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## INFOGEST static *in vitro* simulation of gastrointestinal food digestion

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## Abstract

Developing a mechanistic understanding of the impact of food structure and composition on human health has increasingly involved simulating digestion in the upper gastrointestinal tract. These simulations have used a wide range of different conditions that have often very little physiological relevance and this impedes the meaningful comparison of results. The standardised protocol presented here is based on an international consensus developed by the COST INFOGEST network. The method is designed to be used with the standard laboratory equipment and limited experience to encourage a wide range of researchers to adopt it. It is a static digestion method that uses constant ratios of meal to digestive fluids and a constant pH for each step of digestion. This makes the method simple to use but not suitable for simulating digestion kinetics. Using this method, food samples are subjected to sequential oral, gastric and intestinal digestion while parameters such as electrolytes, enzymes, bile, dilution, pH and time of digestion are based on available physiological data. This amended and improved digestion method (INFOGEST 2.0) addresses a number of ambiguities in the original scheme such as the inclusion of the oral phase and the use of gastric lipase. The method can be used to assess the end points resulting from digestion of foods, to analyse the digestion products (e.g. peptides/amino acids, fatty acids, simple sugars, etc.) and evaluate the release of micronutrients from the food matrix. The whole protocol can be completed in ~7 days including ~5 days required for determination of enzyme activities.



## 40 Introduction

41 The worldwide prevalence of diet-related diseases has been on the increase for the last few  
42 decades.<sup>1</sup> Large scale human intervention trials have been used to correlate diet with the  
43 health of different demographic groups. However, to understand the physiological response  
44 to specific foods, it is necessary to follow the complex digestive processes within the human  
45 digestive tract in more detail. This can be achieved with invasive procedures such as  
46 aspiration from the stomach<sup>2</sup> or small intestine<sup>3</sup> or with less invasive imaging technologies  
47 (e.g. magnetic resonance imaging<sup>4</sup>) and wireless, telemetric systems<sup>2,5</sup>. Animal models are  
48 also widely used, though it generally involves animal death or surgical approaches placing  
49 cannulas into digestive organs to access the contents of the gastrointestinal tract. The  
50 relevance of animal models for understanding food digestion in humans is also regularly  
51 questioned. In summary, *in vivo* (human or animal) intervention trials can be difficult to  
52 undertake, unsuitable, expensive or not justifiable on ethical grounds. For these reasons, *in*  
53 *vitro* models have been used for many decades to simulate the digestion of food.

### 54 Development of the Protocol:

55 There are several types of *in vitro* digestion methods that are commonly used for food, which  
56 can be divided into static and dynamic methods. These models aim to simulate the  
57 physiological conditions of the upper gastrointestinal tract, namely the oral, gastric and small  
58 intestinal phases. Most dynamic models<sup>6-10</sup> have been shown to be suitable for simulating the  
59 digestion of foods and pharmaceutical products in different population groups and for  
60 different purposes<sup>11</sup>. However, these models are relatively complex, expensive to set up and  
61 maintain, and therefore may not be available to the majority of food researchers.

62 Owing to its simplicity, static models, which use a constant ratio of food to enzymes and  
63 electrolytes, and a constant pH for each digestive phase, have been widely used for many  
64 decades for food, animal feed and pharmaceutical purposes<sup>12-14</sup>. Static *in vitro* digestion  
65 models have been shown to be very useful in predicting outcomes of *in vivo* digestion<sup>15,16</sup>.  
66 There are standardised static models<sup>17</sup> that vary in complexity<sup>18,19</sup>, which are used for  
67 simulating the gastrointestinal behaviour of pharmaceutical products (Pharmacopeia  
68 methods)<sup>17</sup>. Other static methods were developed for assessing the *in vitro* bioaccessibility of  
69 soil contaminants<sup>20</sup>, heavy metals in particular, or mycotoxins in food<sup>21</sup>. These methods,  
70 developed and standardised<sup>22</sup> by the Bioaccessibility Research Group of Europe (BARGE)  
71 were based on available physiological data reported by landmark papers such as Dressman  
72 et al.<sup>23</sup> or the Geigy tables<sup>24</sup>. The static methods of the BARGE group and Pharmacopeia

73 procedures were important milestones in the evolution of standardised *in vitro* digestion  
74 methods. However, their experimental conditions, purpose and endpoint were found to be  
75 unsuitable for digesting food due to the complexity and variability of food structures as well  
76 as very different research questions in food science. This resulted in the use of a great  
77 number of digestion methods, reviewed by Hur et al.<sup>25</sup>, with slight but significant variations in  
78 parameters such as pH, duration, enzyme concentration and activity, composition of  
79 simulated digestive fluids, etc.

80 Hence, the need for a harmonisation of digestion conditions was identified and the  
81 international INFOGEST<sup>26</sup> network ([www.cost-infogest.eu](http://www.cost-infogest.eu)) of multidisciplinary experts (food  
82 science, nutrition, gastroenterology, engineering, enzymology, etc.) from more than 35  
83 countries was established. One of the primary outcomes of this network was an international  
84 consensus on a set of digestion parameters for a static *in vitro* simulation of adult digestion  
85 suitable for food. The method, generally referred to as the INFOGEST method, was  
86 published<sup>27</sup> and experimental parameters were justified and discussed in great detail in  
87 relation to available *in vivo* physiological data. Some of the previous digestion methods  
88 outlined above were used as a starting point. Since its publication in 2014, this *in vitro*  
89 digestion method has received a *Highly Cited Paper* status for Agricultural Sciences with  
90 more than 550 citations in Web of Science and has been extensively used all over the world  
91 for numerous purposes, with a variety of foods and different endpoints. The current article  
92 builds on that publication and clarifies a number of aspects of the original protocol, leading to  
93 an improved INFOGEST 2.0 protocol described here.

## 94 **Overview of the Procedure**

95 The digestion procedure is summarised in **Figure 1**. It can be divided into three phases:  
96 preparation, digestion procedure and sample treatment with subsequent analysis. For  
97 preparation of the *in vitro* digestion, the activity of all digestive enzymes and the  
98 concentration of bile salts should be determined experimentally, using the recommended  
99 standardised assays for amylase, pepsin, lipase (both gastric and pancreatic), trypsin and  
100 chymotrypsin, outlined in **Box 1**, described in detail in the Supplementary Information. This  
101 first preparation step is of the utmost importance and failure to correctly assay enzyme  
102 activity will lead to incorrect rates of digestion of components (e.g. proteins)<sup>28</sup>, potentially  
103 changing the overall digestion of the food.

104 The digestion involves the exposure of the food to three successive digestive phases: oral,  
105 gastric and intestinal. For static *in vitro* digestion methods, the experimental conditions are  
106 constant, during each phase. The oral phase involves dilution of the food 1:1 (w/w) with

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107 simulated salivary fluid (SSF), with or without salivary amylase, and for solids or semi-solids  
108 simulated mastication of the food. If used, exposure of the food to salivary amylase is limited  
109 to two minutes at pH 7. The oral phase needs to be included in all simulated digestion  
110 procedures, regardless of the state of the food (liquid or solid) in order to provide consistency  
111 of dilution. Further clarification regarding the preparation of the food and the oral phase can  
112 be found in the Experimental Design.

113 The oral bolus is then diluted 1:1 (v/v) with simulated gastric fluid (SGF) and gastric enzymes  
114 (pepsin and gastric lipase) and incubated under agitation at pH 3.0 for two hours. The gastric  
115 chyme is then diluted 1:1 (v/v) with simulated intestinal fluid (SIF), bile salts and pancreatic  
116 enzymes (pancreatin based on the activity of trypsin or as individual enzymes) and incubated  
117 at pH 7 for a further two hours.

118 The experimental conditions for the digestion procedure such as pH, time of digestion and  
119 enzyme activity etc. were based on available physiological data of the fed state for a typical  
120 meal and were described and justified in detail in Minekus et al.<sup>27</sup> For this improved  
121 INFOGEST 2.0 method, the use of gastric lipase is recommended, hence a detailed  
122 justification of the type and activity of the gastric lipase is provided in the Experimental  
123 Design section.

124 The last step of the digestion procedure involves sampling, sample treatment, storage and  
125 subsequent analysis of samples. This step should be carefully considered prior to digestion  
126 as it may differ from case to case due to different endpoints, purposes of the digestion  
127 experiment and type of analysis. A description of sample treatment can be found in the  
128 Experimental Design and Table 1.

129

## 130 **Advantages and limitations**

131 Static *in vitro* digestions are the simplest methods to simulate *in vivo* food digestion. While  
132 there are clear weaknesses in these simple models, they have obvious advantages over  
133 more complex methods. The main strengths of static *in vitro* models is the good intra- and  
134 inter-laboratory reproducibility, robustness, simplicity, relatively low cost and easy  
135 assessment of each digestion phase. This latter point makes them very suitable for  
136 mechanistic studies, hypothesis building and screening. It was one of the aims of the  
137 INFOGEST network not just to standardise *in vitro* methods but to agree on experimental  
138 conditions that are based on available physiological data to be as close as possible to the *in*  
139 *vivo* equivalent, while keeping the method sufficiently simple to reproduce all over the world.  
140 The clear definition of standardised experimental conditions and procedures is one of the  
141 major advantages of the INFOGEST method. Egger et al. <sup>28</sup> showed very good lab to lab  
142 reproducibility of results from the *in vitro* digestion of skim milk from powder, in regards to  
143 peptide patterns. Some weaknesses were identified and have been addressed subsequently.  
144 The recommendation of standardised enzyme assays (including units) significantly added to  
145 the precision and reproducibility of the digestion procedure as previously, a number of  
146 common but slightly different enzyme assays were being used, resulting in the application of  
147 a wide range of enzyme activities during digestion experiments. The end point of this  
148 INFOGEST method was recently compared to digests obtained in human jejunum after  
149 casein and whey protein ingestion<sup>16</sup> showing excellent correlation in protein degradation and  
150 peptide patterns, as explained below in Applications.

151 However, static digestion methods have known limitations and cannot mimic the complex  
152 dynamics of the digestion process or the physiological interaction with the host. For example  
153 for the gastric phase, the pH is kept constant, there is a lack of the gradual addition of gastric  
154 fluid (acid, minerals, pepsin) and an absence of gradual gastric emptying. In addition, the  
155 enzyme activity in each digestive phase is kept constant, regardless of the type of food and  
156 whether the food contains high or low amount of substrate e.g. proteins, lipids and  
157 carbohydrates. The intestinal phase is treated as one phase rather than those of the  
158 sequential duodenal, jejunal and ileal phases, which exhibit different dilutions, mineral  
159 content, pH, enzyme activities, microbial content, etc. These shortcomings render the  
160 method unsuitable for detailed kinetic analysis of the different stages of the digestion  
161 process. However, *in vivo* comparison shows good correlation with the INFOGEST method  
162 at the end points of each digestion phase.<sup>16,29</sup> For this reason, the static model should only  
163 be used to assess digestion endpoints and not kinetics.



164 In some cases, a slight alteration of the procedure may be considered to more accurately  
165 reflect physiological conditions. For example, during the gastric *in vivo* digestion of food  
166 containing probiotic bacteria, the bacteria are exposed to a range of pHs, as low 1 at the end  
167 of the gastric emptying. Hence, a static method with a constant pH of 3.0 for the gastric  
168 phase may fail to accurately predict probiotic survival and a lower pH or a dynamic gastric  
169 model should be chosen. Studying the bioaccessibility of phytochemicals such as  
170 polyphenols and carotenoids, the model allows the realistic release from a food into the  
171 aqueous phase. However, specific hydrolytic processes occurring at the brush-border are  
172 currently not simulated, and additional steps such as centrifugation of the digesta are needed  
173 to separate the bioaccessible phases. An extension including colonic fermentation, an  
174 important step in the bioactivation of several phytochemicals, would further enhance the  
175 physiological appropriateness. Finally, for the assessment of the bioaccessibility of small  
176 amounts of contaminants in food, such as heavy metals, environmental pollutants, or  
177 mycotoxins, alternative methods reflecting extensive digestion and “worst-case scenarios”<sup>20</sup>  
178 can be applied.

## 179 **Applications**

180 The method described has been used to assess the release of carotenoids and phenolic  
181 compounds from different matrices, such as, carotenoids in fruits<sup>30,31</sup>, carotenoids in  
182 tomatoes compared to tomatoes subjected to pulsed electric fields<sup>32</sup>,  $\beta$ -carotene protected by  
183 microencapsulation<sup>33</sup> and resveratrol encapsulated in protein nanoparticules<sup>34</sup>. However,  
184 most studies have been dedicated to the evaluation of protein, lipid and starch digestion in  
185 foods or modified carriers. Protein digestion has been widely assessed in different dairy  
186 products<sup>35,36</sup>, or in isolated milk proteins, such as lactoferrin with different iron contents and  
187 after mild heat treatment<sup>37</sup>. The stability of proteins to gastrointestinal digestion has been  
188 proposed as an additional piece of information for the allergenicity assessment of novel  
189 proteins<sup>38</sup>. With this focus, the INFOGEST method was also applied to the study of the  
190 immunogenic potential of peptides from pasta<sup>39</sup>, hazelnut<sup>40</sup>, and peanut<sup>41</sup>, which are resistant  
191 to gastrointestinal digestion. Using a pH-stat to monitor enzymatic hydrolysis, it was shown  
192 that solid emulsions led to a lesser extent of lipolysis but a greater degree of proteolysis  
193 compared to liquid emulsions due to the higher sensitivity of denatured whey proteins to  
194 gastrointestinal enzymes<sup>42</sup>. The tendency of dairy rennet gels to form compact protein  
195 aggregates during gastric digestion has also been assessed<sup>43</sup>. Other applications of this  
196 protocol include the evaluation of novel biopolymers designed for a controlled nutrient  
197 release<sup>44,45</sup>, or the digestive stability of transgenic microRNAs in genetically modified plants<sup>46</sup>.

198 An inter-laboratory trial applying different *in vitro* digestion protocols clearly demonstrated a  
199 good reproducibility obtained by using the standardised INFOGEST protocol. It also  
200 highlighted the importance of correctly applying standardised pepsin activity assays, which is  
201 a key factor for proper gastric protein hydrolysis<sup>28</sup>. A special effort was made to validate and  
202 compare the results from this *in vitro* digestion protocol with *in vivo* data. For instance,  $\beta$ -  
203 cryptoxanthin bioavailability from pasteurised orange juice was found to be higher than from  
204 fresh oranges in a randomised crossover human study, and from the *in vitro* digestion an  
205 increased bioaccessibility could also be inferred<sup>47</sup>. Several studies have focused on protein  
206 digestion and the comparison with *in vivo* digestion in human or animal models. The results  
207 from the *in vitro* gastrointestinal digestion of skim milk powder were compared with *in vivo*  
208 porcine samples collected from the stomach and several sites in the intestine<sup>29</sup>. Protein  
209 degradation and peptides generated at the end of the gastric phase correlated well with *in*  
210 *vivo* gastric peptides while the *in vitro* intestinal phase correlated well with the *in vivo*  
211 samples taken in the median jejunum. Human jejunal digests after the oral ingestion of  
212 casein and whey protein were compared with the intestinal digests obtained using the  
213 standardised INFOGEST method<sup>16</sup>. *In vivo* and *in vitro* intestinal digests showed common  
214 protein regions that are resistant to digestion and a high number of identical peptide  
215 sequences, concluding that the INFOGEST *in vitro* method is a good approximation to the  
216 end points of gastrointestinal digestion of milk proteins *in vivo*.

## 217 **Alternative methods**

218 A wide variety of static *in vitro* digestion models can be found in the literature<sup>25</sup> but they all  
219 exhibit different conditions (pH, duration of each step, ratio enzymes/substrate...) making the  
220 comparison between studies impossible. The static methods published by Versantvoort et  
221 al.<sup>21</sup>, Garrett et al.<sup>48</sup> and Oomen et al.<sup>20</sup> are amongst the most used, based on their citations.  
222 However, most of the of static *in vitro* digestion methods found in the literature simulate the  
223 fasted state, which is quite far from the physiological conditions when food is digested in the  
224 gastrointestinal tract. Advantages and limitations of static *in vitro* digestion models have been  
225 recently reviewed by a group of experts within the INFOGEST network<sup>15</sup>. While static  
226 methods can be useful for understanding trends or performing a screening of samples, it falls  
227 short in terms of some of the important dynamic processes occurring during gastrointestinal  
228 digestion, namely the pH gradients and the gradual addition of enzymes and gastric fluid as  
229 well as continuous gastric emptying. More physiologically relevant dynamic digestion  
230 methods<sup>6-10</sup> take these and other factors into account. However these models are highly  
231 complex, require substantial hard- and software and are still expensive to set up and  
232 maintain, hence are often not available to food researchers. It has recently been shown that,

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233 when human data are available to set up the system, these models can be physiologically-  
234 relevant<sup>11</sup>. In an effort to improve *in vitro* digestion methods, a low-cost semi-dynamic  
235 method was recently developed<sup>49</sup> and described in detail<sup>50</sup>, where parameters were based on  
236 the equivalent *in vivo* data from the digestion of dairy products. Here, the simulated gastric  
237 fluid (SGF) and pepsin are slowly added to the food in a suitable reaction vessel with  
238 manual, stepwise gastric emptying. A harmonisation of experimental conditions is currently  
239 on-going and a standardised semi-dynamic method will be published shortly by INFOGEST  
240 members, coordinated by A.R Mackie.

241 Even though they are expensive and must be ethically justifiable, *in vivo* models have been  
242 widely used for studying the digestive process. The pig model can closely simulate the upper  
243 part of the human digestive tract (stomach and small intestine)<sup>51</sup>. Conventional pigs or mini-  
244 pigs can be used for this purpose and can be equipped with cannulas in order to sample the  
245 effluents throughout digestion and a catheter to collect blood, whereas piglets can be used  
246 for all the questions related to neonatal nutrition<sup>29,52,53</sup>.

247 Finally, human volunteers can be equipped with naso-gastric or naso-intestinal probes to  
248 access and sample the digestive effluents<sup>3</sup>. Ileostomy patients have been used to study  
249 digestion<sup>54-56</sup> but can hardly be considered as a model of a healthy human since they are  
250 affected by digestive pathologies.

251

## 252 **Experimental Design**

### 253 *Enzyme assays*

254 The determination of the standard units of activity of the enzyme used in the protocol is a  
255 crucial step and one of the main sources of variation in results with the digestion periods or  
256 between different laboratories.<sup>37</sup> Enzyme activity determination is recommended for each  
257 new batch of enzyme or after prolonged storage.

258 Enzyme and bile assays were previously described in protocol format in the Supplementary  
259 Materials of Minekus et al.<sup>27</sup>, namely:  $\alpha$ -amylase (EC 3.2.1.1), pepsin (EC 3.4.23.1), trypsin  
260 (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1), pancreatic lipase (EC 3.1.1.3) and bile salts  
261 (according to supplier's protocol). In order to improve the reproducibility of the pepsin activity  
262 assay for this revised INFOGEST 2.0 protocol, it is now recommended to dissolve pepsin in  
263 10 mM Tris buffer (tris-hydroxymethyl-aminomethane), 150 mM NaCl, (pH 6.5), instead of in  
264 sodium chloride solution adjusted with sodium hydroxide. The buffering capacity of Tris  
265 buffer reduces the variability in the measurement of the pepsin activity, as shown  
266 previously<sup>37</sup>. The detailed protocols for the complete set of enzyme and bile assays, including



267 that of the gastric lipase assay (EC 3.1.1.3), can be found in the Supplementary Information  
268 and is summarise in **Box 1**.

269 Spreadsheets for the enzyme assays and the volumes for the digestion procedure are  
270 provided in the Supplementary Information of this manuscript. The enzyme assay  
271 spreadsheets (Supplementary spreadsheets 1) can be used to calculate the enzyme  
272 activities of all digestive enzymes. The digestion spreadsheets (Supplementary spreadsheets  
273 2) provides help in calculating all volumes of simulated digestive fluids, enzyme and bile  
274 solutions based on the initial amount of digested food; one example is shown in **Table 3**. The  
275 corresponding online spreadsheets can also be used, and are available here:  
276 [www.proteomics.ch/IVD](http://www.proteomics.ch/IVD) and on the INFOGEST website <https://www.cost-infoigest.eu/>. In  
277 addition, videos of the digestion procedures (Supplementary Video 1 and 2) and all enzyme  
278 activity assays (Supplementary Video 3 to 7) are available in the Supplementary Information.  
279 In addition, the videos are also available online on the YouTube channel “In vitro food  
280 digestion - COST action INFOGEST” [https://www.youtube.com/channel/UCdc-](https://www.youtube.com/channel/UCdc-NPx9kTDGyH_kZCgpQWg)  
281 [NPx9kTDGyH\\_kZCgpQWg](https://www.youtube.com/channel/UCdc-NPx9kTDGyH_kZCgpQWg) and on the INFOGEST website <https://www.cost-infoigest.eu/>.

#### 282 *Food preparation and oral phase*

283 It is important to plan the preparation of the food and the oral phase prior to *in vitro*  
284 gastrointestinal digestion to determine the food to digestive enzyme ratio throughout the *in*  
285 *vitro* digestion process. Firstly, consideration should be given as to whether the food to be  
286 digested *in vitro* is consumed as a meal, a meal portion or even a food ingredient. Some  
287 foods such as milk are often consumed on their own or as part of a meal. Other foods or food  
288 ingredients are nearly always consumed as part of a meal rather than on its own (e.g.  
289 coconut milk, spices, pure proteins, oils). Hence these foods should be prepared in a way  
290 that reflects real food or a meal, i.e. dilution, emulsification, integration into other foods, etc.  
291 High solid foods such as powders need to be reconstituted in liquids to make them a  
292 consumable food.

293 An optional oral phase with a standardised 1:1 (w/w) ratio of food to simulated oral fluid for all  
294 foods (solid and liquid foods) was recommended by the INFOGEST method<sup>27</sup> in 2014. While  
295 *in vivo* data varies greatly (Supplementary **Figure 1**), this dilution ratio enables the formation  
296 a swallowable bolus with almost all types of foods. For this revised INFOGEST 2.0 protocol a  
297 standardised, easy-to-follow approach for the oral phase is necessary. Hence, it is now  
298 recommended to dilute all food 1:1 (w/w) with simulated oral fluid to achieve a swallowable  
299 bolus that is no thicker than a paste-like consistency similar to that of tomato paste or



300 mustard at the end of the oral phase. If the consistency of the bolus is thicker than paste-like,  
301 add water to achieve it (see also **Table 3** and **Table 4** Troubleshooting).

### 302 *Use of lipase in the gastric phase*

303 Lipid digestion starts in the stomach with the action of preduodenal lipase (gastric lipase in  
304 humans, lingual lipase in rodents) on triacylglycerides (TAG) and some other esters<sup>57</sup>.  
305 Gastric lipolysis not only contributes to the overall digestion of TAG (10% with a solid-liquid  
306 test meal to 25% with an emulsified liquid test meal) but it also triggers the subsequent action  
307 of pancreatic lipase on lipid substrates that may be poorly digested by pancreatic lipase  
308 alone; examples include milk fat droplets and lecithin-stabilised TAG emulsions<sup>58</sup>. It is  
309 therefore recommended to add gastric lipase during the gastric phase of *in vitro* digestion.  
310 The mean gastric lipase concentration in human gastric juice is 100 µg/mL, which is  
311 equivalent to 120 U/mL using tributyrin as the reference substrate for gastric lipase<sup>59,60</sup>. In  
312 some static digestion models, a concentration of approx. 16 µg gastric lipase/mL (20 U/mL)  
313 has been used to reproduce gastric conditions at half time of gastric emptying<sup>61,62</sup>, which  
314 corresponds to a gastric juice to meal ratio of 1:5 v/v. In the INFOGEST method, the gastric  
315 phase of digestion includes a 1:1 dilution of the oral bolus by simulated gastric fluid, which  
316 would correspond to a dilution of gastric juice by half and thus a gastric lipase concentration  
317 of 60 U/mL. To date, access to commercially available gastric lipase, or an appropriate  
318 equivalent has been limited, hence gastric lipase has been omitted or lipases from alternative  
319 sources have been widely used. However, caution should be applied regarding the specific  
320 biochemical properties of these alternative lipases. Human gastric lipase (HGL), encoded by  
321 the LIPF gene, is stable and active between pH 2 and 7 with an optimum activity between pH  
322 4 to 5.4. HGL displays a  $S_N3$  stereospecificity for TAG hydrolysis leading to the preferential  
323 release of short/medium chain fatty acids from milk TAG<sup>61</sup>. It is resistant to pepsin hydrolysis  
324 and is not inhibited by bile salts. HGL can however be replaced by other preduodenal lipases  
325 from the acid lipase gene family of various mammalian species like dog<sup>63</sup> and rabbit<sup>64</sup>. Rabbit  
326 gastric lipase is now commercially available (Lipolytech, www.lipolytech.com). Pre-duodenal  
327 lipases originating from the oro-pharyngeal tissues of young ruminants (pharyngeal lipase of  
328 calf, kid goat, lamb) may also be used and are commercially available for applications in the  
329 dairy industry (DSM for Capalase<sup>®</sup> K and Capalase<sup>®</sup> KL lipases; CHR Hansen for Lipase Kid-  
330 Goat ST20, Lipase Calf 57 LFU, Spice IT<sup>™</sup> AC and Spice IT<sup>™</sup> AG; DuPont Danisco, Clerici-  
331 Sacco). These preduodenal lipases are however less resistant to acid denaturation  
332 (threshold at around pH 3.5<sup>65</sup>) than gastric lipase and pH conditions may have to be  
333 adapted. Their contents and activity should be estimated before use in *in vitro* digestion  
334 experiments, using the recommended standard gastric lipase assay<sup>27</sup>, see Supplementary

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335 Information Section. So far, no commercially available lipase of microbial origin combines all  
336 the above properties of gastric lipase<sup>61,66</sup>, and their use is not recommended at this time. For  
337 this revised INFOGEST 2.0 protocol, the authors recommend using rabbit gastric lipase,  
338 commercially available as rabbit gastric extracts (RGE) at 60 U/mL in the final gastric  
339 digestion mixture. However, since these extracts also contain pepsin<sup>67</sup>, the pepsin  
340 concentration/activity in the gastric phase has to be accordingly adjusted to the  
341 recommended value.

#### 342 *Sampling, controls and test tube*

343 Before performing the protocol (time-lagged before the digestion experiment or one day prior  
344 to the digestion experiment), it is recommended to run one preliminary experiment, the **pH-**  
345 **test adjustment experiment**, with the relevant amount of food, enzymes and bile for the  
346 entire digestion process. The aim of this pH-test adjustment experiment is to measure and  
347 record the amounts of HCl and NaOH used to reach the target pH in order to perform more  
348 efficient pH adjustments when running the digestion protocol. These volumes are indicative  
349 of the necessary volume of acids and bases needed for the gastric and intestinal phase. It  
350 has to be noted that for solid food, the pH changes are generally slower in response to  
351 addition of HCl or NaOH – it is important to remain patient and wait long enough for the pH to  
352 become stable - >5 min depending on food particle size and buffering capacity.

353 If it is intended to take samples at different time points during digestion, it is recommended to  
354 prepare one tube per time point, e.g. prepare six digestion tubes for six time points. Because  
355 most foods are heterogeneous mixtures during digestion, sampling is more reproducible by  
356 starting digestion with individual tubes per time point. If the food sample has special  
357 requirements in terms of nutrient stability (e.g. light sensitivity, oxidation) the characteristics  
358 of the tubes should be adapted to these particular situations (opaque tubes, maintenance of  
359 the food samples on ice, etc). The end volume of the digest should be calculated to use the  
360 most suitable reaction vessel, e.g. 50 mL tubes, which allow properly mixing during all  
361 digestion phases.

362 Optionally, a replicate test tube (**stability test tube**) can be prepared to evaluate food  
363 stability during exposure to simulated digestive fluids without enzymes or bile, for example  
364 after oral, gastric and intestinal phase. It can also be advisable to prepare an **enzyme-blank**  
365 **tube**, i.e., a digestion tube with all enzymes and bile but without food. This may be helpful to  
366 identify enzyme, bile salts or degradation products thereof during analysis of the digests. It is  
367 important to highlight that due to proteolytic enzyme autolysis, especially pepsin, enzyme-



368 derived peptides can be detected in digesta which can be easily monitored with this blank-  
369 enzyme tube.

370 *Intestinal phase, stop reaction and read out*

371 The intestinal phase of the protocol starts with the mixing of the gastric chyme with the same  
372 volume of the pre-warmed SIF. The pH is adjusted with the amount of NaOH previously  
373 calculated in the *pH-test adjustment experiment*. In this phase, two different options are  
374 given, (i) the use of pancreatin or (ii) the use of individual enzymes: porcine trypsin (100  
375 U/mL), bovine chymotrypsin (25 U/mL), porcine pancreatic  $\alpha$ -amylase (200 U/mL), porcine  
376 pancreatic lipase (2,000 U/mL) and porcine pancreatic colipase in molar excess to lipase.  
377 The amount of pancreatin to be used in the intestinal phase of digestion is based on trypsin  
378 activity to achieve 100 U/mL in the final mixture. This calculation may result in low lipase  
379 activity for high fat containing foods or if fat digestion is the aim of the study. In this case, it is  
380 recommended to include additional lipase to get 2000 U/mL of lipase activity in the final  
381 mixture and colipase in a molar ratio 2:1 colipase to lipase, which corresponds approximately  
382 to a mass ratio 1:2 colipase to lipase. Since this will require the measurement of the lipase  
383 activity in the pancreatic extract and in the lipase preparation, the use of individual enzymes  
384 could be a preferred option. Similarly, because the activity of amylase in pancreatin can vary  
385 between batches and the activity can be too low to digest starch rich foods, the use of  
386 individual enzymes could also be a good option when following carbohydrate digestion. Bile  
387 salts are added to the intestinal mixture to reach 10 mM in the final mixture, after  
388 determination of the bile salt concentration in the commercial product (see Enzymatic  
389 Assays). There are several commercial options for bile salts but bovine bile is preferred  
390 because its composition is similar to that in humans<sup>64</sup>. Bile solubilisation requires exhaustive  
391 mixing which can be achieved, for instance, in a rotating wheel mixer at 37°C for 30 min.

392 *In vitro* digestion is carried out for a wide range of purposes and with different endpoints. In  
393 all cases, sampling, sample preservation and the post-treatment of samples after food  
394 digestion are critical and some adaptations could be needed depending on the particular  
395 requirements of each experiment (**Table 1**). For example, to stop pepsin activity, the pH of  
396 gastric samples must be raised to 7.0, either by the addition of 1 M sodium bicarbonate or 1  
397 N NaOH solution. The pH shift after the gastric phase is very effective in stopping pepsin  
398 activity and similar to *in vivo* conditions found in the duodenum<sup>56</sup>. If the pH increase is not  
399 desired, the use of pepstatin A, a highly selective inhibitor of aspartyl proteases like pepsin  
400 ( $K_i = 0.1$  nM) has also been suggested<sup>68</sup>. When gastric digestion is considered as an end  
401 point, sample snap freezing in liquid nitrogen followed by freeze-drying are recommended.





402 Raising the pH to 7.0 strongly reduces the activity of gastric lipase on long chain  
403 triglycerides<sup>58-60</sup>. Alternatively, the use of Orlistat<sup>®</sup> (tetrahydrolipstatin) is also recommended  
404 (gastric lipase half-inhibition time of < 1 min) to block gastric lipolysis<sup>61</sup>. Add Orlistat at a final  
405 concentration of 0.6 mg/mL (1 mM) to obtain an inhibitor to lipase molar ratio of 1,000, taking  
406 into account that the gastric lipase activity of 60 U/mL corresponds to 50 µg/mL or 1 µM  
407 lipase.

408 After gastrointestinal digestion and in order to inhibit the different enzymatic activities of the  
409 digested samples, immediate snap freezing after sampling is necessary. However, when  
410 thawing the sample for subsequent analysis, residual enzymatic activities could significantly  
411 affect the stability of the samples. Therefore, addition of sufficient amounts of enzyme  
412 inhibitors against target digestive enzymes is strongly recommended. In the case of  
413 proteases, the addition of 5 mM of Pefabloc<sup>®</sup> SC (4-(2-Aminoethyl) benzenesulfonyl fluoride  
414 hydrochloride, AEBSF) with ability to irreversibly inhibit trypsin and chymotrypsin is  
415 recommended due to its lower toxicity in comparison with phenylmethylsulfonyl fluoride  
416 (PMSF)<sup>40</sup>. Alternatively, the use of Bowman-Birk inhibitor from soybean, a potent inhibitor  
417 against both trypsin and chymotrypsin having  $K_i$  values at nanomolar level, has been also  
418 recommended<sup>62</sup>. In order to inhibit lipolysis by pancreatic lipase, the use of 5 mM of 4-  
419 bromophenylboronic acid has been reported<sup>63</sup>. Inhibition of pancreatic lipase by Orlistat is too  
420 slow (half-inhibition time > 5 min) to be used here<sup>61</sup>. For amylase inhibition heat-shock  
421 treatment, inactivation by ethanol or inhibition with 12% TCA have been used<sup>64</sup>, depending  
422 on the downstream sample analysis. Once the target inhibition occurs, the digests should be  
423 immediately snap frozen in liquid nitrogen and freeze-dried.

424 When biological activity of digested samples has been evaluated, heat-shock treatment (in  
425 boiling water for 5 min) to irreversibly inactivate proteases may also be considered<sup>28</sup>.  
426 However, it should be noted that heat treatment is detrimental to the food structure, proteins  
427 in particular as heat treatment generally causes irreversible denaturation and aggregation.  
428 For cell culture assays, consider whether the use of Pefabloc or other enzyme inhibitors can  
429 affect the read out of the experiment, and whether the osmolarity needs to be corrected by  
430 dilution to physiological values (285-300 mOsm/kg H<sub>2</sub>O, pH 7-7.5) in order to avoid cell  
431 osmotic shock. Other combined procedures for removal or enrichment of certain food  
432 components such as defatting, centrifugation, dialysis, filtration and size exclusion  
433 chromatography are also commonly used.

434

435



436  
437  
438

## Materials

### 439 Reagents:

- 440 - Ultrapure type I water, generated by a Milli-Q<sup>®</sup> system or similar (referred in text as  
441 water)
- 442 - Human salivary  $\alpha$ -amylase (Sigma-Aldrich, 1031)
- 443 - Porcine pepsin (Sigma -Aldrich, P7012 or P6887)
- 444 - Rabbit gastric extract (RGE) for gastric lipase (see section on gastric lipase above,  
445 currently supplied by e.g. Lipolytech RGE 25-100MG) **Critical:** RGE contains both  
446 gastric lipase and pepsin.
- 447 - Bovine bile (Sigma-Aldrich, B3883, preferred option as composition in closest to that  
448 in humans), alternatively Porcine Bile (Sigma-Aldrich, B8631),
- 449 - Porcine pancreatin (Sigma-Aldrich, P7545) or individual intestinal porcine enzymes  
450 (trypsin, chymotrypsin, amylase, lipase and co-lipase), see below optional reagents
- 451 -  $\text{CaCl}_2(\text{H}_2\text{O})_2$  (Merck 2382)
- 452 - NaOH (Merck 9141) **! Caution: corrosive, causes severe skin burns and eye damage**
- 453 - HCl (J. T. Baker 6081) **! Caution: corrosive, causes burns, irritating to respiratory**  
454 **system**
- 455 - KCl (Merck 4936)
- 456 -  $\text{KH}_2\text{PO}_4$  (J. T. Baker 0240)
- 457 -  $\text{NaHCO}_3$  (Merck 6329)
- 458 - NaCl (Merck 6404)
- 459 -  $\text{MgCl}_2(\text{H}_2\text{O})_6$  (Merck 5833)
- 460 -  $(\text{NH}_4)_2\text{CO}_3$  (Sigma-Aldrich, 207861)
- 461 - Enzyme inhibitors options (see Experimental Design and **Table 1**) :
- 462     o Pefabloc<sup>®</sup> SC (4-(2-Aminoethyl)benzenesulfonyl fluoride, Sigma-Aldrich,  
463     76307) **! Caution: corrosive;**
- 464     o Pepstatin A (Sigma-Aldrich, P5318)
- 465     o Bowman-Birk inhibitor (Sigma Aldrich, T9777)
- 466     o 4-bromophenylboronic acid (Sigma Aldrich, B75956) **! Caution: hazardous,**  
467     **corrosive, causes eye damage, harmful for respiratory system**

468 Chemicals for enzyme and bile tests:

- 469 - Pepsin test
- 470     o Haemoglobin from bovine blood (Sigma-Aldrich, H6525-25G),
- 471     o Trichloroacetic acid (Sigma-Aldrich, T6399-5G) **! Caution: Corrosive, causes**
- 472         **severe burns to skin and eyes. Soluble in water with release of heat.**
- 473 - Gastric lipase test:
- 474     o Taurodeoxycholate (Sigma-Aldrich, T0875-1G)
- 475     o Tributyrin (Sigma-Aldrich, T8626; ≥99%)
- 476     o Bovine serum albumin (Sigma-Aldrich, A7030; ≥98%)
- 477 - Trypsin test:
- 478     o TAME (p-Toluene-Sulfonyl-L-arginine methyl ester, Sigma-Aldrich, T4626-5G)
- 479 - Amylase test:
- 480     o Maltose Std. (Sigma-Aldrich, M5885-100G)
- 481     o Soluble Potato Starch (Sigma-Aldrich, S5651-500G)
- 482     o DNS (3,5-Dinitrosalicylic acid, Sigma-Aldrich, D0550-10G), **! Caution:**
- 483         **Harmful if swallowed, Acute oral toxicity**
- 484 - Chymotrypsin test:
- 485     o BTEE (N-Benzoyl-L-Tyrosine Ethyl Ester, Sigma-Aldrich, B6125-5G)
- 486 - Pancreatic lipase test:
- 487     o Sodium taurodeoxycholate (Sigma-Aldrich, T0875-1G)
- 488     o Tributyrin (Sigma-Aldrich, W222305-1KG)
- 489 - Bile acid determination
- 490     o Bile acid kit (Sigma-Aldrich, MAK 309) or ECOLINE Acides Biliaires, Diasys,
- 491         122129990313) or equivalent assay
- 492 Reagents for optional protocol with individual enzymes:
- 493 - Porcine trypsin (Sigma-Aldrich, T0303)
- 494 - Bovine chymotrypsin (Sigma-Aldrich, C7762)
- 495 - Porcine pancreatic α-amylase (Sigma-Aldrich, A3176)
- 496 - Porcine pancreatic lipase (Sigma-Aldrich, L3126)
- 497 - Porcine pancreatic co-lipase (Sigma-Aldrich, C3028)
- 498 Food (for further examples see Anticipated Results Section)
- 499 - Skim milk powder (SMP, Fonterra, NZ, low-heat organic, protein 42.34%, fat 0.89%,
- 500     lactose 49.8% (w/w)<sup>28</sup>
- 501



502

503 **Equipment:**

- 504 - Standard laboratory centrifuge suitable for 50 mL tubes, 5,000 × *g* (e.g. Heraeus  
505 Megafuge 40R, 75004519, Thermo Fisher, Switzerland)
- 506 - Standard laboratory vortex (e.g. Genius 3, IKA, 17.1377.01, HuberLab, Switzerland)
- 507 - Standard laboratory pH Meter (e.g. 827 pH lab, 2.827.0214, Metrohm, Switzerland),  
508 electrode, designed for food systems (e.g. Sentek, P17/S7, pH electrode for food and  
509 dairy, 11981656, Fisher Scientific)
- 510 - Overhead shaker/rotator; small volume up to 50mL (Rotator SB Stuart, 17.0014.02,  
511 Huberlab, Switzerland)
- 512 - Incubator large enough to hold the above rotator (e.g. Termaks, B9000, Labtec,  
513 Switzerland), adjustable at 37°C
- 514 - Electric or manual mincer (Eddingtons Mincer Pro, 86001, Amazon, or similar)
- 515 - Eppendorf tubes (2 mL, 211-2120, VWR, Deutschland)
- 516 - Centrifuge Plastic tubes (15 mL, 391-3450, 50 mL, 525-0399, VWR, Deutschland)
- 517 - Micropipettes (e.g. Gilson P10 - P1000, VWR) and tips
- 518 - Volumetric flasks for solutions
- 519 - Glass beakers

520 **Reagent setup:**

521 Minimum volumes of stock solutions needed for the preparation of 400 mL of simulated  
522 digestion fluids 1.25× concentration:

- 523 - 0.5 mL of  $\text{CaCl}_2(\text{H}_2\text{O})_2$  (0.3M)
- 524 - 30 mL of KCl (0.5M)
- 525 - 6 mL of  $\text{KH}_2\text{PO}_4$  (0.5M)
- 526 - 65 mL of  $\text{NaHCO}_3$  (1M)
- 527 - 25 mL of NaCl (2M)
- 528 - 2 mL of  $\text{MgCl}_2(\text{H}_2\text{O})_6$  (0.15M)
- 529 - 2 mL of  $(\text{NH}_4)_2\text{CO}_3$  (0.5M)
- 530 1 M NaOH and 1 M HCl: for pH adjustment of stock solutions of simulated digestion  
531 fluids

532 Stock solutions can be prepared and stored in aliquots at -20°C for one year.

533 Preparation of simulated digestion fluids at a 1.25× concentration

534  
535 Simulated digestion fluids for oral (SSF), gastric (SGF), and intestinal (SIF) digestion phase  
536 are mixed at a 1.25× concentration using the electrolyte stock solutions and water according  
537 to **Table 2** and can be stored at -20°C for one year. **Critical:** CaCl<sub>2</sub> should be added  
538 immediately prior to the digestion experiment to avoid precipitation upon storage. **Critical:** All  
539 the volumes (**Table 2**) are calculated for 400 mL of a 1.25× concentrated storage solution  
540 and just before use they are mixed with the necessary quantities of enzyme and finally  
541 diluted to a 1× concentrated working solution (i.e. 4 parts of electrolyte solution + 1 part  
542 consisting of enzymes and water result in a 1× concentration of the digestion fluids).  
543 Simulated digestion fluids (1.25× concentrates) can be stored at -20°C for one year in small  
544 aliquots of appropriate size; e.g. for the experiment shown in **Box 1**, using 5 g of food, at  
545 least 48 mL of SSF, 88 mL of SGF, and 96 mL of SIF are needed. **Critical:** Dilute enzymes  
546 in cold solutions and keep them on ice until used. This will keep enzyme activity to a  
547 minimum. **Critical:** Pre-warm electrolyte solutions (SSF, SGF, SIF) to 37°C prior to using  
548 them in the digestion procedures.

549

## 550 Procedure

### 551 Preparation reagents and digestion tubes (5 days):

- 552 1. Perform all enzyme and bile assays (**Box 1**) according to the protocols in the  
553 Supplementary Information for each new batch of enzymes or after prolonged storage;  
554 **TIMING** 4-5 days for all assays  
555 **Critical Step:** For the pepsin assay, dissolve pepsin in 10 mM Tris, 150 mM NaCl, pH  
556 6.5, which improves the reproducibility of the assay (see Supplementary Information).  
557 **Critical Step:** Spreadsheets for the enzyme assays and the volumes for the digestion  
558 procedure are provided in the Supplementary Information of this manuscript  
559 (Supplementary spreadsheets 1 and 2). In addition, the corresponding online  
560 spreadsheets are available here: [www.proteomics.ch/IVD](http://www.proteomics.ch/IVD) and on the INFOGEST website  
561 <https://www.cost-infogest.eu/>.  
562 **Critical Step:** Prepare one tube per time point and food; e.g. for one food and six time  
563 points, prepare six tubes
- 564 2. Pre-warm the electrolyte stock solutions at 37 °C, initially only SSF and SGF, SIF  
565 3. Prepare all enzyme and bile solutions immediately before the digestion experiment  
566 **Critical Step:** Keep all enzyme solutions on ice
- 567 4. In order to perform more efficient pH adjustments during the digestive phases, prepare  
568 one replicate tube (pH-test adjustment experiment) with the relevant amount of food,  
569 enzymes and bile for the entire digestion process (time-lagged before the digestion  
570 experiment or one day prior to the digestion experiment) and measure and record the  
571 volumes of HCl and NaOH used to reach the target pH. These volumes are indicative of  
572 the necessary volume of acids and bases needed for the gastric and intestinal phase  
573 **TIMING** 5h
- 574 5. Optional: Prepare one replicate test as a food stability control to assess the behaviour of  
575 the food during exposure to simulated digestive fluids without enzymes or bile, for  
576 example after oral, gastric and intestinal phase
- 577 6. Prepare one replicate test tube as a blank, digestion without food (replaced by water) but  
578 with all required enzymes and bile. See videos of enzyme assays (supplementary videos  
579 3 to 7) as well as the digestion procedures (supplementary videos 3 and 4). Videos are  
580 also available online on the YouTube channel “In vitro food digestion - COST action  
581 INFOGEST” [https://www.youtube.com/channel/UCdc-NPx9kTDGyH\\_kZCgpQWg](https://www.youtube.com/channel/UCdc-NPx9kTDGyH_kZCgpQWg)  
582 and on the INFOGEST website <https://www.cost-infogest.eu/>

583

584 **Digestion procedure**

585 **TIMING** depending on number of food samples and time points, for example: 1 food sample  
 586 and 5 time points - approximately 5h; 2 food samples and 5 time points (2 gastric and 3  
 587 intestinal points) - approximately 8h

588 **Oral phase (30 min)**

- 589 7. Dilute food with SSF at a ratio of 1:1 (w/w) to achieve a swallowable bolus with a paste-  
 590 like consistency similar to that of tomato paste or mustard at the end of the oral phase. If  
 591 the consistency of the bolus is thicker than paste-like, add water to achieve it. Salivary  
 592 amylase is only needed to digest starch containing food. It can be omitted if the food  
 593 does not contain starch. Do not use lower purity salivary amylase or pancreatic amylase.
- 594 8. Mix food with SSF at a 1:1 ratio (w/w), e.g. 5 g of food to 5 g of SSF
- 595 9. Measure the volume of the final digestion mixture of the food + SSF mixture. Record this  
 596 volume as it will be used in step 17.
- 597 10. If necessary, simulate mastication by mincing the food in an electric or manual mincer.
- 598 11. Depending on the food (e.g. bread), mincing can be done together with the SSF  
 599 electrolyte (without enzymes)
- 600 12. Add SSF electrolyte stock solution to the food, if not done in the previous step
- 601 13. Add CaCl<sub>2</sub> in order to achieve a total concentration of 1.5 mM in SSF
- 602 14. Add the salivary amylase, if necessary, prepared in water to achieve an activity of 75  
 603 U/mL in the final mixture.
- 604 15. Add the remaining water in order to achieve 1× concentration of the SSF.
- 605 16. Incubate while mixing for 2 minutes at 37°C.

606 **Critical step:** Electrolyte concentrations are given for the simulated digestive fluids  
 607 (SSF, SGF and SIF) and accumulation in consecutive digestion phases is not  
 608 considered whereas enzyme activities are expressed U/mL in the final digestion mixture.

609

610 **Gastric phase (3h)**

- 611 17. Pre-warm the SGF electrolyte stock solution at 37°C. Add SGF electrolyte stock solution  
 612 to the oral bolus to a final ratio of 1:1 (v/v)
- 613 18. Adjust the pH to 3.0 by adding a defined volume of HCl previously determined during a  
 614 pH-test adjustment experiment, see Experimental Design

615 **Critical step:** For solid food, the pH changes are generally slower in response to the  
 616 addition of HCl – it is important to remain patient and wait until the pH is stable, usually,  
 617 this takes >5 min depending on food particle size and buffering capacity.



- 618 19. Add CaCl<sub>2</sub> solution in order to achieve a final concentration of 0.15 mM in SGF.
- 619 20. Add the porcine pepsin solution prepared in water to achieve an activity of 2,000 U/mL in  
620 the final digestion mixture.
- 621 21. Add the gastric lipase solution prepared in water to achieve an activity of 60 U/mL in the  
622 final digestion mixture.
- 623 22. Verify the pH and adjust to 3.0 if necessary
- 624 23. Add water in order to achieve 1× concentration of the SGF
- 625 24. Incubate the samples at 37 °C, mixing the digestive mixture sufficiently (e.g. rotating  
626 wheel, shaking incubator) for 2 h from the point when pepsin was added. In case of large  
627 precipitates and formation of clogs, see Troubleshooting.
- 628 **Critical step:** Rabbit gastric extracts (RGE) contains both gastric lipase and pepsin<sup>67</sup>.  
629 The pepsin activity in RGE needs to be determined and taken into account together with  
630 the porcine pepsin to reach a combined pepsin activity of 2,000 U/mL in the final  
631 digestion mixture.
- 632 **Critical step:** The use of carbonate salts in the electrolyte solutions requires that sealed  
633 containers with limited headspace are used. In open vessels, CO<sub>2</sub> will be release and  
634 the pH will progressively increase with time. If open vessels are to be used, such as  
635 when using the “pH-stat” approach or for sampling purposes, it is suggested to replace  
636 sodium bicarbonate (NaHCO<sub>3</sub>), the main source of carbonates, by NaCl at the same  
637 molar ratio in order to maintain the ionic strength of the electrolyte solutions (oral, gastric  
638 and intestinal). Such adjustment has already proven effective in avoiding unwanted pH  
639 drift in open vessels in both gastric<sup>69</sup> and intestinal<sup>42</sup> phases of digestion (see **Table 2**).

640

**Intestinal phase (3h):**

- 641 **Intestinal phase (3h):**
- 642 25. Pre-warm the SIF electrolyte stock solution in a 37°C water bath. Add SIF electrolyte to  
643 the gastric chyme and achieve a final ratio of 1:1 (v/v).
- 644 26. Adjust to pH 7.0 by adding a defined volume of NaOH previously determined during a  
645 pH-test adjustment experiment, see Experimental Design.
- 646 **Critical step:** For solid food, the pH changes are slower in response to the addition of  
647 NaOH, see remarks in step 18; this may take several minutes.
- 648 27. Add the bile solution to the SIF: gastric chime solution in order to reach a final  
649 concentration of 10 mM. Place the solution in a rotating wheel mixer at 37 °C for at least  
650 30 min to achieve complete bile solubilisation.
- 651 28. Add CaCl<sub>2</sub> solution in order to reach concentration of 0.6 mM in SIF.
- 652 29. Perform intestinal phase with option (A) pancreatin or option (B) with individual enzymes

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- 653 A.
- 654 i. Add the pancreatin suspension in SIF solution to achieve a trypsin activity
- 655 of 100 U/mL in the final mixture. Additional pancreatic lipase may be
- 656 needed for the digestion of fat containing food to reach the required lipase
- 657 activity to achieve a lipase activity of 2,000 U/mL in the final mixture.
- 658 **Critical step:** Measure trypsin activity in pancreatic lipase powder and subtract it
- 659 from the needed trypsin activity
- 660 B.
- 661 i. Add trypsin, chymotrypsin, pancreatic  $\alpha$ -amylase, pancreatic lipase and
- 662 the co-lipase solutions in SIF, in order to reach 100, 25, 200 and 2,000
- 663 U/mL, respectively, in the final digestion mixture
- 664 30. Verify the pH and adjust to 7.0 if necessary
- 665 31. Add water in order to achieve 1 $\times$  concentration of the SIF
- 666 32. Incubate the samples at 37 °C, mixing the digestive mixture sufficiently using a rotating
- 667 wheel or shaking incubator for 2h starting at the point when pancreatic enzymes were
- 668 added. For difficulties with sampling, see **Table 4** Troubleshooting.
- 669 **Critical step:** If open vessels are used (“pH-stat” approach), NaHCO<sub>3</sub> should be
- 670 replaced by NaCl in the electrolyte solutions to avoid unwanted pH drift (see the step 24
- 671 critical step).
- 672



## 673 Anticipated Results

### 674 Protein digestion

675 Without the use of standardised digestion methods, the main difficulties were (i) the absence  
676 of comparable results from different laboratories and (ii) the physiological relevance of  
677 experimental data in the field of food digestion. The INFOGEST method was tested with  
678 respect to these two aspects focusing on protein digestion.

679 (i) *Robustness of the protocol* and comparability of experimental data were assessed in  
680 several inter-laboratory trials where the participants were asked to digest a standardised  
681 skim milk powder (SMP) by applying their existing in-house protocols first, then by using the  
682 harmonised protocol<sup>28</sup>. The first critical step in protein hydrolysis is the pepsin activity in the  
683 gastric phase. The heterogeneous pattern observed with the in-house digestion protocols  
684 (**Figure 2a**, gastric phase) was improved significantly by the correct implementation of the  
685 harmonised protocol (**Figure 2b**, gastric phase), except for laboratories 6 and 7, which  
686 showed incomplete casein hydrolysis. Adjustments in the pepsin assay (addition of Tris  
687 buffer, see Step 1 Critical Step and **Box 1**) improved the reproducibility and reduced lab-to-  
688 lab variability<sup>28</sup>. This improved pepsin assay is now recommended for the INFOGEST 2.0  
689 method. **Figure 2b** shows improved homogeneity between samples, compared to the gastric  
690 phase when the harmonised protocol was applied. Increased protein degradation in the  
691 intestinal phase was observed in laboratories 4 and 7. Subsequent recommendation on the  
692 correct sample preparation, in particular the correct inhibition of enzymes at the end of the  
693 digestion experiment (see **Table 1**), improved lab-to-lab variability<sup>28</sup>.

694 (ii) *Physiological relevance* was evaluated by comparing *in vitro* SMP digestion with that of  
695 an *in vivo* pig trial<sup>29</sup>. Pigs were fed reconstituted SMP from the same batch as applied in the  
696 *in vitro* tests and samples were collected from the stomach and in several sections of the  
697 small intestine (jejunum, I1- I3 to ileum, I4) after sacrifice. Milk peptides were identified with  
698 mass spectrometry and overall peptide patterns were visualised by summing up the number  
699 of times each individual amino acid was identified within a milk peptide. Overlay of the  
700 average peptide patterns for  $\alpha_{s2}$ -casein from the harmonised *in vitro* digestion (n=7) and *in*  
701 *in vivo* pig digestion (n=8) showed that at the end of the gastric phase, the peptide pattern  
702 corresponded well to that of the pig sample collected from the stomach; the peptide pattern  
703 in the *in vitro* intestinal phase sample was most similar to that of the pig sample collected in  
704 the median jejunum (I3). This comparison showed that protein hydrolysis at the endpoints of

705 the harmonised INFOGEST digestion method were in agreement with that of the *in vivo*  
706 digestion (**Figure 3**).

707 In conclusion, both critical points, inter-laboratory comparability and physiological relevance  
708 were improved by the correct application of the harmonised *in vitro* digestion protocol.

709

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710

**711 Lipid Digestion**

712 To date, most published digestion experiments using this INFOGEST method did not include  
713 a gastric lipase because of the lack of commercially available, acceptable substitutes for  
714 human gastric lipase (HGL). This situation has changed with the availability of rabbit gastric  
715 extracts containing gastric lipase, see Experimental Design in the Introduction: *Use of lipase*  
716 *in the gastric phase*. Both HGL and rabbit gastric lipases exhibit, at the recommended gastric  
717 pH of 3.0, approximately 50% of their maximum activity measured at pH 4 to 5.4<sup>70,71</sup>.  
718 Moreover, the *in vitro* gastric lipolysis of infant formula by rabbit gastric lipase were  
719 consistent with *in vivo* data, with a degree of lipolysis reaching 10% after 60 min of gastric  
720 digestion<sup>72</sup>. These data therefore suggest that gastric lipolysis could be studied using this  
721 INFOGEST 2.0 method with rabbit gastric extract as a source of gastric lipase<sup>64</sup> or human  
722 gastric lipase if available<sup>61</sup>.

723 The INFOGEST method has also been used to study intestinal lipid digestion, for example in  
724 oil-in-water emulsions stabilised by milk or soya lecithin<sup>73</sup>. However, human gastric analogue  
725 and phospholipases A2 (PLA<sub>2</sub>) were added in this procedure. The degree of hydrolysis (%  
726 TAG disappearance) ranged between 73 and 87 % ( $\pm 5$  %) at the end of the intestinal phase  
727 (120 min). In addition, *in vitro* digestion was also performed with more complex systems such  
728 as whole fat dairy products or protein/polysaccharide emulsions. Depending on the structure  
729 of the food matrix and the state of dispersion of the lipids, the reported degrees of hydrolysis  
730 at the end of the intestinal phase ranged from moderate (66% of remaining lipids in poorly  
731 digestible raw oat flakes due to limiting matrix structure)<sup>74</sup> to an almost complete  
732 disappearance of triglycerides<sup>75</sup>.

733 Intestinal lipid digestion can be assessed by chemical analyses of collected samples. The  
734 protocol recommends analysing the entire volume of digestive tubes to prevent sampling  
735 errors (see Procedure Step 1 Critical Step, one tube per time point and food). This  
736 precaution is particularly useful in the presence of lipids<sup>74</sup> as they often tend to destabilise  
737 and phase-separate (cream) during the gastric and/or intestinal phases of digestion. If  
738 aliquots are taken as sample points, great care should be taken to represent the whole  
739 digested solution. The best way to analyse the extent of lipolysis is to conduct the Folch  
740 extractions<sup>76</sup> on the samples in the presence of internal standards before the analysis of  
741 classes of the lipids (residual triglycerides, free fatty acids, diglycerides and monoglycerides)  
742 by thin layer chromatography<sup>75</sup> combined with densitometry or gas chromatography with a  
743 flame ionization detector (GC-FID)<sup>77</sup> or HPLC coupled to a light scattering detector<sup>78</sup>. Free  
744 fatty acids can also be quantified after solid phase extraction with GC-FID, using fatty acids

745 (typically C11:0, C15:0, C17:0 or C23:0) as internal standards<sup>72,79</sup>. The pH-stat method, one  
746 of the most commonly used methods for monitoring pancreatic lipolysis, can also be used,  
747 but three sources of errors should be taken into consideration: (i) the pH-stat measurements  
748 can be impaired by the high concentrations of carbonate salts, recommended for the  
749 simulated digestion fluids (see the step 24 critical step It is therefore advised to replace  
750 NaHCO<sub>3</sub> salts with NaCl at the same molarity in all electrolyte solutions (oral, gastric and  
751 intestinal) when planning to use pH-stat experiments during the intestinal phase of  
752 digestion<sup>42</sup>; (ii) protein hydrolysis also contributes to the pH-stat signal in the intestinal  
753 conditions (pH = 7), meaning that this approach is only suitable for studying pancreatic  
754 lipolysis when the contribution of proteins is either neglected or subtracted<sup>42</sup>; (iii) some fatty  
755 acids, especially long chain fatty acids, are not ionised at pH7. A back titration at pH 9.0  
756 should be performed to measure all the free fatty acids released<sup>80</sup>.

757

### 758 **Digestion of starch**

759 The structure of starch in a ready-to-eat plant-based food is a function of a multitude of  
760 factors. These include its botanical origin, growing conditions, processing, food preparation  
761 (mainly cooking), and not least storage. These all have a major impact on salivary and  
762 pancreatic amylase catalysed starch digestion. The rate of the loss of starch and the  
763 appearance of the digestion product (maltose and maltooligosaccharides) are the most  
764 common measures of *in vitro* starch digestibility. To help in the understanding of the  
765 physiological effects of starch digestion such as on glycaemic response in humans,  
766 measurements should also include (i) the accurate dose and nature of the starch in the food  
767 as eaten, (ii) the characterisation of the food matrix (microstructure, macro and micronutrient  
768 composition) and (iii) a measure of the degree of starch gelatinisation and/or retrogradation.

769 It is recommended that starch amylolysis is quantified *only* in the intestinal phase by  
770 measuring the appearance of the starch digestion products over time, e.g. the concentration  
771 of reducing sugars in the liquid phase. Salivary amylase will have a minor impact on starch  
772 digestion in the static model were the gastric pH is instantaneously adjusted to 3. After  
773 terminating amylase activity by mixing the sample with 4 volumes of ethanol (final conc. 80%  
774 w/v) to the sample, for example (see different options in **Table 1**), undigested starch is often  
775 separated from digested starch by centrifugation. Analysis of reducing sugar concentration in  
776 the supernatant is often done with common colorimetric assays (e.g. using DNS or PAHBAH  
777 (4-Hydroxybenzhydrazide) reagents). Another more common method is to treat an aliquot of  
778 the amylase digestion products from the 80% w/v ethanol supernatant with buffered  
779 amyloglucosidase to convert all amylase digestion products to glucose. Glucose can then be



780 determined through a whole host of methods including colorimetric and enzymatic assays (e.  
781 g. GOPOD) or by direct chromatography analysis to name just a few. The data collected can  
782 then be used as input variables to a wide variety of simple to complex kinetic-based  
783 mathematical models that seek to quantify starch digestion and give predictions on the  
784 physiological effects of the food under.

785

### 786 **Bioaccessibility of phytochemicals**

787 The main challenges for investigating common dietary phytochemicals such as hydrophilic  
788 polyphenols and hydrophobic carotenoids are: i) the physiological appropriateness of the  
789 digestion conditions, such as reproducible matrix-release and the sufficient presence of  
790 enzymes required for cleavage and cellular uptake and ii) separating the bioaccessible  
791 phase from unavailable phytochemicals (e.g. precipitated or in complexed form), which can  
792 be achieved by centrifugation and/or filtration/dialysis.

793 (i) *Physiological appropriateness and pitfalls*: Good correlations between bioaccessibility and  
794 *in vivo* bioavailability have been obtained for certain phytochemicals, such as  
795 carotenoids<sup>81,82</sup>. However, slight alterations of the digestion parameters suggested by the  
796 original INFOGEST method<sup>27</sup> can drastically influence bioaccessibility. For instance,  
797 increasing the amount of pancreatin and/or bile<sup>83</sup> or increasing the speed of shaking/stirring  
798 can considerably enhance the bioaccessibility of carotenoids by improving mixing, disrupting  
799 oil droplets and increasing micellisation. Thus, careful consideration and the possible further  
800 standardisation of these parameters are vital. Additional important factors to consider are  
801 light and oxygen, as they can result in the oxidative degradation of carotenoids<sup>84</sup> and  
802 polyphenols<sup>85</sup> and polymerisation of the latter<sup>86</sup>. It is recommended to flush samples with Ar  
803 or N<sub>2</sub> for a few minutes prior to small intestinal digestion to remove oxygen<sup>82,87</sup> or to use  
804 pyrogallol. However, the latter is unsuitable for polyphenolic samples as this is a potential  
805 metabolite. Another often neglected factor is the potential effect of brush border membrane  
806 enzymes (e.g. lactase-phlorizin-hydrolase) on phytochemical bioaccessibility, especially for  
807 polyphenols<sup>88,89</sup>. The inclusion of brush border membranes (BBM) vesicles in *in vitro*  
808 gastrointestinal digestion may increase the physiological relevance of the model, especially  
809 for polyphenols<sup>90</sup>. However, BBM are not commercially available nor is there any standard  
810 method available to date.

811 (ii) *Bioaccessible phase and pitfalls*: For polyphenols, dialysis is often performed to remove  
812 macromolecular-bound compounds<sup>91</sup>, but for carotenoids a combination of centrifugation  
813 (e.g. 4,000×g for at least 30 minutes) and a filtration step (0.2 µm) has become the most

814 widely used method<sup>31</sup> to separate the bioaccessible aqueous phase from larger lipid droplets  
815 or crystals that would not be taken up by the enterocytes.

816 When combining *in vitro* digestion with cellular assays (e.g. cellular uptake/transport), the  
817 toxicity of the bile salts must be accounted for, by including a clean-up step, e.g. solid phase  
818 extraction<sup>92-94</sup>, or at least the sufficient dilution of samples (e.g. 4× dilution).

819 Finally, it should be considered that the colon may play an important role for the bioavailable  
820 fraction. While it is well known that polyphenols can undergo many changes in the colon<sup>88</sup>,  
821 and may be absorbable in the colon, little is known for carotenoids, though a significant  
822 fraction would be bioaccessible in the colon<sup>95</sup>.

### 823 **On-going developments and future perspectives for *in vitro* food digestion**

824 The establishment of the INFOGEST digestion protocol is a good starting point in the  
825 standardisation and harmonisation of food digestion methods. Henceforth, results from  
826 different research groups can be compared in a meaningful manner. However, users have to  
827 be aware of the shortcomings of this method and considerable efforts are being made  
828 around the world to improve or add to the existing method.

829 The INFOGEST method is for adult digestion only. However, there is a strong need to apply  
830 this method to specific human population groups, the most important being infants and the  
831 elderly, but also adolescents and patients with cystic fibrosis or gastric bypass surgery, to  
832 name but a few. A recent review<sup>96</sup> summarised the existing literature and provides some  
833 recommendations on experimental digestion parameters, with the INFOGEST method being  
834 the starting point for all other methods.

835 While static methods can be useful, they can be inadequate to simulate the dynamic  
836 processes during digestion (e.g. pH gradients, gradual addition of enzymes and gastric fluid,  
837 continuous gastric emptying, etc.). As mentioned earlier, various dynamic digestion  
838 methods<sup>6-10</sup> account for some of these factors. A low-cost semi-dynamic method was recently  
839 developed<sup>49</sup> and described in detail<sup>50</sup>, based on equivalent *in vivo* data from the digestion of  
840 dairy products. International INFOGEST members are currently working on a consensus  
841 method.

842 Enzymes from the small intestinal brush border membranes are recognised as playing a  
843 major role in the activation of trypsinogen (enterokinase) and the further degradation of  
844 proteins/peptides and carbohydrates as well as improving the bioaccessibility of  
845 phytochemicals. The use of brush border enzymes falls into the grey area between  
846 bioaccessibility (potentially absorbable) and bioavailability (available at the site of action) and  
847 to date, it is not clear how they should be applied. BBM of animal origin have recently been  
848 included in static digestion methods<sup>39,97,98</sup> and can provide physiologically consistent  
849 information<sup>99</sup>. However, to date BBM enzymes are not commercially available and are

850 extracted from fresh animal intestines<sup>100</sup> or used as intestinal extracts. There is still a lack of  
851 reliable information on the correct enzymatic activities, enzyme substrate ratio and diversity  
852 of enzymes, which further limits the use of BBM in standardised digestion methods at the  
853 moment. However, given the importance of BBM in the digestive process, further progress in  
854 terms of defining digestive parameters is anticipated.

855

## 856 **TIMING**

857 Step 1, enzyme activity and bile assays: 4 to 5 days for all assays

858 Steps 2 and 3, preparation of solutions: 2 hours

859 Step 4, pH-adjustment experiment: 5 hours (time-lagged before the digestion experiment)

860 Steps 5 and 6, preparation of replicate tests as control: 20 min

861 Steps 7 to 32, whole digestion experiment: 5 to 8 hours, depending on number of food  
862 samples and time points, for example: 1 food sample and 5 time points - approximately 5h; 2  
863 food samples and 5 time points (2 gastric and 3 intestinal points) - approximately 8h

864 Steps 7 to 16, oral phase: 30 min

865 Steps 17 to 24, gastric phase: 3 hours

866 Steps 25 to 32, intestinal phase: 3 hours

867

868

869

## 870 **TROUBLESHOOTING**

871 Troubleshooting advice can be found in **Table 4**.

872

873

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884

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986 AB, LE and IR wrote the article. MA, SB, TB, FC, AC, DD, CD, CE, SLF, UL, AdM, AIM, OM,  
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 988 RB, FC, AC, MC, DD, CD, CE, MG, SK, BK, SLF, UL, AdM, AIM, SM, OM, MM, RP, CNS, IS,  
 989 GEV, MSJW, WW and IR contributed to the definition of digestion parameters. RP wrote the

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990 online tools. RA and CM prepared the videos. MG, DJMcC and RPS contributed to the  
991 manuscript by critical revision of digestion parameters and manuscript.

992

### 993 **Competing interests**

994 Rabbit lipase from rabbit gastric extract is available commercially from Lipolytech, a start-up  
995 company founded by a researcher who had previously worked at the group of F. Carrière  
996 (co-author of this manuscript). The laboratory of F. Carrière, a joint unit of Centre National de  
997 la Recherche Scientifique (CNRS) and Aix Marseille University (AMU), has a research  
998 collaboration contract with Lipolytech (CNRS reference number: 163451; signed on June  
999 30th, 2017). However, the co-author F. Carrière does not financially benefit from this contract  
1000 and, as an employee of CNRS and civil servant of the French state, is not allowed to have  
1001 private consulting activity for a company contracting with his own laboratory.

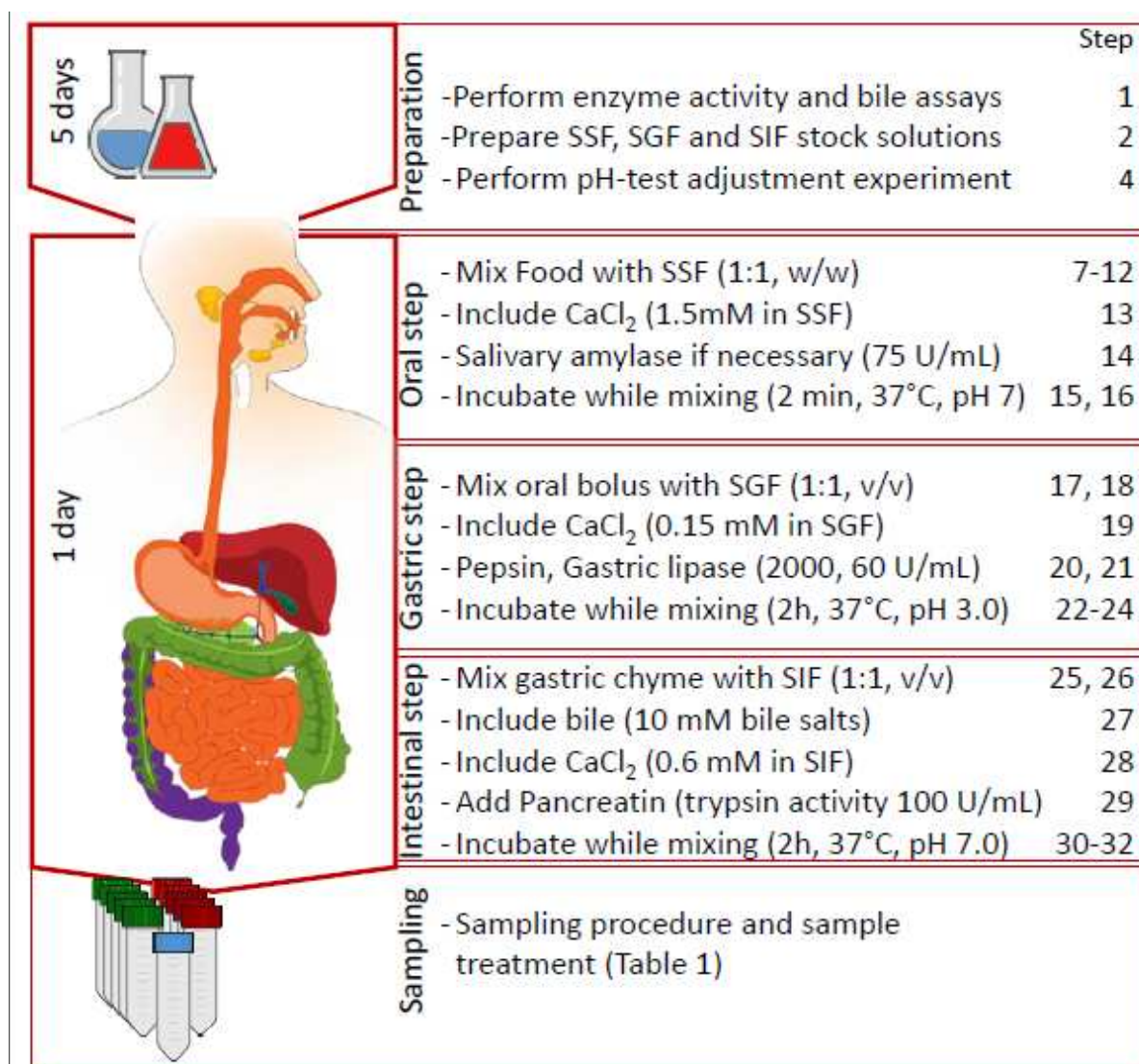
1002

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1007 **Figures**

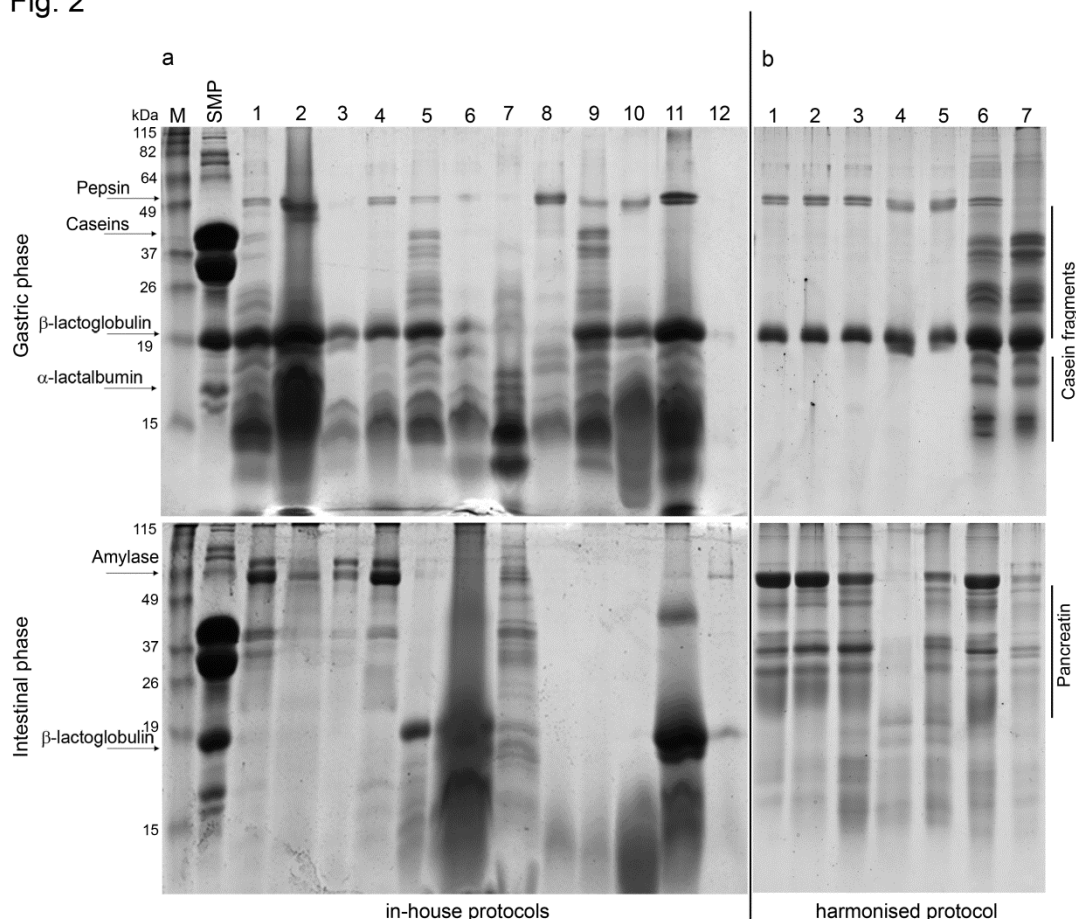
1008

1009 **Figure 1: Flow diagram of the INFOGEST 2.0 digestion method**

1010 Timing and flow diagram of the INFOGEST2.0 *in vitro* digestion method for food. SSF, SGF  
 1011 and SIF stand for simulated salivary, gastric and intestinal fluid, respectively. Expected time  
 1012 frame (left) and steps (right) corresponding to the step numbers in the Procedure section.

1013

Fig. 2

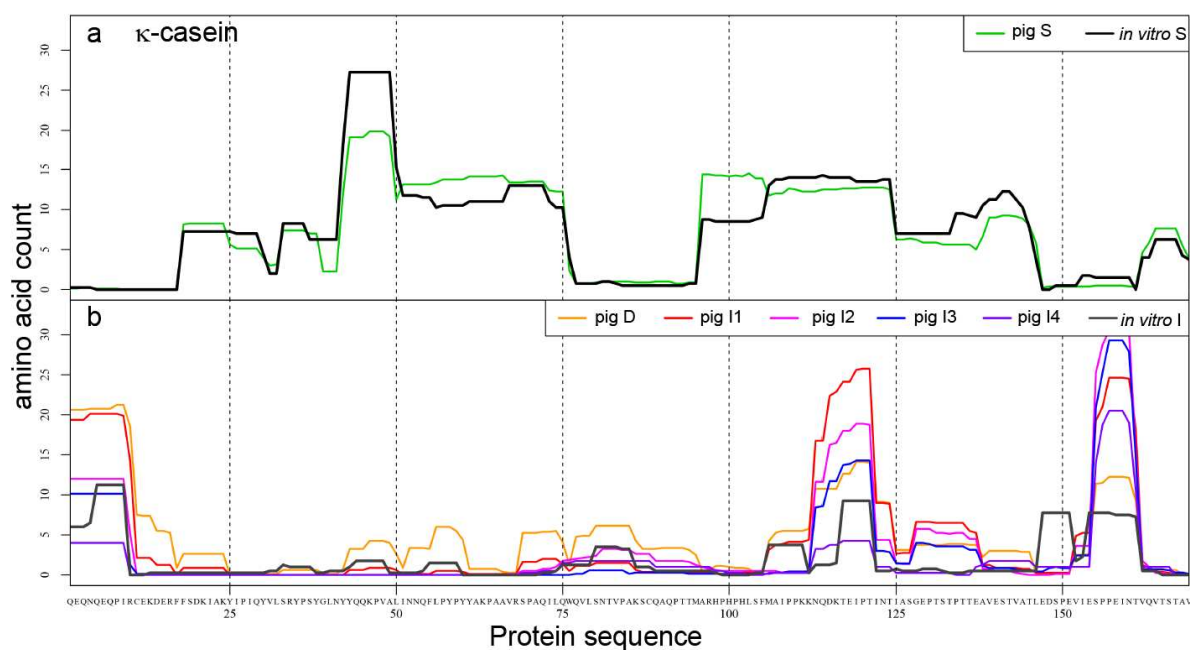


1014

1015 **Figure 2: Protein separation by gel electrophoresis of *in vitro* digested skim milk**  
 1016 **powder (SMP)**

1017 Comparing results from in-house protocols performed in individual laboratories 1-12 (a), with  
 1018 the harmonised protocol, performed in 7 different laboratories (b) after the gastric and the  
 1019 intestinal phase of *in vitro* digestion. Undigested skim milk powder (SMP) is shown as a  
 1020 control, specific protein bands are highlighted with arrows: casein fragments, partly  
 1021 hydrolysed casein; pancreatin, bands originating from pancreatin. Figure adapted from Egger  
 1022 et al.<sup>28</sup>

1023

1024  
1025

1026 **Figure 3: Comparison of *in vitro* digested skim milk powder (SMP) peptide patterns of**  
1027  **$\kappa$ -casein with *in vivo* (pig) digestion**

1028 (a) Gastric *in vitro* digestion samples (*in vitro* S) were compared to gastric pig samples (pig  
1029 S, n = 8, as previously published by Egger et al.<sup>29</sup>, approval number 2015\_04\_FR;26115). (b)  
1030 Intestinal *in vitro* digestion samples were compared to pig sampling sections collected along  
1031 the digestive tube from duodenum (D), proximal- (I1), median- (I2), distal jejunum (I3), and to  
1032 ileum (I4)<sup>29</sup>. The x-axis shows the amino acid (AA) sequence of  $\kappa$ -casein and the y-axis  
1033 shows the number of times each amino acid was identified within a  $\kappa$ -casein peptide of  $\geq 5$   
1034 AA in length.

1035  
1036

1037

1038 **Related links**1039 **Key references using this protocol**1040 1. Egger, L. et al. *Food Res. Int.* **88**, 217–225 (2016):1041 <https://doi.org/10.1016/j.foodres.2015.12.006>1042 2. Egger, L. et al. *Food Res. Int.* **102**, 567–574 (2017):1043 <https://doi.org/10.1016/j.foodres.2017.09.047>1044 3. Sanchón, J. et al. *Food Chem.* 239, 486–494 (2018):1045 <https://doi.org/10.1016/j.foodchem.2017.06.134>

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1047 REFERENCES  
1048

- 1049 1 WHO. Global Health Observatory - world health statistics. . 172pp, doi: (2013).  
1050 2 Sullivan, L. M. *et al.* Gastric digestion of  $\alpha$ -lactalbumin in adult human subjects using capsule  
1051 endoscopy and nasogastric tube sampling. *Br. J. Nutr.* **112**, 638–646, doi:  
1052 10.1017/S0007114514001196 (2014).  
1053 3 Boutrou, R. *et al.* Sequential release of milk protein–derived bioactive peptides in the  
1054 jejunum in healthy humans. *Am. J. Clin. Nutr.* **97**, 1314–1323, doi: 10.3945/ajcn.112.055202  
1055 (2013).  
1056 4 Mackie, A. R., Rafiee, H., Malcolm, P., Salt, L. & van Aken, G. Specific food structures suppress  
1057 appetite through reduced gastric emptying rate. *Am. J. Physiol. Gastrointest. Liver Physiol.*  
1058 **304**, G1038–G1043, doi: 10.1152/ajpgi.00060.2013 (2013).  
1059 5 Koziolok, M. *et al.* Intra-gastric pH and pressure profiles after intake of the high-caloric, high-  
1060 fat meal as used for food effect studies. *J. Control. Release* **220**, 71–78, doi:  
1061 10.1016/j.jconrel.2015.10.022 (2015).  
1062 6 Minekus, M., Marteau, P., Havenaar, R. & Huis In't Veld, J. H. J. A multicompartimental  
1063 dynamic computer-controlled model simulating the stomach and small intestine. *ATLA.*  
1064 *Alternatives to laboratory animals* **23**, 197–209, doi: - (1995).  
1065 7 Wickham, M., Faulks, R. & Mills, C. In vitro digestion methods for assessing the effect of food  
1066 structure on allergen breakdown. *Mol. Nutr. Food Res.* **53**, 952–958, doi:  
1067 10.1002/mnfr.200800193 (2009).  
1068 8 Ménard, O. *et al.* Validation of a new in vitro dynamic system to simulate infant digestion.  
1069 *Food Chem.* **145**, 1039–1045, doi: 10.1016/j.foodchem.2013.09.036 (2014).  
1070 9 Molly, K., Woestyne, M. V. & Verstraete, W. Development of a 5-step multi-chamber reactor  
1071 as a simulation of the human intestinal microbial ecosystem. *Appl. Microbiol. Biotechnol.* **39**,  
1072 254–258, doi: 10.1007/BF00228615 (1993).  
1073 10 Kong, F. & Singh, R. P. A Human Gastric Simulator (HGS) to Study Food Digestion in Human  
1074 Stomach. *J. Food Sci.* **75**, E627–E635, doi: 10.1111/j.1750-3841.2010.01856.x (2010).  
1075 11 Dupont, D. *et al.* Can dynamic in vitro digestion systems mimic the physiological reality? *Crit.*  
1076 *Rev. Food Sci. Nutr.*, 1–17, doi: 10.1080/10408398.2017.1421900 (2018).  
1077 12 Kaukonen, A. M., Boyd, B. J., Charman, W. N. & Porter, C. J. Drug solubilization behavior  
1078 during in vitro digestion of suspension formulations of poorly water-soluble drugs in  
1079 triglyceride lipids. *Pharm. Res.* **21**, 254–260, doi: (2004).  
1080 13 Maldonado-Valderrama, J., Gunning, A. P., Wilde, P. J. & Morris, V. J. In vitro gastric digestion  
1081 of interfacial protein structures: visualisation by AFM. *Soft Matter* **6**, 4908–4915, doi: (2010).  
1082 14 Boisen, S. & Fernández, J. A. Prediction of the total tract digestibility of energy in feedstuffs  
1083 and pig diets by in vitro analyses. *Anim. Feed Sci. Technol.* **68**, 277–286, doi: 10.1016/S0377-  
1084 8401(97)00058-8 (1997).  
1085 15 Bohn, T. *et al.* Correlation between in vitro and in vivo data on food digestion. What can we  
1086 predict with static in vitro digestion models? *Crit. Rev. Food Sci. Nutr.* **58**, 2239–2261 doi:  
1087 10.1080/10408398.2017.1315362 (2017).  
1088 16 Sanchón, J. *et al.* Protein degradation and peptide release from milk proteins in human  
1089 jejunum. Comparison with in vitro gastrointestinal simulation. *Food Chem.* **239**, 486–494, doi:  
090 10.1016/j.foodchem.2017.06.134 (2018).  
091 17 Inc., U. S. P. C. Canada (National Publishing for the United States Pharmacopeial  
092 Convention, Inc., Rockville, MD, 2003, 2003).  
093 18 McCarthy, C. A. *et al.* In vitro dissolution models for the prediction of in vivo performance of  
094 an oral mesoporous silica formulation. *J. Control. Release* **250**, 86–95, doi:  
095 10.1016/j.jconrel.2016.12.043 (2017).



- 1096 19 Griffin, B. T. *et al.* Comparison of in vitro tests at various levels of complexity for the  
1097 prediction of in vivo performance of lipid-based formulations: Case studies with fenofibrate.  
1098 *Eur. J. Pharm. Biopharm.* **86**, 427-437, doi: 10.1016/j.ejpb.2013.10.016 (2014).
- 1099 20 Oomen, A. G. *et al.* Development of an In Vitro Digestion Model for Estimating the  
1100 Bioaccessibility of Soil Contaminants. *Arch. Environ. Contam. Toxicol.* **44**, 0281-0287, doi:  
1101 10.1007/s00244-002-1278-0 (2003).
- 1102 21 Versantvoort, C. H. M., Oomen, A. G., Van de Kamp, E., Rompelberg, C. J. M. & Sips, A. J. A.  
1103 M. Applicability of an in vitro digestion model in assessing the bioaccessibility of mycotoxins  
1104 from food. *Food Chem. Toxicol.* **43**, 31-40, doi: 10.1016/j.fct.2004.08.007 (2005).
- 1105 22 Wragg, J. *et al.* Inter-laboratory trial of a unified bioaccessibility testing procedure; Chemical  
1106 & Biological Hazards Programme; Open Report OR/07/027. (2009).
- 1107 23 Dressman, J. B. *et al.* Upper gastrointestinal (GI) pH in young, healthy men and women.  
1108 *Pharm. Res.* **7**, 756-761, doi: 10.1023/A:1015827908309 (1990).
- 1109 24 Lentner, C. *Geigy Scientific tables. Vol. 1, Units of measurement, body fluids, composition of*  
1110 *the body, nutrition.* 8th edn, (Ciba-Geigy Basel, Switzerland, 1981).
- 1111 25 Hur, S. J., Lim, B. O., Decker, E. A. & McClements, D. J. In vitro human digestion models for  
1112 food applications. *Food Chem.* **125**, 1-12, doi: 10.1016/j.foodchem.2010.08.036 (2011).
- 1113 26 Dupont, D. *et al.* An International Network for Improving Health Properties of Food by  
1114 Sharing our Knowledge on the Digestive Process. *Food Digestion* **2**, 23-25, doi:  
1115 10.1007/s13228-011-0011-8 (2011).
- 1116 27 Minekus, M. *et al.* A standardised static in vitro digestion method suitable for food - an  
1117 international consensus. *Food & Function* **5**, 1113-1124, doi: 10.1039/C3FO60702J (2014).
- 1118 28 Egger, L. *et al.* The harmonized INFOGEST in vitro digestion method: From knowledge to  
1119 action. *Food Res. Int.* **88**, 217-225, doi: 10.1016/j.foodres.2015.12.006 (2016).
- 1120 29 Egger, L. *et al.* Physiological comparability of the harmonized INFOGEST in vitro digestion  
1121 method to in vivo pig digestion. *Food Res. Int.* **102**, 567-574, doi:  
1122 10.1016/j.foodres.2017.09.047 (2017).
- 1123 30 Hempel, J. *et al.* Ultrastructural deposition forms and bioaccessibility of carotenoids and  
1124 carotenoid esters from goji berries (*Lycium barbarum* L.). *Food Chem.* **218**, 525-533, doi:  
1125 10.1016/j.foodchem.2016.09.065 (2017).
- 1126 31 Rodrigues, D. B., Mariutti, L. R. B. & Mercadante, A. Z. An in vitro digestion method adapted  
1127 for carotenoids and carotenoid esters: moving forward towards standardization. *Food &*  
1128 *Function* **7**, 4992-5001, doi: 10.1039/c6fo01293k (2016).
- 1129 32 Bot, F. *et al.* The effect of pulsed electric fields on carotenoids bioaccessibility: The role of  
1130 tomato matrix. *Food Chem.* **240**, 415-421, doi: 10.1016/j.foodchem.2017.07.102 (2018).
- 1131 33 Gomez-Mascaraque, L. G., Perez-Masia, R., Gonzalez-Barrio, R., Periago, M. J. & Lopez-Rubio,  
1132 A. Potential of microencapsulation through emulsion-electrospraying to improve the  
1133 bioaccessibility of beta-carotene. *Food Hydrocolloids* **73**, 1-12, doi:  
1134 10.1016/j.foodhyd.2017.06.019 (2017).
- 1135 34 Davidov-Pardo, G., Perez-Ciordia, S., Marin-Arroyo, M. R. & McClements, D. J. Improving  
1136 Resveratrol Bioaccessibility Using Biopolymer Nanoparticles and Complexes: Impact of  
1137 Protein-Carbohydrate Maillard Conjugation. *J. Agric. Food. Chem.* **63**, 3915-3923, doi:  
1138 10.1021/acs.jafc.5b00777 (2015).
- 1139 35 Ferreira-Lazarte, A. *et al.* Study on the digestion of milk with prebiotic carbohydrates in a  
1140 simulated gastrointestinal model. *J. Funct. Foods* **33**, 149-154, doi: 10.1016/j.jff.2017.03.031  
1141 (2017).
- 1142 36 El, S. N. *et al.* In vitro digestibility of goat milk and kefir with a new standardised static  
1143 digestion method (INFOGEST cost action) and bioactivities of the resultant peptides. *Food &*  
1144 *Function* **6**, 2322-2330, doi: 10.1039/c5fo00357a (2015).

- 1145 37 Wang, B., Timilsena, Y. P., Blanch, E. & Adhikari, B. Mild thermal treatment and in-vitro  
1146 digestion of three forms of bovine lactoferrin: Effects on functional properties. *Int. Dairy J.*  
1147 **64**, 22-30, doi: 10.1016/j.idairyj.2016.09.001 (2017).
- 1148 38 Naegeli, H. *et al.* Guidance on allergenicity assessment of genetically modified plants. *Efsa*  
1149 *Journal* **15**, doi: 10.2903/j.efsa.2017.4862 (2017).
- 1150 39 Mamone, G. *et al.* Tracking the fate of pasta (T. durum semolina) immunogenic proteins by in  
1151 vitro simulated digestion. *J. Agric. Food. Chem.* **63**, 2660–2667, doi: 10.1021/jf505461x  
1152 (2015).
- 1153 40 Korte, R., Bracker, J. & Brockmeyer, J. Gastrointestinal digestion of hazelnut allergens on  
1154 molecular level: Elucidation of degradation kinetics and resistant immunoactive peptides  
1155 using mass spectrometry. *Mol. Nutr. Food Res.* **61**, doi: 10.1002/mnfr.201700130 (2017).
- 1156 41 Di Stasio, L. *et al.* Peanut digestome: Identification of digestion resistant IgE binding peptides.  
1157 *Food Chem. Toxicol.* **107**, 88-98, doi: 10.1016/j.fct.2017.06.029 (2017).
- 1158 42 Mat, D. J. L., Le Feunteun, S., Michon, C. & Souchon, I. In vitro digestion of foods using pH-  
1159 stat and the INFOGEST protocol: Impact of matrix structure on digestion kinetics of  
1160 macronutrients, proteins and lipids. *Food Res. Int.* **88**, Part B, 226-233, doi:  
1161 10.1016/j.foodres.2015.12.002 (2016).
- 1162 43 Flourey, J. *et al.* Exploring the breakdown of dairy protein gels during in vitro gastric digestion  
1163 using time-lapse synchrotron deep-UV fluorescence microscopy. *Food Chem.* **239**, 898-910,  
1164 doi: 10.1016/j.foodchem.2017.07.023 (2018).
- 1165 44 Sarkar, A. *et al.* In vitro digestion of Pickering emulsions stabilized by soft whey protein  
1166 microgel particles: influence of thermal treatment. *Soft Matter* **12**, 3558-3569, doi:  
1167 10.1039/C5SM02998H (2016).
- 1168 45 Fernandez-Avila, C., Arranz, E., Guri, A., Trujillo, A. & Corredig, M. Vegetable protein isolate-  
1169 stabilized emulsions for enhanced delivery of conjugated linoleic acid in Caco-2 cells. *Food*  
1170 *Hydrocolloids* **55**, 144-154, doi: 10.1016/j.foodhyd.2015.10.015 (2016).
- 1171 46 Yang, J., Primo, C., Elbaz-Younes, I. & Hirschi, K. D. Bioavailability of transgenic microRNAs in  
1172 genetically modified plants. *Genes and Nutrition* **12**, doi: 10.1186/s12263-017-0563-5 (2017).
- 1173 47 Aschoff, J. K. *et al.* Bioavailability of beta-cryptoxanthin is greater from pasteurized orange  
1174 juice than from fresh oranges - a randomized cross-over study. *Mol. Nutr. Food Res.* **59**,  
1175 1896-1904, doi: 10.1002/mnfr.201500327 (2015).
- 1176 48 Garrett, D. A., Failla, M. L. & Sarama, R. J. Development of an in vitro digestion method to  
1177 assess carotenoid bioavailability from meals. *J. Agric. Food. Chem.* **47**, 4301-4309, doi:  
1178 10.1021/jf9903298 (1999).
- 1179 49 Mulet-Cabero, A.-I., Rigby, N. M., Brodkorb, A. & Mackie, A. R. Dairy food structures influence  
1180 the rates of nutrient digestion through different in vitro gastric behaviour. *Food*  
1181 *Hydrocolloids* **67**, 63-73, doi: 10.1016/j.foodhyd.2016.12.039 (2017).
- 1182 50 Mulet-Cabero, A.-I., Mackie, A., Wilde, P., Fenelon, M. A. & Brodkorb, A. Structural  
1183 mechanism and kinetics of in vitro gastric digestion are affected by process-induced changes  
1184 in bovine milk. *Food Hydrocolloids* **86**, 172-183, doi: 10.1016/j.foodhyd.2018.03.035 (2019).
- 1185 51 Roura, E. *et al.* Critical review evaluating the pig as a model for human nutritional physiology.  
1186 *Nutrition Research Reviews* **29**, 60-90, doi: 10.1017/S0954422416000020 (2016).
- 1187 52 Le Huërou-Luron, I. *et al.* A mixture of milk and vegetable lipids in infant formula changes gut  
1188 digestion, mucosal immunity and microbiota composition in neonatal piglets. *Eur. J. Nutr.* **57**,  
1189 463-476, doi: 10.1007/s00394-016-1329-3 (2018).
- 1190 53 Barbe, F. *et al.* The heat treatment and the gelation are strong determinants of the kinetics  
1191 of milk proteins digestion and of the peripheral availability of amino acids. *Food Chem.* **136**,  
1192 1203-1212, doi: 10.1016/j.foodchem.2012.09.022 (2013).
- 1193 54 Evenepoel, P. *et al.* Digestibility of Cooked and Raw Egg Protein in Humans as Assessed by  
1194 Stable Isotope Techniques. *J. Nutr.* **128**, 1716-1722, doi: 10.1093/jn/128.10.1716 (1998).

- 1195 55 Normén, L. *et al.* Phytosterol and phytostanol esters are effectively hydrolysed in the gut and  
 1196 do not affect fat digestion in ileostomy subjects. *Eur. J. Nutr.* **45**, 165-170, doi:  
 1197 10.1007/s00394-006-0578-y (2006).
- 1198 56 Edwards, C. H. *et al.* Manipulation of starch bioaccessibility in wheat endosperm to regulate  
 1199 starch digestion, postprandial glycemia, insulinemia, and gut hormone responses: a  
 1200 randomized controlled trial in healthy ileostomy participants. *Am. J. Clin. Nutr.* **102**, 791-800,  
 1201 doi: 10.3945/ajcn.114.106203 (2015).
- 1202 57 Bakala N'Goma, J. C., Amara, S., Dridi, K., Jannin, V. & Carriere, F. Understanding the lipid-  
 1203 digestion processes in the GI tract before designing lipid-based drug-delivery systems. *Ther.*  
 1204 *Deliv.* **3**, 105-124, doi: 10.4155/tde.11.138 (2012).
- 1205 58 Gargouri, Y. *et al.* Importance of human gastric lipase for intestinal lipolysis: an in vitro study.  
 1206 *Biochim. Biophys. Acta* **879**, 419-423, doi: 10.1016/0005-2760(86)90234-1 (1986).
- 1207 59 Ville, E., Carriere, F., Renou, C. & Laugier, R. Physiological study of pH stability and sensitivity  
 1208 to pepsin of human gastric lipase. *Digestion* **65**, 73-81, doi: 10.1159/000057708 (2002).
- 1209 60 Carrière, F., Barrowman, J. A., Verger, R. & Laugier, R. Secretion and contribution to lipolysis  
 1210 of gastric and pancreatic lipases during a test meal in humans. *Gastroenterology* **105**, 876-  
 1211 888, doi: (1993).
- 1212 61 Sams, L., Paume, J., Giallo, J. & Carriere, F. Relevant pH and lipase for in vitro models of  
 1213 gastric digestion. *Food & Function* **7**, 30-45, doi: 10.1039/C5FO00930H (2016).
- 1214 62 Carrière, F. *et al.* The specific activities of human digestive lipases measured from the in vivo  
 1215 and in vitro lipolysis of test meals. *Gastroenterology* **119**, 949-960, doi:  
 1216 10.1053/gast.2000.18140 (2000).
- 1217 63 Bakala-N'Goma, J. C. *et al.* Toward the establishment of standardized in vitro tests for lipid-  
 1218 based formulations. 5. Lipolysis of representative formulations by gastric lipase. *Pharm. Res.*  
 1219 **32**, 1279-1287, doi: 10.1007/s11095-014-1532-y (2015).
- 1220 64 Capolino, P. *et al.* In vitro gastrointestinal lipolysis: replacement of human digestive lipases  
 1221 by a combination of rabbit gastric and porcine pancreatic extracts. *Food Digestion* **2**, 43-51,  
 1222 doi: 10.1007/s13228-011-0014-5 (2011).
- 1223 65 Moreau, H., Gargouri, Y., Lecat, D., Junien, J.-L. & Verger, R. Screening of preduodenal lipases  
 1224 in several mammals. *Biochim. Biophys. Acta* **959**, 247-252, doi: 10.1016/0005-  
 1225 2760(88)90197-X (1988).
- 1226 66 De Caro, J., Ferrato, F., Verger, R. & de Caro, A. Purification and molecular characterization of  
 1227 lamb pregastric lipase. *Biochim. Biophys. Acta* **1252**, 321-329, doi: 10.1016/0167-  
 1228 4838(95)00134-G (1995).
- 1229 67 Sams, L. *et al.* Characterization of pepsin from rabbit gastric extract, its action on  $\beta$ -casein  
 1230 and the effects of lipids on proteolysis. *Food & Function* **Accepted Manuscript** doi:  
 1231 10.1039/C8FO01450G (2018).
- 1232 68 Rich, D. H. *et al.* Inhibition of aspartic proteases by pepstatin and 3-methylstatine derivatives  
 1233 of pepstatin. Evidence for collected-substrate enzyme inhibition. *Biochemistry* **24**, 3165-  
 1234 3173, doi: 10.1021/bi00334a014 (1985).
- 1235 69 Mat, D. J. L., Cattenoz, T., Souchon, I., Michon, C. & Le Feunteun, S. Monitoring protein  
 1236 hydrolysis by pepsin using pH-stat: In vitro gastric digestions in static and dynamic pH  
 1237 conditions. *Food Chem.* **239**, 268-275, doi: 10.1016/j.foodchem.2017.06.115 (2018).
- 1238 70 Gargouri, Y. *et al.* Kinetic assay of human gastric lipase on short- and long-chain  
 1239 triacylglycerol emulsions. *Gastroenterology* **91**, 919-925, doi:  
 1240 10.5555/uri:pii:0016508586906955 (1986).
- 1241 71 Moreau, H., Gargouri, Y., Lecat, D., Junien, J.-L. & Verger, R. Purification, characterization and  
 1242 kinetic properties of the rabbit gastric lipase. *Biochimica et Biophysica Acta (BBA)-Lipids and*  
 1243 *Lipid Metabolism* **960**, 286-293, doi: 10.1016/0005-2760(88)90036-7 (1988).

- 1244 72 Ménard, O. *et al.* A first step towards a consensus static in vitro model for simulating full-  
1245 term infant digestion. *Food Chem.* **240**, 338-345, doi: 10.1016/j.foodchem.2017.07.145  
1246 (2018).
- 1247 73 Lecomte, M. *et al.* Milk Polar Lipids Affect In Vitro Digestive Lipolysis and Postprandial Lipid  
1248 Metabolism in Mice. *J. Nutr.* **145**, 1770-1777, doi: 10.3945/jn.115.212068 (2015).
- 1249 74 Grundy, M. M. L. *et al.* The impact of oat structure and beta-glucan on in vitro lipid digestion.  
1250 *J Funct Foods* **38**, 378-388, doi: 10.1016/j.jff.2017.09.011 (2017).
- 1251 75 Salvia-Trujillo, L. *et al.* Lipid digestion, micelle formation and carotenoid bioaccessibility  
1252 kinetics: Influence of emulsion droplet size. *Food Chem.* **229**, 653-662, doi:  
1253 10.1016/j.foodchem.2017.02.146 (2017).
- 1254 76 Bligh, E. G. & Dyer, W. J. A rapid method of total lipid extraction and purification. *Can. J.*  
1255 *Biochem. Physiol.* **37**, 911-917, doi: 10.1139/y59-099 (1959).
- 1256 77 Cavalier, J.-F. *et al.* Validation of lipolysis product extraction from aqueous/biological  
1257 samples, separation and quantification by thin-layer chromatography with flame ionization  
1258 detection analysis using O-cholesteryl ethylene glycol as a new internal standard. *J.*  
1259 *Chromatogr. A* **1216**, 6543-6548, doi: 10.1016/j.chroma.2009.07.061 (2009).
- 1260 78 Carriere, F. *et al.* Purification and biochemical characterization of dog gastric lipase. *The FEBS*  
1261 *Journal* **202**, 75-83, doi: 10.1111/j.1432-1033.1991.tb16346.x (1991).
- 1262 79 Bourlieu, C. *et al.* The structure of infant formulas impacts their lipolysis, proteolysis and  
1263 disintegration during in vitro gastric digestion. *Food Chem.* **182**, 224-235, doi:  
1264 10.1016/j.foodchem.2015.03.001 (2015).
- 1265 80 Chatzidaki, M. D., Mateos-Diaz, E., Leal-Calderon, F., Xenakis, A. & Carriere, F. Water-in-oil  
1266 microemulsions versus emulsions as carriers of hydroxytyrosol: an in vitro gastrointestinal  
1267 lipolysis study using the pHstat technique. *Food & Function* **7**, 2258-2269, doi:  
1268 10.1039/C6FO00361C (2016).
- 1269 81 Tyssandier, V. *et al.* Processing of vegetable-borne carotenoids in the human stomach and  
1270 duodenum. *Am. J. Physiol. Gastrointest. Liver Physiol.* **284**, G913-G923, doi:  
1271 10.1152/ajpgi.00410.2002 (2003).
- 1272 82 Reboul, E. *et al.* Bioaccessibility of carotenoids and vitamin E from their main dietary sources.  
1273 *J. Agric. Food. Chem.* **54**, 8749-8755, doi: 10.1021/jf061818s (2006).
- 1274 83 Biehler, E., Kaulmann, A., Hoffmann, L., Krause, E. & Bohn, T. Dietary and host-related factors  
1275 influencing carotenoid bioaccessibility from spinach (*Spinacia oleracea*). *Food Chem.* **125**,  
1276 1328-1334, doi: 10.1016/j.foodchem.2010.09.110 (2011).
- 1277 84 Boon, C. S., McClements, D. J., Weiss, J. & Decker, E. A. Factors influencing the chemical  
1278 stability of carotenoids in foods. *Crit. Rev. Food Sci. Nutr.* **50**, 515-532, doi:  
1279 10.1080/10408390802565889 (2010).
- 1280 85 Jorgensen, E. M., Marin, A. B. & Kennedy, J. A. Analysis of the oxidative degradation of  
1281 proanthocyanidins under basic conditions. *J. Agric. Food. Chem.* **52**, 2292-2296, doi:  
1282 10.1021/jf035311i (2004).
- 1283 86 Talcott, S. T. & Howard, L. R. Phenolic autoxidation is responsible for color degradation in  
1284 processed carrot puree. *J. Agric. Food. Chem.* **47**, 2109-2115, doi: 10.1021/jf981134n (1999).
- 1285 87 Bermúdez-Soto, M. J., Tomás-Barberán, F. A. & García-Conesa, M. T. Stability of polyphenols  
1286 in chokeberry (*Aronia melanocarpa*) subjected to in vitro gastric and pancreatic digestion.  
1287 *Food Chem.* **102**, 865-874, doi: 10.1016/j.foodchem.2006.06.025 (2007).
- 1288 88 Alming, M. *et al.* In vitro models for studying secondary plant metabolite digestion and  
289 bioaccessibility. *Comprehensive Reviews in Food Science and Food Safety* **13**, 413-436, doi:  
290 10.1111/1541-4337.12081 (2014).
- 291 89 Bohn, T. *et al.* Mind the gap-deficits in our knowledge of aspects impacting the bioavailability  
292 of phytochemicals and their metabolites-a position paper focusing on carotenoids and  
293 polyphenols. *Mol. Nutr. Food Res.* **59**, 1307-1323, doi: 10.1002/mnfr.201400745 (2015).



- 1294 90 Amiri, M. & Naim, H. Y. Characterization of mucosal disaccharidases from human intestine. *Nutrients* **9**, doi: 10.3390/nu9101106 (2017).  
1295
- 1296 91 Bouayed, J., Deusser, H., Hoffmann, L. & Bohn, T. Bioaccessible and dialysable polyphenols in  
1297 selected apple varieties following in vitro digestion vs. their native patterns. *Food Chem.* **131**,  
1298 1466-1472, doi: 10.1016/j.foodchem.2011.10.030 (2012).
- 1299 92 Coates, E. M. *et al.* Colon-available raspberry polyphenols exhibit anti-cancer effects on in  
1300 vitro models of colon cancer. *J. Carcinog.* **6**, 4, doi: 10.1186/1477-3163-6-4 (2007).
- 1301 93 Figueira, I. *et al.* Blood-brain barrier transport and neuroprotective potential of blackberry-  
1302 digested polyphenols: an in vitro study. *Eur. J. Nutr.*, doi: 10.1007/s00394-017-1576-y (2017).
- 1303 94 Garcia, G. *et al.* Bioaccessible (poly)phenol metabolites from raspberry protect neural cells  
1304 from oxidative stress and attenuate microglia activation. *Food Chem.* **215**, 274-283, doi:  
1305 10.1016/j.foodchem.2016.07.128 (2017).
- 1306 95 Bohn, T. Bioactivity of carotenoids – chasms of knowledge. *Int. J. Vitam. Nutr. Res.* **10**, 1-5,  
1307 doi: 10.1024/0300-9831/a000400 (2016).
- 1308 96 Levi, C. S. *et al.* Extending in vitro digestion models to specific human populations:  
1309 Perspectives, practical tools and bio-relevant information. *Trends Food Sci. Technol.* **60**, 52-  
1310 63, doi: 10.1016/j.tifs.2016.10.017 (2017).
- 1311 97 Picariello, G. *et al.* Peptides surviving the simulated gastrointestinal digestion of milk  
1312 proteins: Biological and toxicological implications. *Journal of Chromatography B-Analytical  
1313 Technologies in the Biomedical and Life Sciences* **878**, 295-308, doi:  
1314 10.1016/j.jchromb.2009.11.033 (2010).
- 1315 98 Garcia-Campayo, V., Han, S., Vercauteren, R. & Franck, A. Digestion of Food Ingredients and  
1316 Food Using an <i>In Vitro</i> Model Integrating Intestinal Mucosal Enzymes. *Food  
1317 and Nutrition Sciences* **9**, 711-734, doi: 10.4236/fns.2018.96055 (2018).
- 1318 99 Picariello, G., Ferranti, P. & Addeo, F. Use of brush border membrane vesicles to simulate the  
1319 human intestinal digestion. *Food Res. Int.* **88**, Part B, 327-335, doi:  
1320 10.1016/j.foodres.2015.11.002 (2016).
- 1321 100 Cheeseman, C. I. & O'Neill, D. in *Curr. Protoc. Cell Biol.* (John Wiley & Sons, Inc., 2001).
- 1322 101 Lin, X. J. & Wright, A. J. Pectin and gastric pH interactively affect DHA-rich emulsion in vitro  
1323 digestion microstructure, digestibility and bioaccessibility. *Food Hydrocolloids* **76**, 49-59, doi:  
1324 10.1016/j.foodhyd.2017.06.010 (2018).
- 1325 102 Lorieau, L. *et al.* Impact of the dairy product structure and protein nature on the proteolysis  
1326 and amino acid bioaccessibility during in vitro digestion. *Food Hydrocolloids* **82**, 399-411, doi:  
1327 10.1016/j.foodhyd.2018.04.019 (2018).
- 1328 103 Macierzanka, A., Sancho, A., Mills, E. N. C., Rigby, N. & Mackie, A. Emulsification alters  
1329 simulated gastrointestinal proteolysis of  $\beta$ -casein and  $\beta$ -lactoglobulin. *Soft Matter* **5**, 538-  
1330 550, doi: 10.1039/b811233a (2009).
- 1331 104 Carriere, F. *et al.* Inhibition of gastrointestinal lipolysis by Orlistat during digestion of test  
1332 meals in healthy volunteers. *Am. J. Physiol. Gastrointest. Liver Physiol.* **281**, G16-G28, doi:  
1333 10.1152/ajpgi.2001.281.1.G16 (2001).
- 1334 105 Williams, H. D. *et al.* Toward the establishment of standardized in vitro tests for lipid-based  
1335 formulations, part 1: Method parameterization and comparison of in vitro digestion profiles  
1336 across a range of representative formulations. *J. Pharm. Sci.* **101**, 3360-3380, doi:  
1337 10.1002/jps.23205 (2012).
- 1338 106 Edwards, C. H., Maillot, M., Parker, R. & Warren, F. J. A comparison of the kinetics of in vitro  
1339 starch digestion in smooth and wrinkled peas by porcine pancreatic alpha-amylase. *Food  
1340 Chem.* **244**, 386-393, doi: 10.1016/j.foodchem.2017.10.042 (2018).
- 1341 107 Villemejeane, C. *et al.* In vitro digestion of short-dough biscuits enriched in proteins and/or  
1342 fibres using a multi-compartmental and dynamic system (2): Protein and starch hydrolyses.  
1343 *Food Chem.* **190**, 164-172, doi: 10.1016/j.foodchem.2015.05.050 (2016).

- 1344 108 Romano, A. *et al.* Characterisation, in vitro digestibility and expected glycemic index of  
1345 commercial starches as uncooked ingredients. *Journal of Food Science and Technology* **53**,  
1346 4126-4134, doi: 10.1007/s13197-016-2375-9 (2016).
- 1347 109 Bustos, M. C., Vignola, M. B., Perez, G. T. & Leon, A. E. In vitro digestion kinetics and  
1348 bioaccessibility of starch in cereal food products. *Journal of Cereal Science* **77**, 243-250, doi:  
1349 10.1016/j.jcs.2017.08.018 (2017).
- 1350 110 Corte-Real, J., Richling, E., Hoffmann, L. & Bohn, T. Selective factors governing in vitro beta-  
1351 carotene bioaccessibility: negative influence of low filtration cutoffs and alterations by  
1352 emulsifiers and food matrices. *Nutrition Research* **34**, 1101-1110, doi:  
1353 10.1016/j.nutres.2014.04.010 (2014).
- 1354 111 Liu, J. Y. *et al.* Cellular uptake and trans-enterocyte transport of phenolics bound to vinegar  
1355 melanoidins. *J Funct Foods* **37**, 632-640, doi: 10.1016/j.jff.2017.08.009 (2017).
- 1356 112 Hidalgo, A. *et al.* Bioactive compounds and antioxidant properties of pseudocereals-enriched  
1357 water biscuits and their in vitro digestates. *Food Chem.* **240**, 799-807, doi:  
1358 10.1016/j.foodchem.2017.08.014 (2018).
- 1359 113 Eratte, D., Dowling, K., Barrow, C. J. & Adhikari, B. P. In-vitro digestion of probiotic bacteria  
1360 and omega-3 oil co-microencapsulated in whey protein isolate-gum Arabic complex  
1361 coacervates. *Food Chem.* **227**, 129-136, doi: 10.1016/j.foodchem.2017.01.080 (2017).
- 1362 114 Bottari, B. *et al.* Characterization of the peptide fraction from digested Parmigiano Reggiano  
1363 cheese and its effect on growth of lactobacilli and bifidobacteria. *Int. J. Food Microbiol.* **255**,  
1364 32-41, doi: 10.1016/j.ijfoodmicro.2017.05.015 (2017).
- 1365 115 Sanchez-Moya, T. *et al.* In vitro modulation of gut microbiota by whey protein to preserve  
1366 intestinal health. *Food & Function* **8**, 3053-3063, doi: 10.1039/c7fo00197e (2017).
- 1367 116 Watanabe, S. & Dawes, C. The effects of different foods and concentrations of citric acid on  
1368 the flow rate of whole saliva in man. *Arch. Oral Biol.* **33**, 1-5, doi: 10.1016/0003-  
1369 9969(88)90089-1 (1988).
- 1370 117 St-Eve, A., Panouille, M., Capitaine, C., Deleris, I. & Souchon, I. Dynamic aspects of texture  
1371 perception during cheese consumption and relationship with bolus properties. *Food*  
1372 *Hydrocolloids* **46**, 144-152, doi: 10.1016/j.foodhyd.2014.12.015 (2015).
- 1373 118 Motoi, L., Morgenstern, M. P., Hedderley, D. I., Wilson, A. J. & Balita, S. Bolus moisture  
1374 content of solid foods during mastication. *J. Texture Stud.* **44**, 468-479, doi:  
1375 10.1111/jtxs.12036 (2013).
- 1376 119 Moongngarm, A., Bronlund, J. E., Grigg, N. & Sriwai, N. Chewing behavior and Bolus  
1377 Properties as Affected by Different Rice Types. *International Journal of Medical and*  
1378 *Biological Sciences* **6**, 51-56, doi: (2012).
- 1379 120 Loret, C. *et al.* Physical and related sensory properties of a swallowable bolus. *Physiol. Behav.*  
1380 **104**, 855-864, doi: 10.1016/j.physbeh.2011.05.014 (2011).
- 1381 121 Jourdren, S. *et al.* Breakdown pathways during oral processing of different breads: impact of  
1382 crumb and crust structures. *Food & Function* **7**, 1446-1457, doi: 10.1039/c5fo01286d (2016).
- 1383 122 Drago, S. R. *et al.* Relationships between saliva and food bolus properties from model dairy  
1384 products. *Food Hydrocolloids* **25**, 659-667, doi: 10.1016/j.foodhyd.2010.07.024 (2011).
- 1385 123 Doyennette, M. *et al.* Main individual and product characteristics influencing in-mouth  
1386 flavour release during eating masticated food products with different textures: Mechanistic  
1387 modelling and experimental validation. *J. Theor. Biol.* **340**, 209-221, doi:  
1388 10.1016/j.jtbi.2013.09.005 (2014).

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## Box 1 | Enzyme activity assays<sup>1</sup>

### PEPSIN ACTIVITY ASSAY

**Principle:** Haemoglobin + H<sub>2</sub>O  $\xrightarrow{\text{Pepsin}}$  TCA soluble tyrosine peptides

**Unit definition:** One unit produces a  $\Delta A_{280}$  of 0.001 per minute at pH 2.0 and 37°C, measured as trichloroacetic acid (TCA)-soluble products

**Substrate:** 2 % w/v haemoglobin in water at pH 2

**Enzyme solution:** Pepsin in 10 mM Tris buffer, 150 mM NaCl, pH 6.5. Before the assay dilute it in 10mM HCl at concentrations ranging 5-30  $\mu\text{g}/\text{mL}$

Mix 500  $\mu\text{L}$  of haemoglobin with 100  $\mu\text{L}$  of each pepsin solution (5-30  $\mu\text{g}/\text{mL}$ ) and incubate for 10 min at 37°C. To stop the reaction, add 1 mL of 5% w/v TCA. Centrifuge at 6,000  $\times g$  during 30 min and read the absorbance at 280 nm in quartz cuvettes.

### LIPASE ACTIVITY ASSAY

**Principle:** Tributyrin + H<sub>2</sub>O  $\xrightarrow{\text{Lipase}}$  butyric acid + *sn*-2 monobutyrin

**Unit definition:** One unit releases 1  $\mu\text{mol}$  butyric acid per minute at 37°C at the pH of the assay

**Substrate:** Tributyrin purity  $\geq 99\%$

**Enzyme solution:** Lipase 1 mg/mL in H<sub>2</sub>O

**Assay solution for gastric lipase:** 2mM Sodium taurodeoxycholate, 150 mM NaCl, 1 $\mu\text{M}$  BSA

**Assay solution for pancreatic lipase:** 4mM Sodium taurodeoxycholate, 150 mM NaCl, 1.4mM  $\mu\text{M}\text{CaCl}_2$

In a pH-stat at 37°C, mix 14.5 mL of assay solution with 0.5 mL of tributyrin, stir until it forms a fine oil-in-water emulsion. Add 50 or 100  $\mu\text{L}$  of enzyme solution (1 mg/mL) and monitor the rate of titrant (0.1 N NaOH) to maintain pH 6.0 (human gastric lipase) or pH 5.5 (rabbit gastric lipase) or pH 8 (pancreatic lipase) for 5 min.

### TRYPSIN ACTIVITY ASSAY

**Principle:** TAME + H<sub>2</sub>O  $\xrightarrow{\text{Trypsin}}$  p-Toluene-Sulfonyl-L-Arginine + Methanol

**Unit definition:** One unit hydrolyses 1  $\mu\text{mol}$  p-Toluene-Sulfonyl-L-arginine methyl ester (TAME) per minute at pH 8.1 and 25°C

**Substrate:** 10 mM TAME in H<sub>2</sub>O

**Enzyme solution:** Trypsin in 1 mM HCl at concentrations ranging 10-20  $\mu\text{g}/\text{mL}$

Mix 2.6 mL of 46 mM Tris/HCl buffer (pH 8.1) with 300  $\mu\text{L}$  of the substrate at 25°C. Add 100  $\mu\text{L}$  of each trypsin assay solution. Read the absorbance increase at 247 nm during 10 min.

### AMYLASE ACTIVITY ASSAY

**Principle:** Starch + H<sub>2</sub>O  $\xrightarrow{\alpha\text{-Amylase}}$  Reducing Groups (e.g. Maltose)

**Unit definition:** One unit releases 1.0 mg of maltose equivalent from starch in 3 min at pH 6.9 and 20°C

**Substrate:** 1.0 % w/v Soluble potato starch in 20mM sodium phosphate buffer with 6.7 mM NaCl, adjusted to pH 6.9

**Enzyme solution:** 1 mg/mL Amylase in H<sub>2</sub>O

Incubate 1 mL of substrate at 20°C, add the enzyme solution (0.5-1 mL, with estimated activity of 1 unit/mL) and incubate at 20°C for 3 min. Stop reaction with colour reagent (96 mM 3,5-dinitrosalicylic acid, 5.3 M sodium potassium tartrate). Complete enzyme volume with H<sub>2</sub>O to 1 mL, cap the tube and boil it for 15 min. Add 9 mL of H<sub>2</sub>O and read absorbance at 540 nm. Calculate the activity against a maltose standard curve.

<sup>1</sup>Detailed assays for all enzymes in Supplementary Information

1395 **TABLES**

1396

1397 **Table 1:** Examples for the preservation and treatment of samples after *in vitro* digestion

Application	Objectives	Method	Description	Sample preparation	Ref.
Food structure	Microscopy Rheology Particle size		Keep on ice and perform microscopy observations immediately after sampling	Fresh samples for standard microscopy sample preparation (e.g. resin embedding, chemical fixation, drying).	74,101
Breakdown of nutrients: Proteins	Protein hydrolysis or resistant protein analysis	Stop gastric digestion (2 options)	Raise the pH to 7 for partial inactivation of pepsin; pH 8 for complete inactivation.	Addition of 1 M NaHCO <sub>3</sub> or 1N NaOH	28
			Addition of pepstatin A for pepsin inhibition.	Add Pepstatin A at 0.5-1.0 µM final concentration.	102
		Stop intestinal digestion (3 options)	Addition of Pefabloc <sup>®</sup> SC (4-(2-aminoethyl)-benzolsulfonylfluorid-hydrochloride) for serine protease (trypsin and	Add 50 µl of Pefabloc (0.1 M) in water per mL of intestinal digesta. (5 mM final concentration).	28



			chymotrypsin) inhibition.		
			Addition of Bowman-Birk inhibitor (BBI) from soybean with ability to inhibit both trypsin and chymotrypsin.	Add 100µl of a BBI solution 0.05 g/L in water per mL of intestinal digesta.	<sup>103</sup>
			Heat shock treatment	Sample treatment: 100°C, 5 min, but detrimental to food structure, especially protein and carbohydrate structures	<sup>41</sup>
Breakdown of nutrients: Lipids	Lipid hydrolysis	Stop lipase activity in the gastric phase  (2 options)	Addition of Orlistat (tetrahydrolipstatin)	Add 10 µL/mL of a 100 mM Orlistat solution in ethanol (1 mM final concentration)	<sup>104</sup>
			Raise the pH to 8		<sup>59</sup>
	Stop lipase activity in the intestinal phase  (2 options)	Addition of lipase inhibitor (4-bromophenylboronic acid)	Add 5 µL/mL of a 1 M solution of 4-bromophenylboronic acid in methanol to 1 mL of digesta (5 mM final concentration).	<sup>105</sup>	
		Addition of methanol:chloroform	Addition of methanol: chloroform mixture used for Folch extraction	<sup>76</sup>	



Breakdown of nutrients: Carbohydrates	Starch hydrolysis	Stop amylase activity  (4 options)	Addition of NaCO <sub>3</sub>	Dilute digesta in 2 volumes of 0.3 M NaCO <sub>3</sub>	<sup>106</sup>
			Heat shock treatment	100°C for 5 min	
			TCA precipitation	Add 700 µL of 100% TCA to 5 mL digesta	<sup>107</sup>
			Ethanol	Add sample to equal volume of ethanol	<sup>108</sup>
Breakdown of oxygen sensitive phytochemicals	Degradation of polyphenols and carotenoids	Prevent contact with Oxygen	Flushing with Ar or N <sub>2</sub> , pyrogallol addition (carotenoids) prior to small intestinal digestion	Flush sample 1 minute with Ar or N <sub>2</sub>	<sup>87</sup>
Bioaccessibility	Bioaccessibility of digested nutrients	Stop pancreatic activities (see above Stop intestinal digestion)	Use of inhibitors e.g. Pefabloc. Test whether the use of enzyme inhibitors affect the results of the experiment.	See above <i>Stop intestinal digestion</i>	<sup>28</sup>
			Use of dialysis membranes/ centrifugation tubes having		<sup>109</sup>



			cut-off of 3 to 10kDa.		
			To dilute the digested samples to maintain the epithelium integrity of cell monolayers and avoid cytotoxicity	Dilution (several folds) of digested samples to reach osmolarity values at physiological level (285-300 mOsm/kg H <sub>2</sub> O).	110,111
			Extraction of compounds by using either solvents or acidic solutions	Different procedures for a wide range of compounds are employed	112
	Bioaccessibility of digested phytochemicals		Removal of unavailable constituents such as bound to macromolecules or complexed form	Ultracentrifugation and filtration with certain cut-off filters (e.g. 0.2 µm)	110
			Cleavage of glucosides and esters	Addition of brush border vesicles	90
Probiotic survival	To determine the survival rates of probiotic bacteria to digestion conditions		Immediate use of samples after digestion	To serially dilute the digested samples and plate for bacterial growth	113



Colonic fermentation and modulation of intestinal microbiota	Biotransformation of compounds and their effects on bacterial growth	Stop enzymatic activities	By heat shock	Heat treatment: 100 °C for 5 min but detrimental to food structure, especially protein and carbohydrate structures	114
			Immediate storage in ice before batch culture fermentation		115

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1400 **Table 2:** Volumes of electrolyte stock solutions of digestion fluids for a volume of 400 mL  
 1401 diluted with water (1.25× concentrations).

			SSF (pH 7)		SGF (pH 3)		SIF (pH 7)	
Salt solution added	Stock concentrations		mL of Stock added to prepare 0.4 L (1.25x)	Final salt conc. in SSF	mL of Stock added to prepare 0.4 L (1.25x)	Final salt conc. in SGF	mL of Stock added to prepare 0.4 L (1.25x)	Final salt conc. in SIF
	g/L	M	mL	mM	mL	mM	mL	mM
KCl	37.3	0.5	15.1	15.1	6.9	6.9	6.8	6.8
KH <sub>2</sub> PO <sub>4</sub>	68	0.5	3.7	3.7	0.9	0.9	0.8	0.8
NaHCO <sub>3</sub> *	84	1	6.8	13.6	12.5	25	42.5	85
NaCl	117	2	-	-	11.8	47.2	9.6	38.4
MgCl <sub>2</sub> (H <sub>2</sub> O) <sub>6</sub>	30.5	0.15	0.5	0.15	0.4	0.12	1.1	0.33
(NH <sub>4</sub> ) <sub>2</sub> CO <sub>3</sub> *	48	0.5	0.06	0.06	0.5	0.5	-	-
HCl		6	0.09	1.1	1.3	15.6	0.7	8.4

Addition before use (volumes are indicated in **Table 3**, typical experiment of 5 mL of SSF):

CaCl <sub>2</sub> (H <sub>2</sub> O) <sub>2</sub>	44.1	0.3	0.025	1.5	0.005	0.15	0.04	0.6
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1403

1404 **Table 3:** Example of an *in vitro* digestion experiment with 5 g of food

Input	5 g of liquid or solid food				
Digestion phase	Oral (SSF)	Gastric (SGF)		Intestinal (SIF)	
Food or digesta	5 g of food	10 mL from oral phase		20 mL from gastric phase	
1.25× electrolyte stock solutions (mL)	4	8		8**	
CaCl <sub>2</sub> (0.3 M) (mL)	0.025	0.005		0.04	
Enzymes	Salivary amylase	Pepsin	Gastric <sup>#</sup> Lipase	Trypsin in pancreatin	Bile salts
Enzyme activity (U/mL) or bile conc. (mM) in total digesta (final volume in mL at each digestion phase, see row below)	75 U/mL	2,000 U/mL	60 U/mL	100 U/mL	10 mM
Specific activity* (U/mg), Conc. (bile) mmole/g	100 U/mg	3,000 U/mg	25 U/mg	6 U/mg	0.667mmole/g
Conc. of enzyme/bile solution (mg/mL)	10	20	100	133.3	200
Volume of enzyme/bile to be added (mL)	0.75	0.667	0.48	5**	3**
H <sub>2</sub> O (mL)	0.225	0.448		3.16	
HCl (5M) for pH adj. (mL)	-	0.4		-	
NaOH (5M) for pH adj. (mL)	-	-		0.8	
Final volume (mL)	10	20		40	
Remarks	- Use salivary amylase only for food containing starch - 1:1 (w/w) dilution with SSF should result in a paste-like consistency, add more water if necessary - Some foods may not be digested as expected due to high substrate to enzyme		#Rabbit gastric extract (RGE) contains gastric lipase and pepsin, i.e. the pepsin content needs to be accounted for in the total pepsin activity		

	ratio in the static digestion method and may need to be further diluted with water prior the oral phase, see <b>Table 4</b> Troubleshooting		
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1405 \*Specific enzyme activity or bile concentration: measured for each batch of enzymes or bile  
 1406 extract according to standard assays (Supplemental Materials from Minekus et al. <sup>27</sup>), the  
 1407 enzyme assays for gastric lipase and pepsin are described in the supplemental materials of  
 1408 this manuscript

1409 \*\*Total volume of SIF (1.25×): 16 mL including pancreatin and bile, both of which are  
 1410 dissolved in SIF

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1412 **Table 4:** Troubleshooting

Procedure step (number)	Problem	Possible reason	Solution
Enzyme activity (1)	Pepsin activity results in lower activity units than specified	Enzyme activity measurement	Follow the standardised procedure using haemoglobin as substrate. Dissolve pepsin in 10 mM Tris, 150 mM NaCl, pH 6.5
Enzyme activity (1)	Amylase activity very low	DNS (3,5-dinitrosalicylic acid ) does not react with product	DNS solution needs to be freshly prepared
Gastric phase (24)	Food is not digested as expected. It forms a big clog and it is not digested at the end of the gastric phase	Excessive amount of substrate	Revise the amount of food introduced into the system. Realistic food consumption should be targeted. Dilute or suspend food in an appropriate amount of water, if necessary.  For example, to mimic the porcine <i>in vivo</i> digestion of cheese <sup>29</sup> at the end of the gastric phase, the cheese has to be diluted with water at 1:2 (w/w) prior to the oral phase.
Gastric phase (24)	pH difficult to adjust during gastric digestion	Quick pH drift during gastric phase	Run a pH-test adjustment experiment with the same food to determine volumes and times for HCl addition
Gastric/intestinal	Difficulties taking a	Presence of different	Use individual sample tube



phase (24, 32)	homogeneous sample during digestion	phases (lipids, water, solids)	for each time point rather than withdrawal of samples from the digestion vessel.
Gastric/intestinal phase (24, 32)	Poor mixing during digestion	Tube shape, volume or shaking is insufficient	Check the volume of the sample and the tube or vials to allow sufficient mixing of the sample.
Intestinal phase (32)	Intestinal samples affect cell viability in cell culture studies	Presence of bile salts, enzyme inhibitors	Avoid the use of enzyme inhibitors to stop the digestion reaction. Reduce the bile salt concentration during the intestinal phase. Sufficiently dilute the digestion mixture.
Intestinal phase (32)	Presence of insoluble material at the end of the intestinal phase	Non-digestible material	Use individual sample tube for each time point
Intestinal phase (32)	Poor lipid digestion at the end of digestion	Food contains high amount of lipids	Add porcine pancreatic lipase and colipase to achieve 2,000 U/mL lipase activity in the final mixture. Consider additional trypsin activity present in the pancreatic lipase.
Intestinal phase (32)	Starch digestion is too low	Incorrect method for quantification of starch digestion products	Add amyloglucosidase to samples before measuring glucose OR use a reducing sugar assay to measure starch digestion products. Check activity of amylase.



Intestinal phase (32)	Starch digestion product concentration does not change over time	Starch digestion is finished before samples are collected.	Take more samples at earlier time points. Consider using less amylase to slow the reaction down. Check feasibility of results by expressing findings as % of starch digested.
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1416 **Supplementary information**

1417 The Supplementary Information (SI) consists of:

- 1418 1. Supplementary Figure 1
- 1419 2. Supplementary Methods: protocols of enzyme assays
- 1420 3. Supplementary videos
- 1421 4. Supplementary spreadsheets in Excel format

1422

1423 **Supplementary Figure 1: Oral bolus hydration *in vivo***

1424 Bolus hydration (g of saliva / g of foods) *in vivo* just before swallowing, for various foods  
1425 based on published data<sup>116-123</sup>

1426

1427 **Supplementary Methods**

1428 Protocols of enzyme activity assays (summarised in **Box 1**) for  $\alpha$ -amylase (EC 3.2.1.1),  
1429 pepsin (EC 3.4.23.1), gastric lipase (EC 3.1.1.3), trypsin (EC 3.4.21.4), chymotrypsin (EC  
1430 3.4.21.1), pancreatic lipase (EC 3.1.1.3) and bile salts (according to supplier's protocol)

1431

1432 **Supplementary Videos:**

1433 Supplementary Video 1

1434 INFOGEST 2.0 digestion procedure part 1

1435 Supplementary Video 2

1436 INFOGEST 2.0 digestion procedure part 2

1437 Supplementary Video 3

1438 Amylase activity assay

1439 Supplementary Video 4

1440 Pepsin activity assay

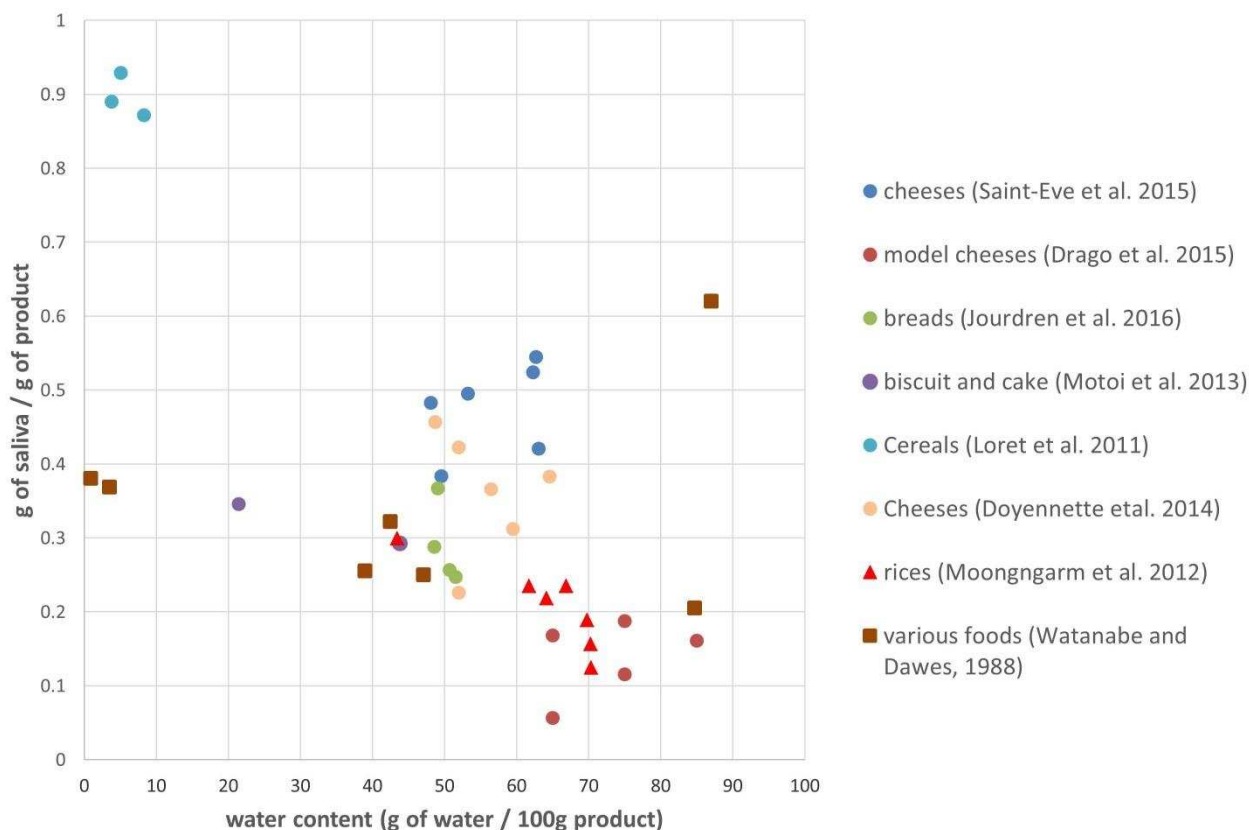
1441 Supplementary Video 5

1442 Lipase activity assay (both gastric and pancreatic)

- 1443           Supplementary Video 6
- 1444           Trypsin activity assay
- 1445           Supplementary Video 7
- 1446           Chymotrypsin activity assay
- 1447
- 1448   **Supplementary spreadsheets**
- 1449           Supplementary spreadsheets 1
- 1450           Excel spreadsheets to calculate the enzyme activities of all digestive
- 1451           enzymes.
- 1452           Supplementary spreadsheets 2
- 1453           Excel spreadsheets to calculate all volumes of simulated digestive fluids,
- 1454           enzyme and bile solutions based on the initial amount of digested food.
- 1455           In addition, the corresponding online spreadsheets and videos of the enzyme assays and
- 1456           digestion procedures are available here: [www.proteomics.ch/IVD](http://www.proteomics.ch/IVD) and on the INFOGEST
- 1457           website <https://www.cost-infogest.eu/>.
- 1458
- 1459



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Supplementary Figure 1

Oral bolus hydration *in vivo*Bolus hydration (g of saliva / g of foods) *in vivo* just before swallowing, for various foods based on published data<sup>1-8</sup>

## References

- 1 Watanabe, S. & Dawes, C. The effects of different foods and concentrations of citric acid on the flow rate of whole saliva in man. *Arch. Oral Biol.* **33**, 1-5, doi: 10.1016/0003-9969(88)90089-1 (1988).
- 2 St-Eve, A., Panouille, M., Capitaine, C., Deleris, I. & Souchon, I. Dynamic aspects of texture perception during cheese consumption and relationship with bolus properties. *Food Hydrocolloids* **46**, 144-152, doi: 10.1016/j.foodhyd.2014.12.015 (2015).
- 3 Motoi, L., Morgenstern, M. P., Hedderley, D. I., Wilson, A. J. & Balita, S. Bolus moisture content of solid foods during mastication. *J. Texture Stud.* **44**, 468-479, doi: 10.1111/jtxs.12036 (2013).
- 4 Moongngarm, A., Bronlund, J., Grigg, N. & Sriwai, N. *Chewing behavior and Bolus Properties as Affected by Different Rice Types*. Vol. 6 (2012).
- 5 Loret, C. *et al.* Physical and related sensory properties of a swallowable bolus. *Physiol. Behav.* **104**, 855-864, doi: 10.1016/j.physbeh.2011.05.014 (2011).
- 6 Jourden, S. *et al.* Breakdown pathways during oral processing of different breads: impact of crumb and crust structures. *Food & Function* **7**, 1446-1457, doi: 10.1039/c5fo01286d (2016).
- 7 Drago, S. R. *et al.* Relationships between saliva and food bolus properties from model dairy products. *Food Hydrocolloids* **25**, 659-667, doi: 10.1016/j.foodhyd.2010.07.024 (2011).
- 8 Doyennette, M. *et al.* Main individual and product characteristics influencing in-mouth flavour release during eating masticated



food products with different textures: Mechanistic modelling and experimental validation. *J. Theor. Biol.* **340**, 209-221, doi:10.1016/j.jtbi.2013.09.005 (2014).

1461

## 1462 **Supplementary Methods - Enzyme assays**

1463 Enzyme and bile assays are adapted from Minekus et al.<sup>1</sup>, namely:  $\alpha$ -amylase (EC 3.2.1.1),  
 1464 pepsin (EC 3.4.23.1), trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1), pancreatic lipase  
 1465 (EC 3.1.13) and bile salts (according to supplier's protocol). The assay for gastric lipase has  
 1466 been adapted from Carrière et al.<sup>2</sup> and merged with that for pancreatic lipase.

1467

### 1468 **$\alpha$ -Amylase Activity Assay (EC 3.2.1.1)**

1469 **References:** according to Bernfeld<sup>3</sup>

1470 **Method:** Spectrophotometric Stop Reaction

1471 **Principle:**

1472 Starch + H<sub>2</sub>O  $\xrightarrow{\alpha\text{-Amylase}}$  Reducing Groups (Maltose)

1473 **Unit definition:** One unit releases 1.0 mg of maltose from (potato) starch in 3 minutes at pH  
 1474 6.9 and 20 °C.

1475 **Conditions:** T = 20 °C, pH = 6.9, A<sub>540nm</sub>, light path = 1 cm

### 1476 **Procedure**

1477 Preparation of reagents

1478 **Substrate:** soluble potato starch (1.0% w/v)

1479 Preparation of substrate solution:

1480 Prepare 100 mL of a 20 mM sodium phosphate buffer containing 6.7 mM NaCl. Adjust the  
 1481 pH to 6.9 at 20 °C with 1 M NaOH. Dissolve 0.25 g soluble potato starch (ref S2630 Sigma-  
 1482 Aldrich) in 20 mL of the sodium phosphate buffer, pH 6.9. Heat the covered beaker while  
 483 stirring and maintain the solution just below boiling temperature for 15 minutes. Cool to room  
 484 temperature and complete the starch solution to the appropriate volume (25 mL) by addition  
 485 of H<sub>2</sub>O.



- 1486 **Standard Curve:** Prepare 10 mL of 0.2 % w/v maltose standard (M5885 Sigma-Aldrich).
- 1487 **Enzyme:** Shortly before the assay, prepare an enzyme solution of an estimated activity of 1  
1488 unit/mL of  $\alpha$ -amylase in purified H<sub>2</sub>O
- 1489 **Assay solution:** Colour reagent solution 3,5-dinitrosalicylic acid (DNS)
- 1490 Prepare a 5.3 M sodium potassium tartrate solution in 2 M NaOH by dissolving 0.8 g NaOH  
1491 in 10 mL H<sub>2</sub>O and heating the solution at a temperature ranging between 50 to 70 °C. Add  
1492 12.0 g of sodium potassium tartrate tetrahydrate (in 8.0 mL of warm 2 M NaOH solution,  
1493 maintain the temperature constant while stirring to dissolve the tartrate but do not boil it.
- 1494 Prepare a 96 mM DNS solution by dissolving 438 mg of DNS in 20 mL of H<sub>2</sub>O. Heat the  
1495 solution at a temperature between 50 to 70 °C. Maintain at this temperature while constant  
1496 stirring to dissolve DNS but do not boil it.
- 1497 Heat 12 mL of purified water to 60 °C and add slowly 8 mL of the 5.3 M the sodium potassium  
1498 tartrate solution. Add 20 mL of the 96 mM 3,5-dinitrosalicylic acid solution and stir until  
1499 complete dissolution. The solution can be stored in an amber flask at room temperature for  
1500 one month.
- 1501 **Assay:**
- 1502 Set the spectrophotometer at 540 nm and 20 °C. Set a bench top shaking incubator fitted with  
1503 a sample holder at 20 °C, a heating bath or block at 100 °C to stop the reaction, and an ice-  
1504 bath to cool the sample.
- 1505 **Test:** Pipette 1 mL of substrate solution (potato starch) into cap covered tubes (15 mL), mix  
1506 and incubate at 20 °C for 5 min to achieve temperature. Add 0.5 – 1 mL of enzyme solution  
1507 (according to the scheme below), mix and incubate at 20 °C for exactly 3 minutes.  
1508 Immediately thereafter, stop the reaction by addition of 1 mL of DNS solution. Complete the  
1509 enzyme volume added to 1 mL, cap the tube, place it at 100 °C (heating bath or block) and  
1510 boil it for exactly 15 minutes. Cool the tube for a few minutes on ice and add 9 mL of H<sub>2</sub>O.  
1511 Mix the reaction and pipette 3 mL in a cuvette and record the absorbance at 540 nm.
- 1512 **Blank:** For blank tests, follow the same procedure but no enzyme is added before the 3  
1513 minutes incubation time.
- 514 **Pipetting scheme for three different enzyme concentrations:**

Volumes in mL	1 <sup>st</sup> enzyme	2 <sup>nd</sup> enzyme	3 <sup>rd</sup> enzyme	Blank
---------------	------------------------	------------------------	------------------------	-------



	concentration	concentration	concentration	
Substrate (potato starch)	1.00	1.00	1.00	1.00
Enzyme solution	0.50	0.70	1.00	-
DNS	1.00	1.00	1.00	1.00
2 <sup>nd</sup> addition of enzyme	0.50	0.30	-	1.00
H <sub>2</sub> O	9.00	9.00	9.00	9.00

1515

1516 **Standard Curve with maltose:**1517 Dilute the maltose solution (0.2% w/v) according to the scheme in H<sub>2</sub>O

Volumes in (mL)	D1	D2	D3	D4	D5	D6	D7	Std. Blank
Maltose solution	0.05	0.20	0.40	0.60	0.80	1.00	2.00	-
H <sub>2</sub> O	1.95	1.80	1.60	1.40	1.20	1.00	-	2.00

1518

1519 1mL DNS reagent solution is added to each maltose standard, thereafter the tubes are boiled  
1520 for 15 minutes, cooled on ice to room temperature and 9mL of H<sub>2</sub>O are added.

1521 **Calculations**

1522 Standard Curve:

$$\Delta A_{540} \text{Standard} = \Delta A_{540} \text{Standard} - \Delta A_{540} \text{Std. Blank}$$

1523 Plot the  $\Delta A_{540 \text{nm}}$  of the Standards versus the quantity of maltose [mg] and establish a linear  
1524 regression:

$$\Delta A_{540} \text{Standard} = a \times [\text{maltose}] - b$$

1525 Enzyme activity:

$$\Delta A_{540} \text{Sample} = \Delta A_{540} \text{Test} - \Delta A_{540} \text{Test Blank}$$

$$\frac{\text{Units}}{\text{mg powder}} = \frac{[A_{540} \text{ Test} - A_{540} \text{ Test Blank}] - b}{(a \times X)}$$

1526

1527 a: slope of the linear regression for standards  $\Delta A_{540 \text{nm}}$  vs the quantity of maltose (mg).1528 b: intercept of the linear regression for standards  $\Delta A_{540 \text{nm}}$  vs the quantity of maltose (mg).

1529 X: quantity of amylase powder (mg) added before stopping the reaction.

1530

### 1531 **Pepsin Activity Assay (EC 3.4.23.1)**

1532 **References:** adapted from Anson *et al.* <sup>4,5</sup>

1533 **Method:** Spectrophotometric Stop Reaction

1534 **Principle:**

1535 Haemoglobin + H<sub>2</sub>O  $\xrightarrow{\text{pepsin}}$  TCA soluble tyrosine containing peptides

1536 **Unit definition:** One unit will produce a  $\Delta A_{280}$  of 0.001 per minute at pH 2.0 and 37°C,  
1537 measured as TCA-soluble products. These units are often referred to “Sigma” or “Anson”  
1538 pepsin units.

1539 **Conditions:** T = 37°C, pH = 2.0,  $A_{280\text{nm}}$ , light path = 1 cm

1540 **Procedure:**

1541 Preparation of reagents

1542 **Substrate:** Prepare a haemoglobin solution by dispersing 0.5 g haemoglobin (bovine blood  
1543 haemoglobin, ref H2500 Sigma-Aldrich) in 20 mL purified water, adjust to pH 2 with 300 mM  
1544 HCl and complete the volume to 25 ml to obtain a solution at 2% w/v haemoglobin at pH 2.

1545 **Enzyme:** Prepare a stock solution of 1 mg/mL pepsin (porcine pepsin, ref. P6887 Sigma-  
1546 Aldrich) in 10 mM Tris buffer, 150 mM NaCl at pH 6.5. The stock solution has to be stored on  
1547 ice or refrigerated at 4°C. Just before the assay, a range of 5 to 10 concentrations of pepsin  
1548 in 10 mM HCl has to be prepared. For instance, dilute the pepsin stock solution to prepare  
1549 the following enzyme assay solutions: 5, 10, 15, 20, 25, 30 µg/mL.

1550 **Assay:**

1551 Set the spectrophotometer at 280 nm and 20°C. Set a bench top shaking incubator fitted with  
1552 a sample holder at 37°C.

553 **Test:** Pipette 500 µL of haemoglobin solution into 2 mL Eppendorf tubes and incubate in a  
554 shaking incubator at 37°C for 3-4 minutes to reach the assay temperature.

555 Add 100 µL of pepsin assay solutions for each concentration and incubate them for 10  
556 minutes exactly. To stop the reaction, 1 mL of 5% w/v TCA (Trichloroacetic Acid) is added in

1557 each tube. In order to get a clear soluble phase available for absorbance measurement,  
 1558 centrifuge the Eppendorf tubes at  $6,000 \times g$  for 30 minutes to precipitate remaining  
 1559 haemoglobin; remove the pellet.

1560 Place the soluble phase into quartz cuvettes and read the absorbance at 280 nm ( $A_{280}$  Test).

1561 **Blank:** For blank tests, the same procedure is followed but the pepsin is added after the  
 1562 addition of TCA, which stops the reaction. The blank absorbance is noted  $A_{280}$  Blank.

1563 Because, the absorbance is a function of the pepsin concentration, a linear curve has to be  
 1564 obtained. If no linear part is found, it can be due to a large amount of enzyme, and therefore  
 1565 it is necessary to use more dilute enzyme assay solutions.

1566 **Calculations:**

$$\text{Units/mg} = \frac{[A_{280} \text{ Test} - A_{280} \text{ Blank}] \times 1,000}{(\Delta t \times X \times 0.001)}$$

1567  $\Delta t$ : duration of the reaction, i.e. 10 minutes

1568 X = amount of pepsin powder ( $\mu\text{g}$ ) in 1mL in the assay solution (i.e., 5, 10, 15, 20, 25, 30  $\mu\text{g}$ )

1569 1,000 = dilution factor to convert  $\mu\text{g}$  to mg

1570 0.001 =  $\Delta A_{280}$  per unit of pepsin

1571 Check that the activity obtained is the same for each tested concentration of pepsin, to make  
 1572 sure that you are in the linear part of the pepsin concentration curve.

1573

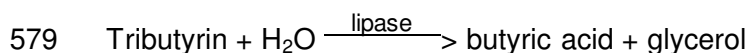
1574

### 1575 **Gastric and pancreatic lipase activity assay (EC 3.1.1.3)**

1576 **References:** Gargouri et al.<sup>6</sup>; Moreau et al.<sup>7</sup>; Carrière et al.<sup>2,8</sup>, Erlanson and Borgström<sup>9</sup>

1577 **Method:** pH titration

1578 **Principle:**



580 The gastric and pancreatic lipase activity assay are conducted by pH titration and tributyrin  
 581 as substrate. The free fatty acids released by the lipases are titrated at a constant pH by

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1582 sodium hydroxide (0.02 - 0.1 N) during at least 5 min. The concentration of NaOH is adjusted  
1583 to allow the titrator to keep the pH as constant as possible during the titration.

1584 **Unit definition:** One unit releases 1  $\mu\text{mol}$  of butyric acid per minute at 37°C at the pH of the  
1585 assay: 6.0 for Human Gastric Lipase, 5.5 for Rabbit Gastric Lipase and 8 for Pancreatic  
1586 Lipase. These units are often referred to International Units. Both, purified Human and Rabbit  
1587 Gastric Lipases show a specific activity of approx. 1,200 U/mg protein on tributyrin<sup>7,10</sup> and  
1588 human Pancreatic Lipase has a specific activity of ca. 8,000 U/mg of protein on tributyrin<sup>2</sup>

1589 **Procedure:**

1590 Preparation of reagents:

1591 **Assay solution:** Prepare 200 mL of the following aqueous solutions which vary for gastric or  
1592 pancreatic lipase:

1593

	Gastric Lipase		Pancreatic Lipase	
	Concentration [mg/L]	Corresponding weight [mg] for 200 mL	Concentration [mg/L]	Corresponding weight [mg] for 200 mL
NaCl	9,000 (150 mM)	1,800	9,000 (150 mM)	1,800
Sodium tauro-deoxycholate	1,000 (2 mM)	200	2,000 (4 mM)	400
BSA	100 (1 $\mu\text{M}$ )	20	-	-
CaCl <sub>2</sub>	-	-	200	40
Tris-(hydroxymethyl)-aminomethane	-	-	36	7.20
pH	adjust with HCl (0.1M) at pH 5.5 (RGE) or pH 6 (HGL)		adjust with HCl (0.1 M) at pH 8	

1594

1595 **Titration Solution:** Prepare a solution of 0.1 N sodium hydroxide (NaOH) by dissolving 2 g  
1596 NaOH in 500 mL of purified water. It is recommended to perform a back titration using 0.1 N  
1597 HCl to confirm the precise molarity of the NaOH titration solution. Alternatively, commercial  
1598 NaOH stock solutions can be used.



1599 **Enzyme:** Prepare a 1 mg/mL solution by dissolving 5 mg of lipase (e.g. rabbit gastric extract  
1600 powder, RGE25-100MG Lipolytech, France) in 5 mL of purified water. Store on ice. Perform  
1601 the assay with at least 2 different amounts of the enzyme solution, i.e. 50 and 100  $\mu$ L, at 1  
1602 mg/mL.

1603 **Substrate:** Use tributyrin of purity grade ( $\geq 99\%$ ; ref T8626 Sigma-Aldrich)

1604 **Assay:**

1605 Set a thermo-regulated pH-stat device to 37°C fitted with a jacketed and capped reaction  
1606 vessel (20-70 mL) and mechanical stirrer, preferentially with a 3-pale propeller.

1607 Pour 14.5 mL of the assay solution and 0.5 mL of tributyrin into the titration vessel. Make  
1608 sure the volume of the assay is enough to ensure adequate pH-measurement, i.e., the pH  
1609 electrode is correctly immersed. By switching on the mechanical stirring of the apparatus,  
1610 tributyrin will get dispersed to form a fine oil-in-water emulsion after 3-5 min at 37°C.

1611  
1612 Switch on the automated delivery of titrant solution (0.1 N NaOH) to monitor the pH and  
1613 adjust it at the selected pH end-point of titration, i.e., pH 5.5 for rabbit gastric lipase, pH 6.0  
1614 for human gastric lipase or pH 8.0 for pancreatic lipase. Add 50 or 100  $\mu$ L of the enzyme  
1615 solution. Monitor the rate of titrant solution (NaOH) which is required to maintain the pH  
1616 constant at 37°C due to the release of free fatty acids. These conditions allow measuring  
1617 linear kinetics of free fatty release for at least 5 minutes.

1618 If pancreatic lipase does not contain colipase, add colipase at a molar excess (ratio of 2:1  
1619 colipase:lipase) before adding the enzyme.

1620 **Calculations:**

$$\frac{\text{Units}}{\text{mg powder}} = \frac{R(\text{NaOH}) \times 1000}{v \times [E]} \times F$$

1621  
1622 R(NaOH): Rate of NaOH delivery in  $\mu$ mol NaOH per minute, i.e.,  $\mu$ mol free fatty acid titrated  
1623 per minute

1624 v: volume [ $\mu$ L] of enzyme solution added in the pH-stat vessel

1625 [E]: concentration of the enzyme solution [mg powder/mL]

1626 F: correction factor to take into account the partial ionization (and titration) of fatty acids at  
 1627 the pH of the assay. Only for the titration of butyric acid at pH 5.5, a correction factor F of  
 1628 1.12 has to be applied.

1629 Check that the activity obtained is the same for each tested concentration of lipase, to make  
 1630 sure that you are in the linear part of the enzyme concentration curve.

1631

### 1632 **Trypsin Activity Assay (EC 3.4.21.4)**

1633 **References:** adapted from Hummel<sup>11</sup> and following recommendations from the Worthington  
 1634 laboratory

1635 **Method:** Kinetic spectrophotometric rate determination

1636 **Principle:**

1637  $\text{TAME} + \text{H}_2\text{O} \xrightarrow{\text{trypsin}} \text{p-Toluene-Sulfonyl-L-Arginine} + \text{Methanol}$

1638 **Unit definition:** One unit hydrolyses 1  $\mu\text{mol}$  of p-toluene-sulfonyl-L-arginine methyl ester  
 1639 (TAME) per minute at 25°C and pH 8.1

1640 Unit conversion: 1 TAME Unit = 19.2 USP/NF Units = 57.5 BAEE Units

1641 **Conditions:** T = 25°C, pH = 8.1,  $A_{247\text{-nm}}$ , Light path = 1 cm

1642 Preparation of reagents

1643 **Substrate:** TAME (ref. T4626 Sigma-Aldrich) at 10 mM is prepared and dissolved in purified  
 1644 water.

1645 **Enzyme:** Prepare at least 2 concentrations of trypsin (porcine trypsin, ref. T0303 Sigma-  
 1646 Aldrich) ranging between 10-20  $\mu\text{g/mL}$  in 1 mM HCl.

1647 **Assay solution:** 46 mM Tris/HCl buffer, containing 11.5 mM  $\text{CaCl}_2$  at pH at 8.1 and 25°C.

1648 **Assay:**

1649 Set the spectrophotometer at 247 nm and 25°C.

650 **Test:** Pipette 2.6 mL of assay solution and 0.3 mL of the substrate (10 mM TAME) into  
 651 quartz cuvettes, mix by inversion and incubate in spectrophotometer at 25°C for 3-4 minutes  
 652 to achieve the temperature.





1653 Add 100 µl of each concentration of trypsin solutions and record in continuum the  
 1654 absorbance increase at 247 nm ( $\Delta A_{247}$ ) during 10 min, until levelling off. Determine the slope  
 1655  $\Delta A_{247}$  from the initial linear portion of the curve. If no linear part is found, repeat the test with  
 1656 a lower or higher amount of enzyme.

1657 **Blank:** For blank assays, follow the same protocol by replacing the enzyme with buffer  
 1658 (equilibration is usually reached faster, 5 min). The blank slope,  $\Delta A_{247}$ , should be close to  
 1659 zero.

1660 **Calculations:**

1661 The slopes  $\Delta A_{247}$  [unit absorbance/minute] are established for both the blank and the test  
 1662 reactions by using the maximum linear rate over at least 5 minutes:

$$\text{Units/mg} = \frac{[\Delta A_{247} \text{ Test} - \Delta A_{247} \text{ Blank}] \times 1000 \times 3}{(540 \times X)}$$

1663  $\Delta A_{247}$ : slope of the initial linear portion of the curve, [unit absorbance/minute] for the Test  
 1664 (with enzyme) and  $\Delta A_{247}$  Blank without enzyme

1665 540: molar extinction coefficient (L/(mol × cm)) of TAME at 247 nm.

1666 3: Volume (in millilitres) of reaction mix

1667 X: quantity of trypsin in the final reaction mixture (quartz cuvette) [mg]

1668 Check that the activity obtained is the same for each tested concentration of trypsin, to make  
 1669 sure that you are in the linear part of the enzyme concentration curve.

1670

1671 **Chymotrypsin activity assay (EC 3.4.21.1)**

1672 **References:** adapted from Hummel<sup>11</sup> and Rick<sup>12</sup>

1673 **Method:** Kinetic spectrophotometric rate determination

1674 **Principle:**

1675  $\text{BTEE} + \text{H}_2\text{O} \xrightarrow{\text{chymotrypsin}} \text{N-Benzoyl-L-Tyrosine} + \text{Ethanol}$

1676 **Unit Definition:** One unit of chymotrypsin hydrolyses 1.0 µmol of N-Benzoyl-L-Tyrosine  
 1677 Ethyl Ester (BTEE) per minute at pH 7.8 and 25°C.

1678 Conditions: T = 25°C, pH = 7.8,  $A_{256\text{nm}}$ , Light path = 1 cm



1679 Preparation of reagents:

1680 **Substrate:** Dissolve the substrate, BTEE (ref. B6125 Sigma-Aldrich), at a concentration of  
1681 1.18 mM in methanol/purified water. Weigh 18.5 mg of BTEE, dissolve it in 31.7 mL of  
1682 absolute methanol and complete to 50 mL with deionized water in a 50 mL volumetric flask.

1683 **Enzyme:** The enzyme is dissolved in 1 mM HCl. Prepare at least 2 concentrations of  
1684 chymotrypsin (porcine chymotrypsin, ref. C7762 Sigma-Aldrich) ranging between 10-30  
1685 µg/mL in 1 mM HCl.

1686 **Assay solution:** 80 mM Tris/HCl buffer, containing 100 mM CaCl<sub>2</sub> at pH at 7.8 and 25°C.

1687 **Assay:**

1688 Set the spectrophotometer at 256 nm and 25°C.

1689 **Test:** Mix 1.5 mL of the assay solution and 0.3 mL of the substrate (1.18 mM BTEE) into  
1690 quartz cuvette, mix by inversion and incubate in spectrophotometer at 25°C for 3-4 minutes  
1691 to achieve temperature equilibration. Add 100 µl of each concentration of the chymotrypsin  
1692 solutions and record the absorbance increase ΔA at 256 nm (ΔA<sub>256</sub>) during 10 min in  
1693 continuum, until levelling off. Determine the slope ΔA<sub>256</sub> from the initial linear portion of the  
1694 curve. If no linear part is found repeat the test with a lower or higher amount of enzyme.

1695 **Blank:** For blank assays, follow the same protocol by replacing the enzyme with buffer only  
1696 (equilibration is usually reached faster, 5 min). The blank slope ΔA<sub>256</sub> Blank should be close  
1697 to zero.

1698 **Calculations:**

1699 The slopes ΔA<sub>256</sub> [unit absorbance/minute] are established for both the blank and the test  
1700 reactions by using the maximum linear rate over at least 5 minutes:

$$\text{Units/mg} = \frac{[\Delta A_{256} \text{ Test} - A_{256} \text{ Blank}] \times 1000 \times 3}{(964 \times X)}$$

1701 ΔA<sub>256</sub>: slope of the initial linear portion of the curve, [unit absorbance/minute] for the Test  
1702 (with enzyme) and ΔA<sub>256</sub> Blank without enzyme

1703 964: molar extinction coefficient L/(mol × cm) of BTEE at 256 nm.

1704 3: Volume (in millilitres) of reaction mix

1705 X: quantity (mg) of chymotrypsin in the final reaction mixture (quartz cuvette)

1706 Check that the activity obtained is the same for each tested concentration of chymotrypsin, to  
1707 make sure that you are in the linear part of the enzyme concentration curve.

1708

### 1709 **Pancreatin**

1710 The amount of pancreatin is normalized to the trypsin activity. However, to digest fat  
1711 containing food, the lipase activity should be recorded as well. Therefore, to measure the  
1712 enzyme activities of the pancreatin (porcine pancreatin 8 x USP specifications, ref P7545  
1713 Sigma-Aldrich), the protocols are the same as described above. For trypsin (or chymotrypsin)  
1714 Pancreatin is dissolved in 1 mM HCl (pH 3). Pancreatin is difficult to dissolve, mix during 10  
1715 minutes using a magnetic stirrer and then keep the solution on ice or at refrigerated  
1716 temperature 4°C to prevent loss of activity. Dilute the pancreatin to a concentration ranging  
1717 between 0.1 to 1 mg/mL and measure at least 3 different dilutions. Vortex pancreatin before  
1718 pipetting it to the enzyme reaction vessel. To measure the lipase activity in pancreatin,  
1719 dissolve it in 150 mM NaCl at pH 6.8 (pancreatic lipase is degraded at low pH), and follow  
1720 the above procedure to record lipase activity.

1721

### 1722 **Bile salts in bile**

1723 The concentration of bile salts in the bile (fresh or commercial) can be measured with a  
1724 commercial kit (bile acid kit, 1 2212 99 90 313, DiaSys Diagnostic System GmbH, Germany,  
1725 MAK309-1KT, Merck or similar) according the supplier's protocol. Measure the bile at  
1726 different concentrations bearing in mind the linearity range of the kit.

1727

1728



## 1729 REFERENCES

- 1730 1 Minekus, M. *et al.* A standardised static in vitro digestion method suitable for food - an  
1731 international consensus. *Food & Function* **5**, 1113-1124, doi:10.1039/C3FO60702J (2014).
- 1732 2 Carrière, F., Barrowman, J. A., Verger, R. & Laugier, R. Secretion and contribution to lipolysis  
1733 of gastric and pancreatic lipases during a test meal in humans. *Gastroenterology* **105**, 876-  
1734 888 (1993).
- 1735 3 Bernfeld, P. in *Methods Enzymol.* Vol. Volume 1 149-158 (Academic Press, 1955).
- 1736 4 Anson, M. L. & Mirsky, A. E. The Estimation of Pepsin with Hemoglobin. *J. Gen. Physiol.* **16**,  
1737 59-63, doi:10.1085/jgp.16.1.59 (1932).
- 1738 5 Anson, M. L. The Estimation of Pepsin, Trypsin, Papain, and Cathepsin with Hemoglobin. *J.*  
1739 *Gen. Physiol.* **22**, 79-89, doi:10.1085/jgp.22.1.79 (1938).
- 1740 6 Gargouri, Y. *et al.* Importance of human gastric lipase for intestinal lipolysis: an in vitro study.  
1741 *Biochim. Biophys. Acta* **879**, 419-423, doi:10.1016/0005-2760(86)90234-1 (1986).
- 1742 7 Moreau, H., Gargouri, Y., Lecat, D., Junien, J.-L. & Verger, R. Purification, characterization and  
1743 kinetic properties of the rabbit gastric lipase. *Biochimica et Biophysica Acta (BBA)-Lipids and*  
1744 *Lipid Metabolism* **960**, 286-293, doi:10.1016/0005-2760(88)90036-7 (1988).
- 1745 8 Carriere, F. *et al.* Purification and biochemical characterization of dog gastric lipase. *The FEBS*  
1746 *Journal* **202**, 75-83, doi:10.1111/j.1432-1033.1991.tb16346.x (1991).
- 1747 9 Erlanson, C. & Borgström, B. Tributyrine as a substrate for determination of lipase activity of  
1748 pancreatic juice and small intestinal content. *Scand. J. Gastroenterol.* **5**, 293 (1970).
- 1749 10 Gargouri, Y. *et al.* Kinetic assay of human gastric lipase on short- and long-chain  
1750 triacylglycerol emulsions. *Gastroenterology* **91**, 919-925,  
1751 doi:10.5555/uri:pii:0016508586906955 (1986).
- 1752 11 Hummel, B. C. W. A modified spectrophotometric determination of chymotrypsin, trypsin,  
1753 and thrombin. *Can. J. Biochem. Physiol.* **37**, 1393-1399, doi:10.1139/o59-157 (1959).
- 1754 12 Rick, W. in *Methods of Enzymatic Analysis (Second Edition)* (ed Hans Ulrich Bergmeyer)  
1755 1006-1012 (Academic Press, 1974).

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