INFOGEST *in vitro* simulation of gastrointestinal food digestion

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EDITORIAL SUMMARY A standardised *in vitro* protocol for study of gastrointestinal food digestion and analysis of digestion products

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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 most basic of 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. The method can be used to analyze 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.

Introduction

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

Development of the Protocol:

There are several types of in vitro digestion methods that are commonly used for food, which can be divided into static and dynamic methods. These models aim to simulate the physiological conditions of the upper gastrointestinal tract, namely the oral, gastric and small intestinal phases. Most dynamic models5-9 have been shown to be suitable for simulating the digestion of foods and pharmaceutical products in different population groups and for different purposes10. However, these models are relatively complex, expensive to set up and maintain, and therefore may not be available to the majority of food researchers.

Owing to its simplicity, static models, which use a constant ratio of food to enzymes and electrolytes, and a constant pH for each digestive phase, have been widely used for many decades for food, animal feed and pharmaceutical purposes. Static *in vitro* digestion models have been shown to be very useful in predicting outcomes of *in vivo* digestion[11]. There are standardised static models[12] that vary in complexity[13,14], which are used for simulating the gastrointestinal behaviour of pharmaceutical products (Pharmacopeia methods)[12]. Other static methods were developed for assessing the *in vitro* bioaccessibility of soil contaminants[15], heavy metals in particular, or mycotoxins in food[16]. These methods, developed and standardised[17] by the Bioaccessibility Research Group of Europe (BARGE) were based on available physiological data reported by landmark papers such as Dressman et al. [18] or the Geigy tables[19]. The static methods of the BARGE group and Pharmacopeia procedures were important milestones in the evolution of standardised *in vitro* digestion methods. However, their experimental conditions, purpose and endpoint were found to be

unsuitable for digesting food due to the complexity and variability of food structures as well as very different research questions in food science. This resulted in the use of a great number of digestion methods, reviewed by Hur et al.[20], with slight but significant variations in parameters such as pH, duration, enzyme concentration and activity, composition of simulated digestive fluids, etc.

Hence, the need for a harmonisation of digestion conditions was identified and the international INFOGEST[21] network (www.cost-infogest.eu) of multidisciplinary experts (food science, nutrition, gastroenterology, engineering, enzymology, etc.) from more than 35 countries was established. One of the primary outcomes of this network was an international consensus on a set of digestion parameters for a static *in vitro* simulation of adult digestion suitable for food. The method, generally referred to as the INFOGEST method, was published[22] and experimental parameters were justified and discussed in great detail in relation to available *in vivo* physiological data. Some of the previous digestion methods outlined above were used as a starting point. Since its publication in 2014, this *in vitro* digestion method has received a *Highly Cited Paper* status for Agricultural Sciences with more than 550 citations in Web of Science and has been extensively used all over the world for numerous purposes, with a variety of foods and different endpoints. The current article builds on that publication and clarifies a number of aspects of the original protocol, leading to an improved INFOGEST 2.0 protocol described here.

Overview of the Procedure

The digestion procedure is summarised in **Figure 1**. It can be divided into three phases: preparation, digestion procedure and sample treatment with subsequent analysis. For preparation of the *in vitro* digestion, the activity of all digestive enzymes and the concentration of bile salts should be determined experimentally, using the recommended standardised assays described in Minekus et al.[22] and in the procedure steps x-x. This first preparation step is of the utmost importance and failure to correctly assay enzyme activity will lead to incorrect rates of digestion of components (e.g. proteins)[23], potentially changing the overall digestion of the food.

The digestion involves the exposure of the food to three successive digestive phases: oral, gastric and intestinal. For static *in vitro* digestion methods, the experimental conditions are constant, during each phase. The oral phase involves dilution of the food 1:1 (w/w) with simulated salivary fluid (SSF), with or without salivary amylase, and for solids or semi-solids simulated mastication of the food. If used, exposure of the food to salivary amylase is limited to two minutes at pH 7. The oral phase needs to be included in all simulated digestion procedures, regardless of the state of the food (liquid or solid) in order to provide consistency

of dilution. Further clarification regarding the preparation of the food and the oral phase can be found in the Experimental Design.

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

The experimental conditions for the digestion procedure such as pH, time of digestion and enzyme activity etc. were based on available physiological data of the fed state for a typical meal and were described and justified in detail in Minekus et al.[22] For this improved INFOGEST 2.0 method, the use of gastric lipase is recommended, hence a detailed justification of the type and activity of the gastric lipase is provided in the Experimental Design section.

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

Advantages and limitations

Static *in vitro* digestions are the simplest methods to simulate *in vivo* food digestion. While there are clear weaknesses in these simple models, they have obvious advantages over more complex methods. The main strengths of static *in vitro* models is the good intra- and inter-laboratory reproducibility, robustness, simplicity, relatively low cost and easy assessment of each digestion phase. This latter point makes them very suitable for mechanistic studies, hypothesis building and screening. It was one of the aims of the INFOGEST network not just to standardise *in vitro* methods but to agree on experimental conditions that are based on available physiological data to be as close as possible to the *in vivo* equivalent, while keeping the method sufficiently simple to reproduce all over the world. The clear definition of standardised experimental conditions and procedures is one of the major advantages of the INFOGEST method. Egger et al. [23] showed very good lab to lab reproducibility of results from the *in vitro* digestion of skim milk from powder, in regards to peptide patterns. Some weaknesses were identified and have been addressed subsequently. The recommendation of standardised enzyme assays (including units) significantly added to the precision and reproducibility of the digestion procedure as previously, a number of common but slightly different enzyme assays were being used, resulting in the application of a wide range of enzyme activities during digestion experiments. The end point of this INFOGEST method was recently compared to digests obtained in human jejunum after casein and whey protein ingestion[24] showing excellent correlation in protein degradation and peptide patterns, as explained below.

However, static digestion methods have known limitations and cannot mimic the complex dynamics of the digestion process or the physiological interaction with the host. For example for the gastric phase, the pH is kept constant, there is a lack of the gradual addition of gastric fluid (acid, minerals, pepsin) and an absence of gradual gastric emptying. In addition, the enzyme activity is kept constant regardless of the food. The intestinal phase is treated as one phase rather than those of the sequential duodenal, jejunal and ileal phases, which exhibit different dilutions, mineral content, pH, enzyme activities, microbial content, etc. These shortcomings render the method unsuitable for detailed kinetic analysis of the different stages of the digestion process. However, *in vivo* comparison shows good correlation with the INFOGEST method at the end points of each digestion phase. For this reason, the static model should only be used to assess digestion endpoints and not kinetics.

In some cases, a slight alteration of the procedure may be considered to more accurately reflect physiological conditions. For example, during the gastric *in vivo* digestion of food containing probiotic bacteria, the bacteria are exposed to a range of pHs, as low 1 at the end of the gastric emptying. Hence, a static method with a constant pH of 3.0 for the gastric

phase may fail to accurately predict probiotic survival and a lower pH or a dynamic gastric model should be chosen. Studying the bioaccessibility of phytochemicals such as polyphenols and carotenoids, the model allows the realistic release from a food into the aqueous phase. However, specific hydrolytic processes occurring at the brush-border are currently not simulated, and additional steps such as centrifugation of the digesta are needed to separate the bioaccessible phases. An extension including colonic fermentation, an important step in the bioactivation of several phytochemicals, would further enhance the physiological appropriateness. Finally, for the assessment of the bioaccessibility of small amounts of contaminants in food, such as heavy metals, environmental pollutants, or mycotoxins, alternative methods reflecting extensive digestion and "worst-case scenarios"[15] can be applied.

Applications

The method described has been used to assess the release of carotenoids and phenolic compounds from different matrices, such as, carotenoids in fruits[25,26], carotenoids in tomatoes compared to tomatoes subjected to pulsed electric fields[27], β-carotene protected by microencapsulation[28] and resveratrol encapsulated in protein nanoparticules[29]. However, most studies have been dedicated to the evaluation of protein, lipid and starch digestion in foods or modified carriers. Protein digestion has been widely assessed in different dairy products[30,31], or in isolated milk proteins, such as lactoferrin with different iron contents and after mild heat treatment[32]. The stability of proteins to gastrointestinal digestion has been proposed as an additional piece of information for the allergenicity assessment of novel proteins[33]. With this focus, the INFOGEST method was also applied to the study of the immunogenic potential of peptides from pasta[34], hazelnut[35], and peanut[36], which are resistant to gastrointestinal digestion. Using a pH-stat to monitor enzymatic hydrolysis, it was shown that solid emulsions led to a lesser extent of lipolysis but a greater degree of proteolysis compared to liquid emulsions due to the higher sensitivity of denatured whey proteins to gastrointestinal enzymes[37]. The tendency of dairy rennet gels to form compact protein aggregates during gastric digestion has also been assessed[38]. Other applications of this protocol include the evaluation of novel biopolymers designed for a controlled nutrient release[39,40], or the digestive stability of transgenic microRNAs in genetically modified plants[41].

An inter-laboratory trial applying different *in vitro* digestion protocols clearly demonstrated a good reproducibility obtained by using the standardised INFOGEST protocol. It also highlighted the importance of correctly applying standardised pepsin activity assays, which is a key factor for proper gastric protein hydrolysis[23]. A special effort was made to validate and compare the results from this *in vitro* digestion protocol with *in vivo* data. For instance, β-cryptoxanthin bioavailability from pasteurised orange juice was found to be higher than from

fresh oranges in a randomised crossover human study, and from the *in vitro* digestion assay an increased bioaccessibility could also be inferred[42]. Several studies have focused on protein digestion and the comparison with *in vivo* digestion in human or animal models. The results from the *in vitro* gastrointestinal digestion of skim milk powder were compared with *in vivo* porcine samples collected from the stomach and several sites in the intestine[43]. Protein degradation and peptides generated at the end of the gastric phase correlated well with *in vivo* gastric peptides while the *in vitro* intestinal phase correlated well with the *in vivo* samples taken in the median jejunum. Human jejunal digests after the oral ingestion of casein and whey protein were compared with the intestinal digests obtained using the standardised INFOGEST method[24]. *In vivo* and *in vitro* intestinal digests showed common protein regions that are resistant to digestion and a high number of identical peptide sequences, concluding that the INFOGEST *in vitro* method is a good approximation to the end points of gastrointestinal digestion of milk proteins *in vivo*.

Alternative methods

A wide variety of static *in vitro* digestion models can be found in the literature [20] but they all exhibit different conditions (pH, duration of each step, ratio enzymes/substrate…) making the comparison between studies impossible. Those published by Versantvoort et al.[16], Garrett et al.[44] and Oomen et al [15] are amongst the most cited. Most of the models found in the literature simulate the fasted state conditions that are quite far from physiological conditions when food enters the gastrointestinal tract. Advantages and limitations of static *in vitro* digestion models have been recently reviewed by a group of experts within the INFOGEST network[11]. While static methods can be useful for understanding trends or performing a screening of samples, it falls short in terms of some of the important dynamic processes occurring during gastrointestinal digestion, namely the pH gradients and the gradual addition of enzymes and gastric fluid as well as continuous gastric emptying. More physiologically relevant dynamic digestion methods[5-9] take these and other factors into account but are often not available to food researchers. It has recently been shown that, when human data are available to set up the system, these models can be physiologically-relevant[10]. In an effort to improve *in vitro* digestion methods, a low-cost semi-dynamic method was recently developed[45] and described in detail[46], where parameters were based on the equivalent *in vivo* data from the digestion of dairy products. Here, the SGF and pepsin are slowly added to the food in a suitable reaction vessel with manual, stepwise gastric emptying. A harmonisation of experimental conditions is currently on-going and a standardised semi-dynamic method will be published by INFOGEST members shortly.

Even though they are expensive and sometimes ethically questionable *in vivo* models have been widely used for studying the digestive process. The pig model can simulate the upper

part of the human digestive tract (stomach and small intestine) more closely[47]. Conventional pigs or mini-pigs can be used for this purpose and can be equipped with cannulas in order to sample the effluents throughout digestion and a catheter to collect blood, whereas piglets can be used for all the questions related to neonatal nutrition[43,48,49].
Finally, human volunteers can be equipped with naso-gastric or naso-intestinal probes to access and sample the digestive effluents[2]. Ileostomy patients have been used to study digestion[50-52] but can hardly be considered as a model of a healthy human since they are affected by digestive pathologies.

Experimental Design

Enzyme assays

The determination of the standard units of activity of the enzyme used in the protocol is a crucial step and one of the main sources of variation in results with the digestion periods or between different laboratories.[37] Enzyme activity determination is recommended for each new batch of enzyme or after prolonged storage.

Enzyme and bile assays are described in protocol format in the supplementary materials of Minekus et al.[22], namely: α-amylase (EC 3.2.1.1), pepsin (EC 3.4.23.1), trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1), pancreatic lipase (EC 3.1.1.3) and bile salts (according to supplier´s protocol). The gastric lipase (EC 3.1.1.3) assay, written in the same format can be found in the Supplementary Material section of this article. In order to improve the reproducibility of the pepsin activity assay for this revised INFOGEST 2.0 protocol, it is now recommended to dissolve pepsin in Tris buffer (tris-hydroxymethyl-aminomethane) and NaCl (pH 6.5), instead of in sodium chloride solution adjusted with sodium hydroxide. The buffering capacity of Tris buffer reduces variability in the measurement of the pepsin activity, as shown previously[37].

Spreadsheets for the enzyme assays and the volumes for the digestion procedure are provided in the Supplementary Material of this manuscript (Supplementary data1 and 2). The corresponding online tools are available here: www.proteomics.ch/IVD and on the INFOGEST website https://www.cost-infogest.eu/. In addition, videos of enzyme assays (supplementary video 5-9) and the digestion procedures (supplementary videos 3and 4) are available in the supplementary materials. The videos are also available online on the YouTube channel “In vitro food digestion - COST action INFOGEST” https://www.youtube.com/channel/UCdc-NPx9kTDGyH_kZCgpQWg and on the INFOGEST website https://www.cost-infogest.eu/.

Food preparation and oral phase

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

An optional oral phase with a standardised 1:1 (w/w) ratio of food to simulated oral fluid for all foods (solid and liquid foods) was recommended by the INFOGEST method[22] in 2014. While *in vivo* data varies greatly (Supplemental **Figure S1**), this dilution ratio enables the formation a swallowable bolus with almost all types of foods. For this revised INFOGEST 2.0 protocol a standardised, easy-to-follow approach for the oral phase is necessary. Hence, it is now recommended to dilute all food 1:1 (w/w) with simulated oral fluid to achieve a swallowable bolus that is no thicker than a paste-like consistency similar to that of tomato paste or mustard at the end of the oral phase. If the consistency of the bolus is thicker than paste-like, add water to achieve it (see also **Table 3** and **Table 4** Troubleshooting).

Use of lipase in the gastric phase

Lipid digestion starts in the stomach with the action of preduodenal lipase (gastric lipase in humans, lingual lipase in rodents) on triacylglycerides (TAG) and some other esters[53]. Gastric lipolysis not only contributes to the overall digestion of TAG (10% with a solid-liquid test meal to 25% with an emulsified liquid test meal) but it also triggers the subsequent action of pancreatic lipase on lipid substrates that may be poorly digested by pancreatic lipase alone; examples include milk fat droplets and lecithin-stabilised TAG emulsions[54]. It is therefore recommended to add gastric lipase during the gastric phase of *in vitro* digestion. The mean gastric lipase concentration in human gastric juice is 100 µg/mL, which is equivalent to 120 U/mL using tributyrin as the reference substrate for gastric lipase[55,56]. In some static digestion models, a concentration of approx. 16 µg gastric lipase/mL (20 U/mL) has been used to reproduce gastric conditions at half time of gastric emptying[57,58], which corresponds to a gastric juice to meal ratio of 1:5 v/v. In the INFOGEST method, the gastric phase of digestion includes a 1:1 dilution of the oral bolus by simulated gastric fluid, which would correspond to a dilution of gastric juice by half and thus a gastric lipase concentration of 60 U/mL. To date, access to commercially available gastric lipase, or an appropriate equivalent has been limited, hence gastric lipase has been omitted or lipases from alternative

sources have been widely used. However, caution should be applied regarding the specific biochemical properties of these alternative lipases. Human gastric lipase (HGL), encoded by the LIPF gene, is stable and active between pH 2 and 7 with an optimum activity between pH 4 to 5.4. HGL displays a S_N3 stereospecificity for TAG hydrolysis leading to the preferential release of short/medium chain fatty acids from milk TAG[57]. It is resistant to pepsin hydrolysis and is not inhibited by bile salts. HGL can however be replaced by other preduodenal lipases from the acid lipase gene family of various mammalian species like dog[59] and rabbit[60]. Rabbit gastric lipase is now commercially available (Lipolytech, www.lipolytech.com). Pre-duodenal lipases originating from the oro-pharyngeal tissues of young ruminants (pharyngeal lipase of calf, kid goat, lamb) may also be used and are commercially available for applications in the dairy industry (DSM for Capalase® K and Capalase® KL lipases; CHR Hansen for Lipase Kid-Goat ST20, Lipase Calf 57 LFU, Spice IT™ AC and Spice IT™ AG; DuPont Danisco, Clerici-Sacco). These preduodenal lipases are however less resistant to acid denaturation (threshold at around pH 3.5 [61]) than gastric lipase and pH conditions may have to be adapted. Their contents and activity should be estimated before use in *in vitro* digestion experiments, using the recommended standard gastric lipase assay[22], see Supplementary Materials Section. So far, no commercially available lipase of microbial origin combines all the above properties of gastric lipase [57,62], and their use is not recommended at this time. For this revised INFOGEST 2.0 protocol, the authors recommend using rabbit gastric lipase, commercially available as rabbit gastric extracts (RGE) at 60 U/mL in the final gastric digestion mixture. However, since these extracts also contain pepsin[63], the pepsin concentration/activity in the gastric phase has to be accordingly adjusted to the recommended value.

Sampling, controls and test tube

Before performing the protocol (time-lagged before the digestion experiment or one day prior to the digestion experiment), it is recommended to run one preliminary experiment, the***pH-test adjustment experiment***, with the relevant amount of food, enzymes and bile for the entire digestion process (**Figure 2** no. 1). The aim of this pH-test adjustment experiment is to measure and record the amounts of HCl and NaOH used to reach the target pH in order to perform more efficient pH adjustments when running the digestion protocol. These volumes are indicative of the necessary volume of acids and bases needed for the gastric and intestinal phase. It has to be noted that for solid food, the pH changes are generally slower in response to addition of HCl or NaOH – it is important to remain patient and wait long enough for the pH to become stable - >5 min depending on food particle size and buffering capacity.

If it is intended to take samples at different time points during digestion, it is recommended to prepare one tube per time point, e.g. prepare six digestion tubes for six time points (**Figure 2**

nos. 2, 3 and 6). Because most foods are heterogeneous mixtures during digestion, sampling is more reproducible by starting digestion with individual tubes per time point. If the food sample has special requirements in terms of nutrient stability (e.g. light sensitivity, oxidation) the characteristics of the tubes should be adapted to these particular situations (opaque tubes, maintenance of the food samples on ice, etc). The end volume of the digest should be calculated to use the more convenient tubes/vessels which allow mixing during all digestion phases.

Optionally, a replicate test tube (***stability test tube***) can be prepared to evaluate food stability during exposure to simulated digestive fluids without enzymes or bile, for example after oral, gastric and intestinal phase (**Figure 2**, nos. 5 and 8). It can also be advisable to prepare an ***enzyme-blank tube***, i.e., a digestion tube with all enzymes and bile but without food (**Figure 2**, nos. 4 and 7). This may be helpful to identify enzyme, bile salts or degradation products thereof during analysis of the digests. It is important to highlight that due to proteolytic enzyme autolysis, especially pepsin, enzyme-derived peptides can be detected in digesta which can be easily monitored with this blank-enzyme tube.

Intestinal phase, stop reaction and read out

The intestinal phase of the protocol starts with the mixing of the gastric chyme with the same volume of the pre-warmed SIF. The pH is adjusted with the amount of NaOH previously calculated in the *pH-test adjustment experiment.* In this phase, two different options are given, (i) the use of pancreatin or (ii) the use of individual enzymes: porcine trypsin (100 U/mL), bovine chymotrypsin (25 U/mL), porcine pancreatic α-amylase (200 U/mL), porcine pancreatic lipase (2000 U/mL) and porcine pancreatic colipase in molar excess to lipase. The amount of pancreatin to be used in the intestinal phase of digestion is based on trypsin activity to achieve 100 U/mL in the final mixture. This calculation may result in low lipase activity for high fat containing foods or if fat digestion is the aim of the study. In this case, it is recommended to include additional lipase to get 2000 U/mL of lipase activity in the final mixture and colipase in a molar ratio 2:1 colipase to lipase, which corresponds approximately to a mass ratio 1:2 colipase to lipase. Since this will require the measurement of the lipase activity in the pancreatic extract and in the lipase preparation, the use of individual enzymes could be a preferred option. Similarly, because the activity of amylase in pancreatin can vary between batches and the activity can be too low to digest starch rich foods, the use of individual enzymes could also be a good option when following carbohydrate digestion. Bile salts are added to the intestinal mixture to reach 10 mM in the final mixture, after determination of the bile salt concentration in the commercial product (see Enzymatic Assays). There are several commercial options for bile salts but bovine bile is preferred

because its composition is similar to that in humans. Bile solubilisation requires exhaustive mixing which can be achieved, for instance, in a rotating wheel mixer at 37ºC for 30 min.

In vitro digestion is carried out for a wide range of purposes and with different endpoints. In all cases, sampling, sample preservation and the post-treatment of samples after food digestion are critical and some adaptations could be needed depending on the particular requirements of each experiment (**Table 1**). For example, to stop pepsin activity, the pH of gastric samples must be raised to 7.0, either by the addition of 1 M sodium bicarbonate or 1 N NaOH solution. The pH shift after the gastric phase is very effective in stopping pepsin activity and similar to *in vivo* conditions found in the duodenum[56]. If the pH increase is not desired, the use of pepstatin A, a highly selective inhibitor of aspartyl proteases like pepsin (K_i = 0.1 nM) has also been suggested[57]. When gastric digestion is considered as an end point, sample snap freezing in liquid nitrogen followed by freeze-drying are recommended. Raising the pH to 7.0 strongly reduces the activity of gastric lipase on long chain triglycerides[58-60]. Alternatively, the use of Orlistat® (tetrahydrolipstatin) is also recommended (gastric lipase half-inhibition time of < 1 min) to block gastric lipolysis[61]. Add Orlistat at a final concentration of 0.6 mg/mL (1 mM) to obtain an inhibitor to lipase molar ratio of 1,000, taking into account that the gastric lipase activity of 60 U/mL corresponds to 50 µg/mL or 1 µM lipase.

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

When biological activity of digested samples has been evaluated, heat-shock treatment (in boiling water for 5 min) to irreversibly inactivate proteases may also be considered[28].

However, it should be noted that heat treatment is detrimental to the food structure, proteins in particular as heat treatment generally causes irreversible denaturation and aggregation. For cell culture assays, consider whether the use of Pefabloc or other enzyme inhibitors can affect the read out of the experiment, and whether the osmolarity needs to be corrected by dilution to physiological values (285-300 mOsm/kg H_2O, pH 7-7.5) in order to avoid cell osmotic shock. Other combined procedures for removal or enrichment of certain food components such as defatting, centrifugation, dialysis, filtration and size exclusion chromatography are also commonly used.

Materials

Reagents:

- Ultrapure type I water, generated by a Milli-Q® system or similar (referred in text as water)
- Human salivary α-amylase (Sigma-Aldrich, 1031)
- Porcine pepsin (Sigma -Aldrich, P7012 or P6887)
- Rabbit gastric extract (RGE) for gastric lipase (see section on gastric lipase above, currently supplied by e.g. Lipolytech RGE 25-100MG) **Critical:** RGE contains both gastric lipase and pepsin.
- Bovine bile (Sigma-Aldrich, B3883, preferred option as composition in closest to that in humans), alternatively Porcine Bile (Sigma-Aldrich, B8631),
- Porcine pancreatin (Sigma-Aldrich, P7545) or individual intestinal porcine enzymes (trypsin, chymotrypsin, amylase, lipase and co-lipase), see below optional reagents
- $CaCl_2(H_2O)_2$ (Merck 2382)
- NaOH (Merck 9141) **! Caution:** corrosive, causes severe skin burns and eye damage
- HCl (J. T. Baker 6081) **! Caution:** corrosive, causes burns, irritating to respiratory system
- KCl (Merck 4936)
- KH_2PO_4 (J. T. Baker 0240)
- $NaHCO_3$ (Merck 6329)
- NaCl (Merck 6404)
- $MgCl_2(H_2O)_6$ (Merck 5833)
- $(NH_4)_2CO_3$ (Sigma-Aldrich, 207861)

- Enzyme inhibitors options (see Experimental Design and **Table 1)** :
    - Pefabloc® SC (4-(2-Aminoethyl)benenesulfonyl fluoride, Sigma-Aldrich, 76307) **! Caution:** corrosive;
    - Pepstatin A (Sigma-Aldrich, P5318)
    - Bowman-Birk inhibitor (Sigma Aldrich, T9777)
    - 4-bromophenylboronic acid (Sigma Aldrich, B75956) **! Caution:** hazardous, corrosive, causes eye damage, harmful for respiratory system

Chemicals for enzyme and bile tests:

- Pepsin test
    - Haemoglobin from bovine blood (Sigma-Aldrich, H6525-25G),
    - Trichloroacetic acid (Sigma-Aldrich, T6399-5G) **! Caution:** Corrosive, causes severe burns to skin and eyes. Soluble in water with release of heat.
- Gastric lipase test:
    - Taurodeoxycholate (Sigma-Aldrich, T0875-1G)
    - Tributyrin (Sigma-Aldrich, T8626; ≥99%)
    - Bovine serum albumin (Sigma-Aldrich, A7030; ≥98%)
- Trypsin test:
    - TAME (p-Toluene-Sulfonyl-L-arginine methyl ester, Sigma-Aldrich, T4626-5G)
- Amylase test:
    - Maltose Std. (Sigma-Aldrich, M5885-100G)
    - Soluble Potato Starch (Sigma-Aldrich, S5651-500G)
    - DNS (3,5-Dinitrosalicylic acid, Sigma-Aldrich, D0550-10G), **! Caution:** Harmful if swallowed, Acute oral toxicity
- Chymotrypsin test:
    - BTEE (N-Benzoyl-L-Tyrosine Ethyl Ester, Sigma-Aldrich, B6125-5G)
- Pancreatic lipase test:
    - Sodium taurodeoxycholate (Sigma-Aldrich, T0875-1G)
    - Tributyrin (Sigma-Aldrich, W222305-1KG)
- Bile acid determination
    - Bile acid kit (Sigma-Aldrich, MAK 309) or ECOLINE Acides Biliaires, Diasys, 122129990313) or equivalent assay

Reagents for optional protocol with individual enzymes:

- Porcine trypsin (Sigma-Aldrich, T0303)
- Bovine chymotrypsin (Sigma-Aldrich, C7762)
- Porcine pancreatic α-amylase (Sigma-Aldrich, A3176)

- Porcine pancreatic lipase (Sigma-Aldrich, L3126)
- Porcine pancreatic co-lipase (Sigma-Aldrich, C3028)

Food (for further examples see Anticipated Results Section)

- Skim milk powder (SMP, Fonterra, NZ, low-heat organic, protein 42.34%, fat 0.89%, lactose 49.8% (w/w)[23]

Equipment:

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

Reagent setup:

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

- 0.5 mL of $CaCl_2(H_2O)_2$ (0.3M)
- 30 mL of KCl (0.5M)
- 6 mL of KH_2PO_4 (0.5M)
- 65 mL of $NaHCO_3$ (1M)
- 25 mL of NaCl (2M)
- 2 mL of $MgCl_2(H_2O)_6$ (0.15M)
- 2 mL of $(NH_4)_2CO_3$ (0.5M)

1 M NaOH and 1 M HCl: for pH adjustment of stock solutions of simulated digestion fluids

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

Preparation of simulated digestion fluids at a 1.25× concentration

Simulated digestion fluids for oral (SSF), gastric (SGF), and intestinal (SIF) digestion phase are mixed at a 1.25× concentration using the electrolyte stock solutions and water according to **Table 2** and can be stored at -20°C for one year. **Critical:** $CaCl_2$ should be added immediately prior to the digestion experiment to avoid precipitation upon storage. **Critical**: All the volumes (**Table 2**) are calculated for 400 mL of a 1.25× concentrated storage solution and just before use they are mixed with the necessary quantities of enzyme and finally diluted to a 1× concentrated working solution (i.e. 4 parts of electrolyte solution + 1 part consisting of enzymes and water result in a 1× concentration of the digestion fluids). Simulated digestion fluids (1.25× concentrates) can be stored at -20°C for one year in small aliquots of appropriate size; e.g. for the experiment shown in Box 1, using 5 g of food, at least 48 mL of SSF, 88 mL of SGF, and 96 mL of SIF are needed. **Critical:** Dilute enzymes in cold solutions and keep them on ice until used. This will keep enzyme activity to a minimum. **Critical:** Pre-warm electrolyte solutions (SSF, SGF, SIF) to 37°C prior to using them in the digestion procedures.

Procedure

Preparation reagents and digestion tubes:

1. Perform all enzyme and bile assays according to the protocols in Supplementary Materials of this article and Minekus et al.[22] for each new batch of enzymes or after prolonged storage; the gastric lipase and pepsin assay can be found in the Supplementary Materials of this manuscript. TIMING 4-5 days for all assays
   CRITICAL STEP: For the pepsin assay, dissolve pepsin in 10 mM Tris, 150 mM NaCl, pH 6.5, which improves the reproducibility of the assay (see Supplementary Materials).
   CRITICAL STEP Spreadsheets for the enzyme assays and the volumes for the digestion procedure are provided in the Supplementary Materials of this manuscript (Supplementary data 1 and 2). In addition, the corresponding online tools are available here: www.proteomics.ch/IVD and on the INFOGEST website https://www.cost-infogest.eu/.

Critical Step: Prepare one tube per time point and food; e.g. for one food and six time points, prepare six tubes

2. Pre-warm the electrolyte stock solutions at 37°C, initially only SSF and SGF, later SIF
3. Prepare all enzyme and bile solutions immediately before the digestion experiment

Critical Step: Keep all enzyme solutions on ice

4. In order to perform more efficient pH adjustments during the digestive phases, prepare one replicate tube (pH-test adjustment experiment) with the relevant amount of food, enzymes and bile for the entire digestion process (time-lagged before the digestion experiment or one day prior to the digestion experiment) and measure and record the volumes of HCl and NaOH used to reach the target pH. These volumes are indicative of the necessary volume of acids and bases needed for the gastric and intestinal phase TIMING 5h
5. Optional: Prepare one replicate test as a food stability control to assess the behaviour of the food during exposure to simulated digestive fluids without enzymes or bile, for example after oral, gastric and intestinal phase
6. Prepare one replicate test tube as a blank, digestion without food (replaced by water) but with all required enzymes and bile. Seevideos of enzyme assays (supplementary videos 5-9) as well as the digestion procedures (supplementary videos 3 and 4). Videos are also available online on the YouTube channel “In vitro food digestion - COST action INFOGEST” https://www.youtube.com/channel/UCdc-NPx9kTDGyH_kZCgpQWg and on the INFOGEST website https://www.cost-infogest.eu/

Digestion procedure

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

Oral phase

7. Always include the oral phase, also for liquid foods. Dilute food with SSF at a ratio of 1:1 (w/w) to achieve a swallowable bolus with a paste-like consistency similar to that of tomato paste or mustard at the end of the oral phase. If the consistency of the bolus is thicker than paste-like, add water to achieve it. Salivary amylase is only needed to digest starch containing food. It can be omitted if the food does not contain starch. Do not use lower purity salivary amylase or pancreatic amylase.
8. Mix food with SSF at a 1:1 ratio (w/w), e.g. 5 g of food to 5 g of SSF
9. Measure the volume of the final digestion mixture of the food + SSF mixture; this can be done in a test experiment prior to the digestion experiment
10. If necessary, simulate mastication by mincing the food in an electric or manual mincer.
11. Depending on the food (e.g. bread), mincing can be done together with the SSF electrolyte (without enzymes)
12. Add SSF electrolyte stock solution to the food, if not done already, see above
13. Add $CaCl_2$ in order to achieve a total concentration of 1.5 mM in SSF
14. Add the salivary amylase, if necessary, prepared in water to achieve an activity of 75 U/mL in the final mixture.
15. Add the remaining water in order to achieve 1× concentration of the SSF.
16. Incubate while mixing for 2 minutes at 37°C

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

Gastric phase:

17. Pre-warm the SGF electrolyte stock solution at 37ºC. Add SGF electrolyte stock solution to the oral bolus to a final ration of 1:1 (v/v)
18. Adjust the pH to 3.0 by adding a defined volume of HCl previously determined during a pH-test adjustment experiment, see above

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

19. Add $CaCl_2$ solution in order to achieve a final concentration of 0.15 mM in SGF.
20. Add the porcine pepsin solution prepared in water to achieve an activity of 2,000 U/mL in the final digestion mixture.
21. Add the gastric lipase solution prepared in water to achieve an activity of 60 U/mL in the final digestion mixture.
22. Verify the pH and adjust to 3.0 if necessary
23. Add water in order to achieve 1×concentration of the SGF
24. Incubate the samples at 37°C, mixing the digestive mixture sufficiently (e.g. rotating wheel, shaking incubator) for 2 h from the point when pepsin was added. In case of large precipitates and formation of clogs, see Troubleshooting.

Critical step: Rabbit gastric extracts (RGE) contains both gastric lipase and pepsin[63]. The pepsin activity in RGE needs to be determined and taken into account together with the porcine pepsin to reach a combined pepsin activity of 2,000 U/mL in the final digestion mixture.

Critical step: The use of carbonate salts in the electrolyte solutions requires that sealed containers with limited headspace are used. In open vessels, CO_2 will be release and the pH will progressively increase with time. If open vessels are to be used, such as when using the "pH-stat" approach or for sampling purposes, it is suggested to replace sodium bicarbonate ($NaHCO_3$), the main source of carbonates, by NaCl at the same molar ratio in order to maintain the ionic strength of the electrolyte solutions (oral, gastric and intestinal). Such adjustment has already proven effective in avoiding unwanted pH drift in open vessels in both gastric[108] and intestinal[37] phases of digestion.

(see to **Table 2**).

Intestinal phase:

25. Pre-warm the SIF electrolyte stock solution in a 37ºC water bath. Add SIF electrolyte to the gastric chyme and achieve a final ratio of 1:1 (v/v).
26. Adjust to pH 7.0 by adding a defined volume of NaOH previously determined during a pH-test adjustment experiment, see above.

Critical step: For solid food, the pH changes are slower in response to the addition of NaOH, see remarks above; this may take several minutes.

27. Add the bile solution to the SIF: gastric chime solution in order to reach a final concentration of 10 mM. Place the solution in a rotating wheel mixer at 37°C for at least 30 min to achieve complete bile solubilisation.
28. Add $CaCl_2$ solution in order to reach concentration of 0.6 mM in SIF.
29. Perform intestinal phase with option (A) pancreatin or option (B) with individual enzymes
    A.
        i. Add the pancreatin suspension in SIF solution to achieve a trypsin activity of 100 U/mL in the final mixture. Additional pancreatic lipase may be needed for the digestion of fat containing food to reach the required lipase activity to achieve a lipase activity of 2,000 U/mL in the final mixture.

    Critical step: Consider trypsin activity in pancreatic lipase powder and adjust accordingly

    B.
        i. Add trypsin, chymotrypsin, pancreatic α-amylase, pancreatic lipase and the co-lipase solutions in SIF, in order to reach 100, 25, 200 and 2,000 U/mL, respectively, in the final digestion mixture
30. Verify the pH and adjust to 7.0 if necessary
31. Add water in order to achieve 1×concentration of the SIF
32. Incubate the samples at 37°C, mixing the digestive mixture sufficiently using a rotating wheel or shaking incubator for 2h starting at the point when pancreatic enzymes were added. For difficulties with sampling, see **Table 4** Troubleshooting.

Critical step: If open vessels are used (“pH-stat” approach), $NaHCO_3$ should be replaced by NaCl in the electrolyte solutions to avoid unwanted pH drift (see the step 25 critical step).

Anticipated Results

Protein digestion

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

(i) *Robustness of the protocol* and comparability of experimental data were assessed in several inter-laboratory trials where the participants were asked to digest a standardised skim milk powder (SMP) by applying their existing in-house protocols first, then by using the harmonised protocol[23]. The first critical step in protein hydrolysis is the pepsin activity in the gastric phase. The heterogeneous pattern observed with the in-house digestion protocols (**Figure 3a**, gastric phase) was improved significantly by the correct implementation of the harmonised protocol (**Figure 3b**, gastric phase), except for laboratories 6 and 7, which showed incomplete casein hydrolysis, most probably due to a mistake in pepsin activity determination. Improved homogeneity between samples was even clearer after the intestinal step when the harmonised protocol was applied. Increased protein degradation in the intestinal phase, observed in laboratories 4 and 7 (**Figure 3b**), could be caused by incorrect inhibition of enzymes at the end of the digestion experiment (see **Table 1**).

(ii) *Physiological relevance* was evaluated by comparing *in vitro* SMP digestion with that of an *in vivo* pig trial [43]. Pigs were fed reconstituted SMP from the same batch as applied in the *in vitro* tests and samples were collected from the stomach and in several sections of the small intestine (jejunum, I1- I3 to ileum, I4) after sacrifice. Milk peptides were identified with mass spectrometry and overall peptide patterns were visualised by summing up the number of times each individual amino acid was identified within a milk peptide. Overlay of the average peptide patterns for α_{s2}-casein from the harmonised *in vitro* digestion (n=7) and *in vivo* pig digestion (n=8) showed that at the end of the gastric phase, the peptide pattern corresponded well to that of the pig sample collected from the stomach; the peptide pattern in the *in vitro* intestinal phase sample was most similar to that of the pig sample collected in the median jejunum (I3). This comparison showed that protein hydrolysis at the endpoints of the harmonised INFOGEST digestion method were in agreement with that of the *in vivo* digestion (**Figure 4**).

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

Lipid Digestion

To date, most published digestion experiments using this INFOGEST method did not include a gastric lipase because of the lack of commercially available, acceptable substitutes for human gastric lipase (HGL). This situation has changed with the availability of rabbit gastric extracts containing gastric lipase, see Section *Use of lipase in the gastric phase*. Both HGL and rabbit gastric lipases exhibit, at the recommended gastric pH of 3.0, approximately 50% of their maximum activity measured at pH 4 to 5.4 [64,65]. Moreover, the *in vitro* gastric lipolysis of infant formula by rabbit gastric lipase were consistent with *in vivo* data, with a degree of lipolysis reaching 10% after 60 min of gastric digestion[66]. These data therefore suggest that gastric lipolysis could be studied using this INFOGEST 2.0 method with rabbit gastric extract as a source of gastric lipase[60] or human gastric lipase if available[57].

The INFOGEST method has also been used to study intestinal lipid digestion, for example in oil-in-water emulsions stabilised by milk or soya lecithin[67]. However, human gastric analogue and phospholipases A2 (PLA_2) were added in this procedure. The degree of hydrolysis (% TAG disappearance) ranged between 73 and 87 % (± 5 %) at the end of the intestinal phase (120 min). In addition, *in vitro* digestion was also performed with more complex systems such as whole fat dairy products or protein/polysaccharide emulsions. Depending on the structure of the food matrix and the state of dispersion of the lipids, the reported degrees of hydrolysis at the end of the intestinal phase ranged from moderate (66% of remaining lipids in poorly digestible raw oat flakes due to limiting matrix structure)[68] to an almost complete disappearance of triglycerides[69].

Intestinal lipid digestion can be assessed by chemical analyses of collected samples. The protocol recommends analysing the entire volume of digestive tubes to prevent sampling errors (see critical step of one tube per time point and food). This precaution is particularly useful in the presence of lipids[68] as they often tend to destabilise and phase-separate (cream) during the gastric and/or intestinal phases of digestion. If aliquots are taken as sample points, great care should be taken to represent the whole digested solution. The best way to analyse the extent of lipolysis is to conduct the Folch extractions[70] on the samples in the presence of internal standards before the analysis of classes of the lipids (residual triglycerides, free fatty acids, diglycerides and monoglycerides) by thin layer chromatography combined with densitometry or gas chromatography with a flame ionization detector (GC-FID)[71] or HPLC coupled to a light scattering detector [72]. Free fatty acids can also be quantified after solid phase extraction with GC-FID, using fatty acids (typically C11:0, C15:0, C17:0 or C23:0) as internal standards [66,73]. The pH-stat method, one of the most commonly used methods for monitoring pancreatic lipolysis, can also be used, but three sources of errors should be taken into consideration: (i) the pH-stat measurements can be impaired by

the high concentrations of carbonate salts, recommended for the simulated digestion fluids (see the step 22 critical step It is therefore advised to replace $NaHCO_3$ salts with NaCl at the same molarity in all electrolyte solutions (oral, gastric and intestinal) when planning to use pH-stat experiments during the intestinal phase of digestion[37]; (ii) protein hydrolysis also contributes to the pH-stat signal in the intestinal conditions (pH = 7), meaning that this approach is only suitable for studying pancreatic lipolysis when the contribution of proteins is either neglected or sustracted[37]; (iii) some fatty acids, especially long chain fatty acids, are not ionised at pH7. A back titration at pH 9.0 should be performed to measure all the free fatty acids released[74].

Digestion of starch

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

It is recommended that starch amylolysis is quantified *only* in the intestinal phase by measuring the appearance of the starch digestion products over time, e.g. the concentration of reducing sugars in the liquid phase. Salivary amylase will have a minor impact on starch digestion in the static model were the gastric pH is instantaneously adjusted to 3. After terminating amylase activity by mixing the sample with 4 volumes of ethanol (final conc. 80% w/v) to the sample, for example (see different options in **Table 1**), undigested starch is often separated from digested starch by centrifugation. Analysis of reducing sugar concentration in the supernatant is often done with common colorimetric assays (e.g. using DNS or PAHBAH (4-Hydroxybenzhydrazide) reagents). Another more common method is to treat an aliquot of the amylase digestion products from the 80% w/v ethanol supernatant with buffered amyloglucosidase to convert all amylase digestion products to glucose. Glucose can then be determined through a whole host of methods including colorimetric and enzymatic assays (e. g. GOPOD) or by direct chromatography analysis to name just a few. The data collected can then be used as input variables to a wide variety of simple to complex kinetic-based mathematical models that seek to quantify starch digestion and give predictions on the physiological effects of the food under.

Bioaccessibility of phytochemicals

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

(i) *Physiological appropriateness and pitfalls:* Good correlations between bioaccessibility and *in vivo* bioavailability have been obtained for certain phytochemicals, such as carotenoids[75,76]. However, slight alterations of the digestion parameters suggested by the original INFOGEST method[22] can drastically influence bioaccessibility. For instance, increasing the amount of pancreatin and/or bile[77] or increasing the speed of shaking/stirring can considerably enhance the bioaccessibility of carotenoids by improving mixing, disrupting oil droplets and increasing micellisation. Thus, careful consideration and the possible further standardisation of these parameters are vital. Additional important factors to consider are light and oxygen, as they can result in the oxidative degradation of carotenoids [78] and polyphenols [79] and polymerisation of the latter [80]. It is recommended to flush samples with Ar or N_2 for a few minutes prior to small intestinal digestion to remove oxygen [76,81] or to use pyrogallol. However, the latter is unsuitable for polyphenolic samples as this is a potential metabolite. Another often neglected factor is the potential effect of brush border membrane enzymes (e.g. lactase-phlorizin-hydrolase) on phytochemical bioaccessibility, especially for polyphenols[82,83]. The inclusion of brush border membranes (BBM) vesicles in *in vitro* gastrointestinal digestion may increase the physiological relevance of the model, especially for polyphenols [84]. However, BBM are not commercially available nor is there any standard method available to date.

(ii) *Bioaccessible phase and pitfalls*: For polyphenols, dialysis is often performed to remove macromolecular-bound compounds[85], but for carotenoids a combination of centrifugation (e.g. 4,000×g for at least 30 minutes) and a filtration step (0.2 µm) has become the most widely used method[26] to separate the bioaccessible aqueous phase from larger lipid droplets or crystals that would not be taken up by the enterocytes.

When combining *in vitro* digestion with cellular assays (e.g. cellular uptake/transport), the toxicity of the bile salts must be accounted for, by including a clean-up step, e.g. solid phase extraction [86-88], or at least the sufficient dilution of samples (e.g. 4× dilution).

Finally, it should be considered that the colon may play an important role for the bioavailable fraction. While it is well known that polyphenols can undergo many changes in the colon [82],

and may be absorbable in the colon, little is known for carotenoids, though a significant fraction would be bioaccessible in the colon [89].

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

The establishment of the INFOGEST digestion protocol is a good starting point in the standardisation and harmonisation of food digestion methods. Henceforth, results from different research groups can be compared in a meaningful manner. However, users have to be aware of the shortcomings of this method and considerable efforts are being made around the world to improve or add to the existing method. These efforts should not be taken as an opportunity to stray from the conditions recommended here.

The INFOGEST method is for adult digestion only. However, there is a strong need to apply this method to specific human population groups, the most important being infants and the elderly, but also adolescents and patients with cystic fibrosis or gastric bypass surgery, to name but a few. A recent review[90] summarised the existing literature and provides some recommendations on experimental digestion parameters, with the INFOGEST method being the starting point for all other methods.

While static methods can be useful, they can be inadequate to simulate the dynamic processes during digestion (e.g. pH gradients, gradual addition of enzymes and gastric fluid, continuous gastric emptying, etc.). As mentioned earlier, various dynamic digestion methods[5-9] account for some of these factors. A low-cost semi-dynamic method was recently developed[45] and described in detail[46], based on equivalent *in vivo* data from the digestion of dairy products. International INFOGEST members are currently working on a consensus method.

Enzymes from the small intestinal brush border membranes are recognised as playing a major role in the activation of trypsinogen (enterokinase) and the further degradation of proteins/peptides and carbohydrates as well as improving the bioaccessibility of phytochemicals. The use of brush border enzymes falls into the grey area between bioaccessibility (potentially absorbable) and bioavailability (available at the site of action) and to date, it is not clear how they should be applied. BBM of animal origin have recently been included in static digestion methods[34,91,92] and can provide physiologically consistent information[93]. However, to date BBM enzymes are not commercially available and are extracted from fresh animal intestines[94] or used as intestinal extracts. There is still a lack of reliable information on the correct enzymatic activities, enzyme substrate ratio and diversity of enzymes, which further limits the use of BBM in standardised digestion methods at the moment. However, given the importance of BBM in the digestive process, further progress in terms of defining digestive parameters is anticipated.

TIMING

TROUBLESHOOTING

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

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Competing interests

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

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Figures

Figure 1: Timing and flow diagram of the INFOGEST *in vitro* digestion method for food. SSF, SGF and SIF stand for simulated salivary, gastric and intestinal fluid, respectively. Expected time frame (left) and steps (right) corresponding to the step numbers in Procedure (Section 2).

Figure 2: Scheme of a typical experiment set up for one food sampling at 3 different time points during gastric phase and intestinal phase. SSF, simulated salivary fluid; SGF, simulated gastric fluid; SIF, simulated intestinal fluid.

Figure 3: Protein separation by gel electrophoresis of *in vitro* digested skim milk powder (SMP)

Comparing results from in-house protocols performed in individual laboratories 1-12 (a), with the harmonised protocol, performed in 7 different laboratories (b) after the gastric and the intestinal phase of in vitro digestion. Undigested skim milk powder (SMP) is shown as control, specific protein bands are highlighted with arrows. Figure adapted from Egger et al.[23]

Figure 4: Comparison of *in vitro* digested SMP peptide patterns of κ-casein with *in vivo* (pig) digestion

Gastric *in vitro* digestion samples (S) were compared to gastric and duodenal pig samples (n = 8). Intestinal *in vitro* digestion samples (I) were compared to pig sampling section I1 – I4, collected along the jejunum (I1-3) and the ileum (I4)[43]. The x-axis shows the amino acid (AA) sequence of κ-casein and the y-axis shows the number of times each amino acid was identified within a κ -casein peptide of ≥ 5 AA in length.

Supplementary information

Supplementary Figure 1: Bolus hydration (g of saliva / g of foods), just before swallowing, for various foods based on published data

Gastric lipase activity assay (EC 3.1.1.3)

Pepsin activity assay (EC 3.4.23.1)

Spreadsheet, online tools and videos of the enzyme assays and digestion procedures can be found in the Supplementary Information and at the following websites: www.proteomics.ch/IVD and https://www.cost-infogest.eu/

Supplementary Videos:

Supplementary Video 1 : INFOGEST 2.0 digestion procedure part 1

Supplementary Video 2 : INFOGEST 2.0 digestion procedure part 2

Supplementary Video 3 - amylase assay

Supplementary Video 4 - pepsin assay

Supplementary Video 5 - lipase assay

Supplementary Video 6 - trypsin assay

Supplementary Video 7 - chymotrypsin assay

Supplementary Data 1: enzyme assays

Supplementary Data 2: digestion procedure

Related links

Key references using this protocol

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TABLES

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).	68,95
Breakdown of nutrients: Proteins	Protein hydrolysis or resistant protein analysis	***To stop gastric digestion:*** Raise the pH to 7 for partial inactivation of pepsin; pH 8 for complete inactivation.	Addition of 1 M $NaHCO_3$ or 1N NaOH	23
		Addition of pepstatin A for pepsin inhibition.	Add Pepstatin A at 0.5-1.0 µM final concentration.	96
		To stop intestinal digestion: Addition of Pefabloc® SC (4-(2-aminoethyl)-benzolsulfonylfluorid-hydrochloride) for serine protease (trypsin and chymotrypsin) inhibition.	Add 50 µl of Pefabloc (0.1 M) in water per mL of intestinal digesta. (5 mM final concentration).	23
		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.	97
		Heat shock treatment	Sample treatment: 100°C, 5 min, but detrimental to food structure,	36

			especially protein and carbohydrate structures	
Breakdown of nutrients: Lipids	Lipid hydrolysis	***To stop lipase activity in the gastric phase:*** Addition of Orlistat (tetrahydrolipstatin), or alternatively raise the pH to 8 ***To stop lipase activity in the intestinal phase:*** Addition of lipase inhibitor (4-bromophenylboronic acid) Addition of methanol: chloroform	Add 10 µL/mL of a 100 mM Orlistat solution in ethanol (1 mM final concentration) Add 5 µL/mL of a 1 M solution in methanol to 1 mL of digesta (5 mM final concentration). Addition of methanol: chloroform mixture used for Folch extraction	98,99 70
Breakdown of nutrients: Carbohydrates	Starch hydrolysis	***To stop amylase activity:*** 'Stop solution' Heat shock treatment TCA precipitation Ethanol	Dilute digesta in equal volume of 0.3 M Na_2CO_3 100°C for 5 min Add 700 µL of 100% TCA to 5 mL digesta Add sample to 4 volumes of ethanol	100
Breakdown of oxygen sensitive phytochemicals	Degradation of polyphenols and carotenoids	Flushing with Ar or N_2, pyrogallol addition (carotenoids) prior to small intestinal digestion	Flush sample 1 minute with Ar or N_2	81

Bioaccessibility	Bioaccessiblibility of digested nutrients	To stop pancreatic activities (see above).	As previously reported	
		Use of inhibitors e.g. Pefabloc. Test whether the use of enzyme inhibitors affect the results of the experiment.		
		Use of dialysis membranes/ centrifugation tubes having cut-off of 3 to 10kDa.		101
		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_2O).	102
		TCA precipitation (15% TCA) for further analysis of soluble peptides and free amino acids.		23
		Extraction of compounds by using either solvents or acidic solutions	Different procedures for a wide range of compounds are employed	103
	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)	104
		Cleavage of glucosides and esters	Addition of brush border vesicles	84
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	105

Colonic fermentation and modulation of intestinal microbiota	Biotransformation of compounds and their effects on bacterial growth	To stop enzymatic activities either by heat shock or immediate storage in ice before batch culture fermentation	Heat treatment: 100 °C for 5 min but detrimental to food structure, especially protein and carbohydrate structures	106,107

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Table 2: Volumes of electrolyte stock solutions of digestion fluids for a volume of 400 mL 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_2PO_4	68	0.5	3.7	3.7	0.9	0.9	0.8	0.8
$NaHCO_3$*	84	1	6.8	13.6	12.5	25	42.5	85
NaCl	117	2	-	-	11.8	47.2	9.6	38.4
$MgCl_2(H_2O)_6$	30.5	0.15	0.5	0.15	0.4	0.12	1.1	0.33
$(NH_4)_2CO_3$ *	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):								
$CaCl_2(H_2O)_2$	44.1	0.3	0.025	1.5	0.005	0.15	0.04	0.6

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	10 mL		20 mL	
1.25× electrolyte stock solutions (mL)	4	8		8**	
$CaCl_2$ (0.3 M) (mL)	0.025	0.005		0.04	
Enzymes	Salivary amylase	Pepsin	Gastric[#] 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) mM/g	100 U/mg	3,000 U/mg	25 U/mg	6 U/mg	0.667mM/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_2O (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 ratio in the static digestion method and may need to be further diluted with water prior the oral phase, see	[#]Rabbit gastric extract (RGE) contains gastric lipase and pepsin, i.e. the pepsin content needs to be accounted for in the total pepsin activity			

	Table 4 Troubleshooting		

*Specific enzyme activity or bile concentration: measured for each batch of enzymes or bile extract according to standard assays (Supplemental Materials from Minekus et al. [22]), the enzyme assays for gastric lipase and pepsin are described in the supplemental materials of this manuscript

**Total volume of SIF (1.25×): 3.2 mL including pancreatin and bile, both of which are dissolved in SIF

1358 **Table 4**: Troubleshooting

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 (28)	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 *in vivo* digestion of cheese [43] 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 (28)	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 phase (28, 37)	Difficulties taking a homogeneous sample during digestion	Presence of different phases (lipids, water, solids)	Use individual sample tube for each time point rather than withdrawal of samples from the digestion vessel.

Gastric/intestinal phase (28, 37)	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 (37)	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 (37)	Presence of insoluble material at the end of the intestinal phase	Non-digestible material	Use individual sample tube for each time point
Intestinal phase (37)	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 (37)	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 (37)	Starch digestion product concentration	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

	does not change over time	feasibility of results by expressing findings as % of starch digested.

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