 meqv/g for whey proteins (Spellman et al., 2003)) and $\alpha(RNH_2)$ is the mean degree of dissociation of α-amino groups, which can be calculated as follows:

$$\alpha(RNH_2) = \frac{10^{(pH-pK)}}{1+10^{(pH-pK)}} \quad (2)$$

where pK is the average dissociation constant for the α-amino groups liberated during hydrolysis.

The value of pK depends on several parameters such as temperature, peptide chain length or the nature of the terminal amino acid. It can therefore vary depending on the protein substrate and enzymes used. To get a proper estimate of the pK value in our conditions, complementary experiments using the OPA (ortho-phtalhadehyde) method were therefore conducted.

Quantification of DH using OPA: Degrees of hydrolysis were measured on LCP at the end an intestinal phase (with no preceding gastric phase) using the protocol proposed by Spellman et al. (2003) except that no SDS were added. Briefly, this method is based on the detection at 340 nm of a complex formed between the α-amino groups of peptides and OPA (ortho-phtalhadehyde) in the presence of N-acetyl-L-cysteine. The difference of absorbance between the hydrolyzed and the corresponding unhydrolyzed samples therefore provide the number of α-amino groups produced during the proteolytic reaction. In this study, a UV-Vis Spectrophotometer (Evolution 201, Thermo Scientific) was used and the unhydrolyzed samples were prepared by mixing LCP with heat-treated (90°C – 10 min) SIF. By comparing the DH measured by the OPA method for LCP to the corresponding volume of added NaOH with pH-stat, a mean pK of the α-amino groups of about 7.85 could be estimated, which leads to a value of 0.12 for $\alpha(RNH_2)$ (Table 1). Note that the OPA method cannot be used to determine DH values with solid matrices because of sampling difficulties. For SCP, the degree of hydrolysis was therefore estimated using Eq. 1 with an $\alpha(RNH_2)$ value of 0.12.

2.7 Degree of Lipolysis

When fully digested, one triglyceride molecule liberates two free fatty acids (FFA) coming from

243 positions sn-1 and sn-3 as well as the sn-2-monoglyceride. Two moles of NaOH are therefore required
244 to neutralize one mole of digested triglyceride. However, as for of proteins, all the FFA released are not
245 necessarily readily ionized depending on the pH of the milieu and the pK of the fatty acids. In a similar
246 way as previously described for proteolysis, the degree of hydrolysis for lipids (DH_{lipid}, in %) can be
247 calculated as follows:

$$DH_{lipid} = 100 \times \frac{V(NaOH) \times N(NaOH) \times M(lipid)}{\alpha(\mathrm{FFA}) \times m(lipid) \times 2} \quad (3)$$

248 where V(NaOH) and N(NaOH) have the same meaning as in (Eq. 1), m(lipid) is the oil mass (g),
249 M(lipid) is the molecular weight (g/mol) of the triglycerides in the oil (930 g/mol in this study), and
250 α(FFA) is the mean degree of dissociation of FFA carboxylic groups.

251 To estimate the value taken by α(FFA) in our experimental conditions, complementary 6h30-long
252 intestinal digestions were performed on reduced quantities (0.5 g) of an emulsion with 10 wt% rapeseed
253 oil and 0.1 wt% WP in order to reach 100 % liberation of fatty acids (controlled by the appearance of a
254 real plateau during pH-stat measurements). Taking into account the auto-digestion of pancreatic juice,
255 α(FFA) was estimated to be 0.54 (Table 1). Note that this value corresponds to a pK of about 6.9 (Eq.
256 2), which is in good agreement with the reported pK values for long chain fatty acids (Fernandez et al.,
257 2008).

3 Results and Discussion

3.1 Characterization of the designed emulsions

Confocal microscopy images and droplet size distributions of the two emulsions are presented in Figure 2. As expected**,** oil droplets are bigger in the solid emulsion (SE) and seem more poly-dispersed (Figure 2B and 2D) than in the liquid emulsion (LE) (Figure 2A and 2C). Mean droplet diameters were 0.748 ± 0.055 µm for LE and 19.842 ± 0.569 µm for the SE emulsion before the first heat treatment (Figures 2C and 2D, respectively). Droplet size changes in SE after the first heat treatment were controlled using laser granulometry and found not significant. In LE, a uniform green background can be seen, suggesting a homogenous protein solution continuous phase (Figure 2A). On the contrary, the continuous phase made of proteins shows irregularities in the SE structure (Figure 2B), suggesting a non-homogenous gelation process during the second heat treatment (30 min at 80°C). The rheology test performed on the SE gave the value of 58 ± 1 kPa, corresponding to a quite high hardness. As a conclusion, the two designed matrices were different in both microstructure (droplet diameter ratio SE / LE = 26.5) and macrostrucure (liquid *vs* solid).

3.2 Digestion of the liquid and solid emulsions

The results from pH-stat experiments during the intestinal digestion of the liquid and solid emulsions are shown in Figure 3A. Both kinetics are similar in shape, with a fast rate at the beginning of the titration followed by a progressive slowing down leading to a pseudo-plateau that was reached earlier for LE (~ 0.1 h) than SE (~ 1 h). The final volume of added NaOH is also similar for both matrices (about 9 mL). A semi-log scale (Figure 3B) was also used to give a complementary view of the data at both short and long digestion times. It confirms the faster liberation of titrated elements during the first minutes (6.3 mL within 0.1 h) for LE. Then the kinetics slow down to reach 8.9 mL at the end of the 2 h of digestion but do not tend to a plateau, indicating that the reactions were not ended at this time. The SE curve

shows a rather reduced rate of hydrolysis at the beginning of the digestion. It starts to increase at a smaller rate than the LE curve and finally crosses it after 23 min. At the end of the 2 h, the SE curve has attained 9.5 mL Like for LE, a real plateau is not reached after 2 h.

The different shapes of the titration curves can be related to the different physical states of the matrices. Protein molecules and oil droplets are moving freely in LE. Therefore, both substrates are rapidly exposed to the enzyme actions. On the contrary, gel pieces were still visible for SE after 2 h of gastric phase, implying that oil droplets were still entrapped in the proteinic network of gel pieces for this matrix. Therefore, enzymes have to diffuse into the SE gel pieces before finding their substrate, which leads to slower digestion kinetics. Luo, Boom, & Janssen (2015) have also compared *in vitro* gastric digestions of WP solutions and gels and explain similar behaviors by a "zipper" type mechanism for the proteins-in-solution reaction (fast initial hydrolysis followed by a steadier stage) opposed to a "one-by-one" mechanism for proteinic gels (slower initial rate followed by a more-sustained trend).

3.3 Digestion of the proteinic continuous phase matrices (SCP and LCP)

In order to estimate the proteolysis contribution during pH-stat experiments performed on SE and LE, lipid-free matrices with the same structures were studied. This implicitly supposes that the absence of oil droplets does not significantly influence the kinetics of proteolysis and the structure of the protein network. Under these hypotheses, one can expect the overall trends and final extents of digestion to be largely unchanged.

It can first be noted that, as for SE, gel pieces of SCP were almost not modified in shape at the end of the gastric phase, implying limited pepsin hydrolysis and the persistence of solid state effects on digestion. The pH-stat results obtained during the intestinal digestion of the liquid and solid proteinic continuous phase matrices (LCP and SCP) are presented in Figures 4A and 4B on a linear and semi-log scale, respectively. Both curves draw similar trends to the ones shown in Figure 3 with a faster reaction

304 rate in the first minutes of digestion, and similar inflexion points and slowdowns. This result validates
305 the major contribution of the continuous phase's physical state on hydrolysis kinetics. In this case,
306 however, a real plateau is reached for both LCP and SCP (Figure 4A and B), indicating that the
307 proteolysis reactions were finished within the 2h of intestinal digestion. The major difference between
308 the liquid and solid matrices lies in the final levels of NaOH added which, after 2 h, were of 4.2 mL and
309 6.5 mL, respectively. This means that the contribution of proteolysis to the overall pH-stat signal
310 measured during digestion of the lipo-proteinic emulsions was important, and represented about 50 %
311 and 70 % of the added NaOH for LE and SE, respectively.

312 At the end of the intestinal phase, the degree of hydrolysis was estimated to be 52 ± 2 % for LCP and
313 80 ± 1% for SCP (Table 1). These results are in good agreement with the recent studies of Picariello et
314 al. (2015) and Egger et al. (this issue) who reported values of about 60 % using the INFOGEST *in vitro*
315 protocol to digest dairy proteins. Besides, the greater final level reached with SCP than LCP can
316 probably be attributed to the greater sensitivity of denatured whey proteins to hydrolysis by intestinal
317 proteases. This finding is indeed consistent with the literature (Macierzanka et al., 2012; T. K. Singh et
318 al., 2014).

319 **3.4 Estimation of the lipolytic kinetics**

320 The contributions of the lipolysis reactions to te SE and LE kinetics were approximated by subtracting,
321 point by point, the pH-stat data collected with proteinic continuous phase (LCP and SCP) from the ones
322 collected with emulsions (LE and SE). Results are presented in Figure 5 (linear and semi-log). The curve
323 shapes are similar to the ones obtained for the digestions of emulsions (LE, SE) and proteinic continuous
324 phases (LCP, SCP) (Figures 3 and 4**)**, meaning a faster rate in the first minutes followed by slower
325 kinetics after an inflexion point. Comparatively to Figure 4 though, the final amount of added NaOH
326 obtained with the liquid matrix is higher than the solid one (4.7 mL *vs*. 2.9 mL). Moreover, a real plateau

was not reached after 2 h for both curves, as previously observed with LE and SE (Figure 3). This demonstrates that the kinetics of lipolysis are slower than the kinetics of proteolysis (Figure 4).

Using Equation 3, a final degree of lipolysis of 81 ± 10 % and of 51 ± 6 % for the lipids of LE and SE were found, respectively (Table 1). The order of magnitude of these values are in good accordance with the literature (Giang et al., 2015; Li & McClements, 2010; Williams et al., 2012). Besides, the higher level of lipolysis for LE than SE may be related to the physical state of the continuous phase and to the sizes of the oil droplets in the emulsions. Indeed, lipids are readily available for hydrolysis as soon as enzyme adjunction has been made for LE, whereas pancreatic lipase have to diffuse into the gel pieces and/or oil droplets must first be liberated from the protein gel network for the reaction to take place. Moreover, the faster rate of titration for LE may also be related to the smaller sizes of oil droplets dispersed in it. Lipolysis is an interfacial enzymatic reaction; so, for the same amount of lipids, the smaller the droplets, the larger the oil/water interfacial area, and the faster the reaction.

The fact that lipolysis is not finished at the end of the experiments is a classical result with long-chain triglycerides (Clifton et al., 2011; Giang et al., 2015; Li & McClements, 2010). The slowdown in the kinetics could be due to an inhibition of the reaction by the lipolysis products (Mun et al., 2007; Pafumi, 2002) or a certain degree of coalescence that decreased the interface area available to the lipase action (Giang et al., 2015; Clifton et al., 2011; Day et al., 2014).

4 Conclusion

This study was aiming at developing a methodology to compare structure-related effects of model lipo-proteinic foods on their digestion. The feasibility and interest of using the pH-stat method in association with the INFOGEST *in vitro* digestion protocol was proved. It resulted in a simple and quantitative method that allows a continuous monitoring of the intestinal digestions of both proteins and lipids. This method is a very valuable add-on to the INFOGEST protocol because it makes possible to follow *in vitro* digestion in real time without the requirement of samplings at specific time points.

Applying this method to model emulsion-type food matrices with the same compositions but different structures allowed observing differences in their digestion kinetics and extents for both proteins and lipids. The physical state of the continuous phase and the native *versus* denatured state of whey proteins were found to be important factors of the overall dynamics for both protein and lipid digestion.

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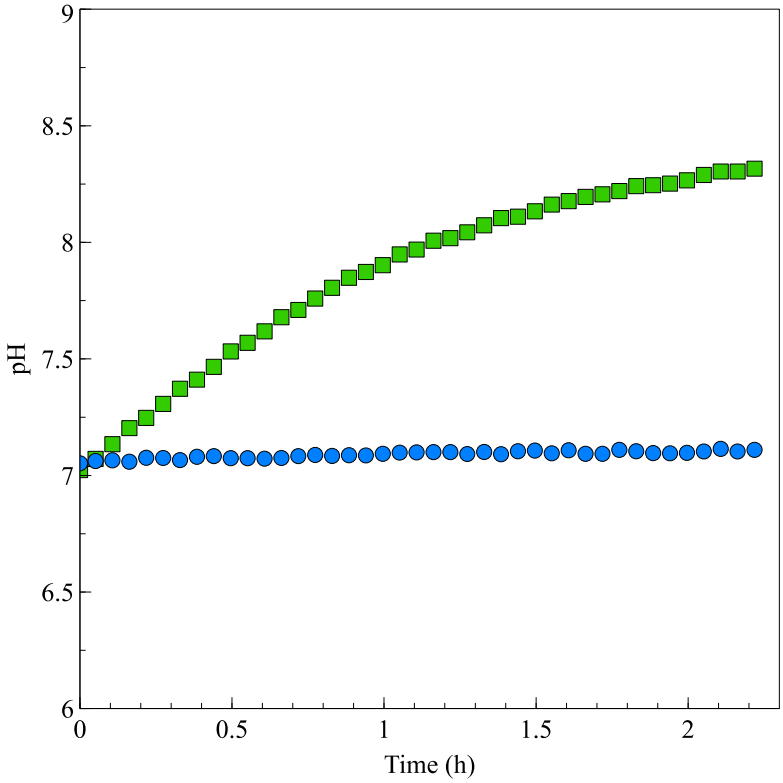


366
367 **Figure 1**: pH evolution of the electrolyte solution used for intestinal phase (eSIF) when placed in an
368 open beaker: (squares) eSIF prepared with respect to the *in vitro* digestion recommendations proposed
369 by Minekus et al. (2014), and (circles) eSIF in which $NaHCO_3$ was replaced by NaCl.

370

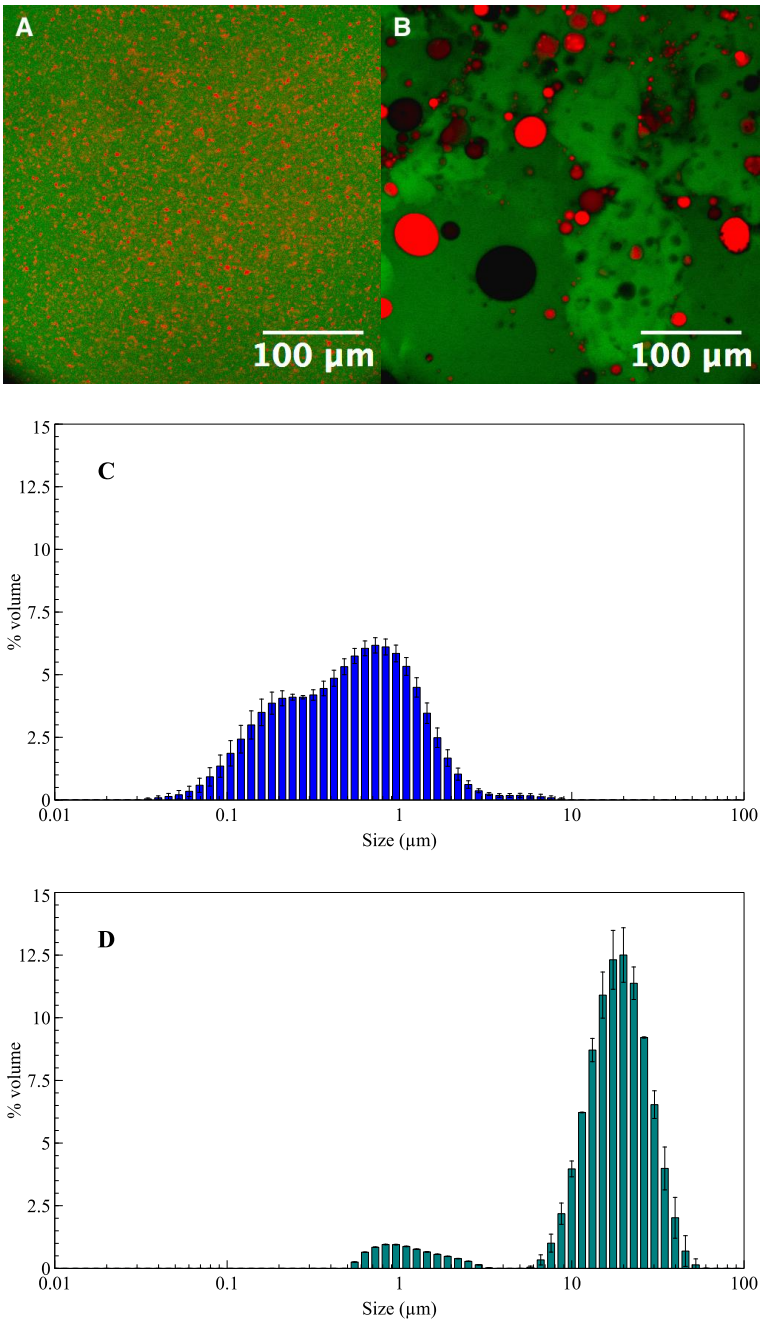
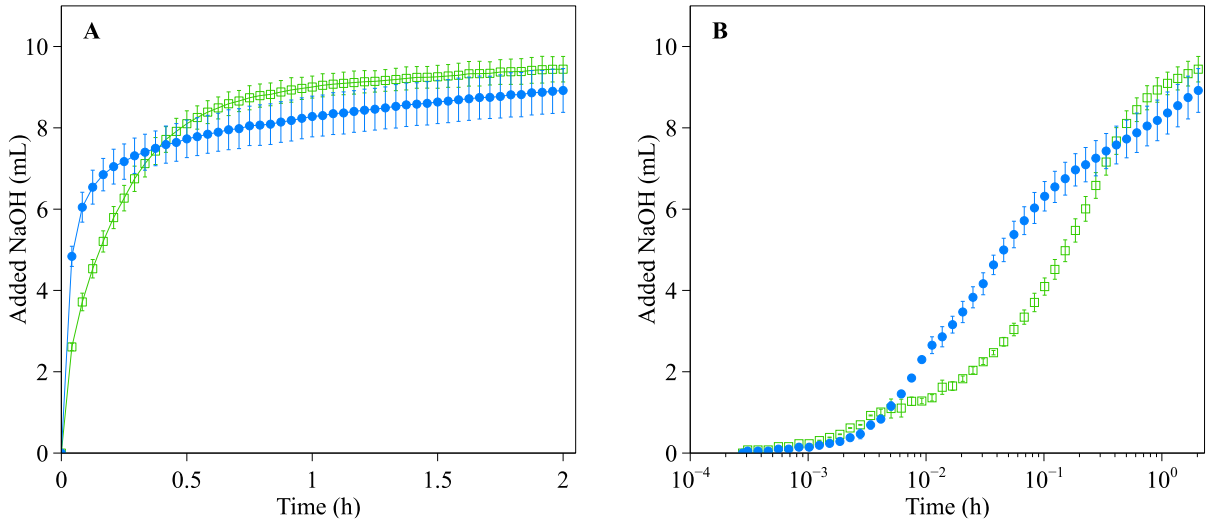


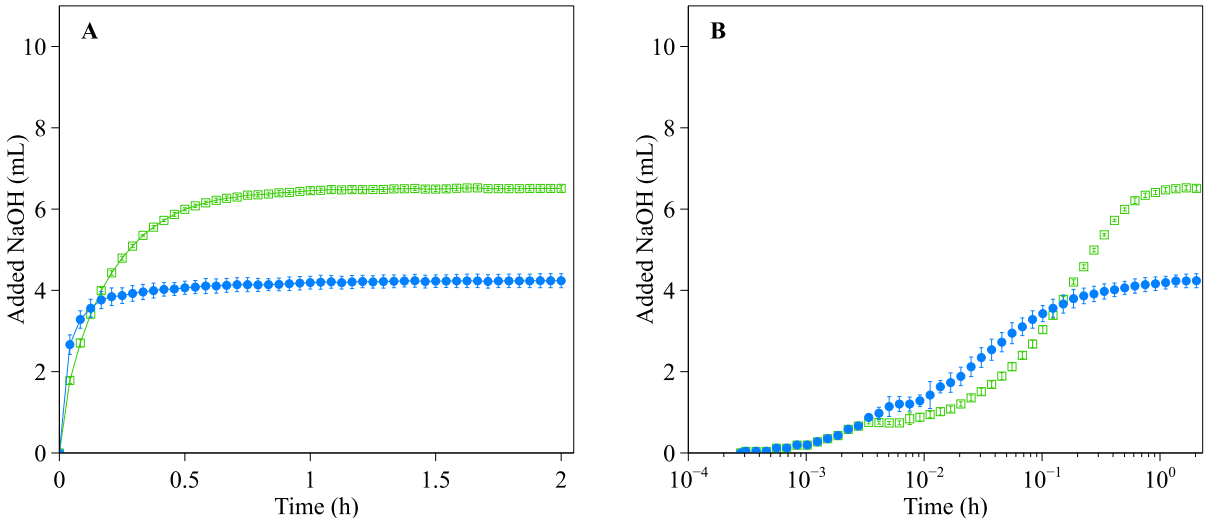
Figure 2: Characterization of the microstructures of emulsions containing 15 wt% rapeseed oil and 10 wt% whey proteins. Confocal micrographs of the liquid emulsion (A) and the solid emulsion (B) at magnification x40 (lipids appear in red and proteins in green). Laser light scattering determination of the sizes of the oil droplets in the liquid emulsion (C) and in the emulsion prepared before heat treatment of the solid matrix (D).



379

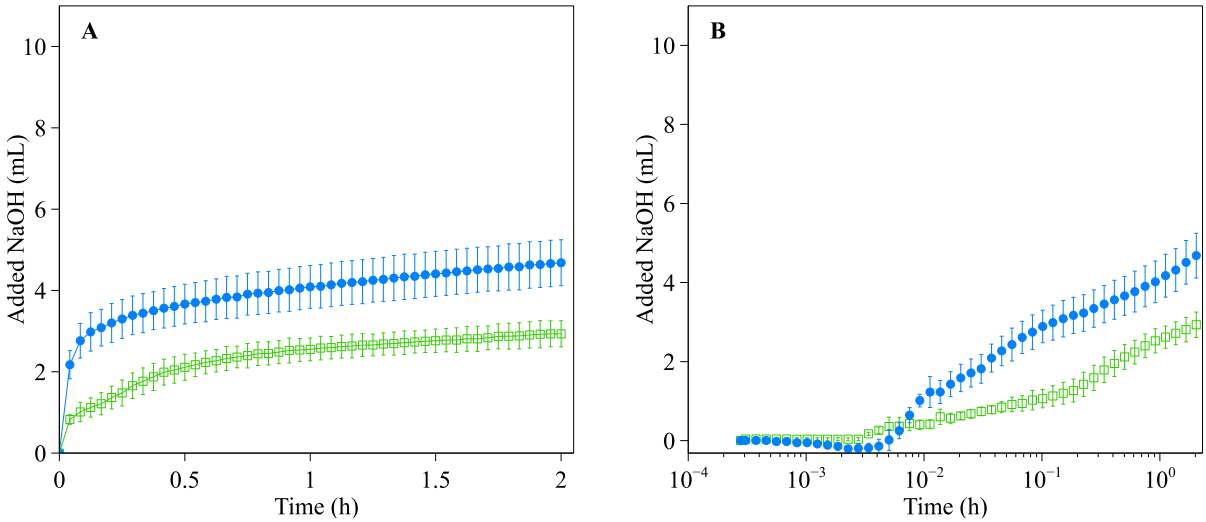
380 **Figure 3**: pH-stat profiles measured during the intestinal phases of digestion for the liquid emulsion
381 (filled circles) and the solid emulsion (open squares) on linear (A) and semi-log (B) scales. Values are
382 means ± SD over 3 replicates.

383



384

385 **Figure 4**: pH-stat profiles measured during the intestinal phases of digestion for the liquid proteinic
386 continuous phase matrix (filled circles) and the solid proteinic continuous phase matrix (open squares)
387 on linear (A) and semi-log (B) scales. Values are means ± SD over 3 replicates.



388

389 **Figure 5**: pH-stat profiles of the lipolysis during the intestinal phases of digestion estimated by
390 subtracting Figure 4 from Figure 3, for lipids in the liquid emulsions (filled circles) and in the solid
391 emulsions (open squares), on linear (A) and semi-log (B) scales. Values are means ± SD over 3
392 replicates.

393

394 **Table 1**: Degree of hydrolysis at the end of 2h of intestinal digestion and mean degree of dissociation
395 of the peptide α-amino groups and fatty acid carboxyl groups.

396
397

Nutrients	Matrix	Degree of hydrolysis (%)	Degree of dissociation (correction factor)
Proteins	Liquid	52 ± 2	0.12 (α in equation 1)
	Solid	80 ± 1	
Lipids	Liquid	81 ± 10	0.54 (α in equation 3)
	Solid	51 ± 6	

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