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Brodkorb, A, Egger, L, Alminger, M et al. (32 more authors) (2019) INFOGEST static in vitro simulation of gastrointestinal food digestion. Nature Protocols, 14 (4). pp. 991-1014. ISSN 1754-2189

https://doi.org/10.1038/s41596-018-0119-1

INFOGEST static *in vitro* simulation of gastrointestinal food digestion

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Keywords:

static *in vitro* digestion, food digestion, physiological digestion, simulated digestion

Manuscript submitted in Word format to Nature Protocols November 28 2018; Figures and Box 1 were subsequently added to the document. Citation: Brodkorb, A., Egger, L., . . . Recio, I. (2019). INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nature Protocols*, 14(4): 991-1014. DOI: 10.1038/s41596-018-0119-1 ; Full text version available here: https://rdcu.be/brEMd

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.

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Introduction

The worldwide prevalence of diet-related diseases has been on the increase for the last few decades.[1] 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 the complex digestive processes within the human digestive tract in more detail. This can be achieved with invasive procedures such as aspiration from the stomach[2] or small intestine[3] or with less invasive imaging technologies (e.g. magnetic resonance imaging[4]) and wireless, telemetric systems[2,5]. 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 also regularly questioned. 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 models[6-10] have been shown to be suitable for simulating the digestion of foods and pharmaceutical products in different population groups and for different purposes[11]. 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[12-14]. Static *in vitro* digestion models have been shown to be very useful in predicting outcomes of *in vivo* digestion[15,16]. There are standardised static models[17] that vary in complexity[18,19], which are used for simulating the gastrointestinal behaviour of pharmaceutical products (Pharmacopeia methods)[17]. Other static methods were developed for assessing the *in vitro* bioaccessibility of soil contaminants[20], heavy metals in particular, or mycotoxins in food[21]. These methods, developed and standardised[22] by the Bioaccessibility Research Group of Europe (BARGE) were based on available physiological data reported by landmark papers such as Dressman et al. [23] or the Geigy tables[24]. The static methods of the BARGE group and Pharmacopeia

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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.[25], 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[26] 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[27] 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 for amylase, pepsin, lipase (both gastric and pancreatic), trypsin and chymotrypsin, outlined in **Box 1**, described in detail in the Supplementary Information. 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)[28], 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

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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.[27] 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.

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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. [28] 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[16] showing excellent correlation in protein degradation and peptide patterns, as explained below in Applications.

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 in each digestive phase is kept constant, regardless of the type of food and whether the food contains high or low amount of substrate e.g. proteins, lipids and carbohydrates. 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.[16,29] For this reason, the static model should only be used to assess digestion endpoints and not kinetics.

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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"[20] 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[30,31], carotenoids in tomatoes compared to tomatoes subjected to pulsed electric fields[32], β-carotene protected by microencapsulation[33] and resveratrol encapsulated in protein nanoparticules[34]. 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[35,36], or in isolated milk proteins, such as lactoferrin with different iron contents and after mild heat treatment[37]. The stability of proteins to gastrointestinal digestion has been proposed as an additional piece of information for the allergenicity assessment of novel proteins[38]. With this focus, the INFOGEST method was also applied to the study of the immunogenic potential of peptides from pasta[39], hazelnut[40], and peanut[41], 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[42]. The tendency of dairy rennet gels to form compact protein aggregates during gastric digestion has also been assessed[43]. Other applications of this protocol include the evaluation of novel biopolymers designed for a controlled nutrient release[44,45], or the digestive stability of transgenic microRNAs in genetically modified plants[46].

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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[28]. 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 an increased bioaccessibility could also be inferred[47]. 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[29]. 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[16]. *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 [25] but they all exhibit different conditions (pH, duration of each step, ratio enzymes/substrate…) making the comparison between studies impossible. The static methods published by Versantvoort et al.[21], Garrett et al.[48] and Oomen et al [20] are amongst the most used, based on their citations. However, most of the of static *in vitro* digestion methods found in the literature simulate the fasted state, which is quite far from the physiological conditions when food is digested in 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[15]. 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[6-10] take these and other factors into account. However these models are highly complex, require substantial hard- and software and are still expensive to set up and maintain, hence are often not available to food researchers. It has recently been shown that,

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when human data are available to set up the system, these models can be physiologically-relevant[11]. In an effort to improve *in vitro* digestion methods, a low-cost semi-dynamic method was recently developed[49] and described in detail[50], where parameters were based on the equivalent *in vivo* data from the digestion of dairy products. Here, the simulated gastric fluid (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 shortly by INFOGEST members , coordinated by A.R Mackie.

Even though they are expensive and must be ethically justifiable, *in vivo* models have been widely used for studying the digestive process. The pig model can closely simulate the upper part of the human digestive tract (stomach and small intestine)[51]. 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[29,52,53].

Finally, human volunteers can be equipped with naso-gastric or naso-intestinal probes to access and sample the digestive effluents[3]. Ileostomy patients have been used to study digestion[54-56] 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 were previously described in protocol format in the Supplementary Materials of Minekus et al.[27], 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). 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 10 mM Tris buffer (tris-hydroxymethyl-aminomethane), 150 mM NaCl, (pH 6.5), instead of in sodium chloride solution adjusted with sodium hydroxide. The buffering capacity of Tris buffer reduces the variability in the measurement of the pepsin activity, as shown previously[37]. The detailed protocols for the complete set of enzyme and bile assays, including

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

Spreadsheets for the enzyme assays and the volumes for the digestion procedure are provided in the Supplementary Information of this manuscript. The enzyme assay spreadsheets (Supplementary spreadsheets 1) can be used to calculate the enzyme activities of all digestive enzymes. The digestion spreadsheets (Supplementary spreadsheets 2) provides help in calculating all volumes of simulated digestive fluids, enzyme and bile solutions based on the initial amount of digested food; one example is shown in **Table 3**. The corresponding online spreadsheets can also be used, and are available here: www.proteomics.ch/IVD and on the INFOGEST website https://www.cost-infogest.eu/ . In addition, videos of the digestion procedures (Supplementary Video 1 and 2) and all enzyme activity assays (Supplementary Video 3 to 7) are available in the Supplementary Information. In addition, 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[27] in 2014. While *in vivo* data varies greatly (Supplementary **Figure 1**), 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

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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[57]. 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[58]. 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[59,60]. 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[61,62], 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[61]. 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[63] and rabbit[64]. 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 [65]) 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[27], see Supplementary

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Information Section. So far, no commercially available lipase of microbial origin combines all the above properties of gastric lipase [61,66], 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[67], 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. 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. 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 most suitable reaction vessel, e.g. 50 mL tubes, which allow properly 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. It can also be advisable to prepare an ***enzyme-blank tube***, i.e., a digestion tube with all enzymes and bile but without food. 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-

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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 α-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[64]. Bile solubilisation requires exhaustive
391 mixing which can be achieved, for instance, in a rotating wheel mixer at 37ºC 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[56]. 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[68]. When gastric digestion is considered as an end
401 point, sample snap freezing in liquid nitrogen followed by freeze-drying are recommended.

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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.

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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:

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- 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)[28]

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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

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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.

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Procedure

Preparation reagents and digestion tubes (5 days):

1. Perform all enzyme and bile assays (**Box 1**) according to the protocols in the Supplementary Information for each new batch of enzymes or after prolonged storage; 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 Information).
   Critical Step: Spreadsheets for the enzyme assays and the volumes for the digestion procedure are provided in the Supplementary Information of this manuscript (Supplementary spreadsheets 1 and 2). In addition, the corresponding online spreadsheets 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, 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. See videos of enzyme assays (supplementary videos 3 to 7) 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/

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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 (30 min)

7. 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. Record this volume as it will be used in step 17.
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 in the previous step
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 (3h)

17. Pre-warm the SGF electrolyte stock solution at 37ºC. Add SGF electrolyte stock solution to the oral bolus to a final ratio 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 Experimental Design

    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.

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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[67]. 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[69] and intestinal[42] phases of digestion (see **Table 2**).

Intestinal phase (3h):

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 Experimental Design.

    Critical step: For solid food, the pH changes are slower in response to the addition of NaOH, see remarks in step 18; 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

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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: Measure trypsin activity in pancreatic lipase powder and subtract it from the needed trypsin activity

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 24 critical step).

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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[28]. 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 2a**, gastric phase) was improved significantly by the correct implementation of the harmonised protocol (**Figure 2b**, gastric phase), except for laboratories 6 and 7, which showed incomplete casein hydrolysis. Adjustments in the pepsin assay (addition of Tris buffer, see Step 1 Critical Step and **Box 1**) improved the reproducibility and reduced lab-to-lab variability[28]. This improved pepsin assay is now recommended for the INFOGEST 2.0 method. **Figure 2b** shows improved homogeneity between samples, compared to the gastric phase when the harmonised protocol was applied. Increased protein degradation in the intestinal phase was observed in laboratories 4 and 7. Subsequent recommendation on the correct sample preparation, in particular the correct inhibition of enzymes at the end of the digestion experiment (see **Table 1**), improved lab-to-lab variability[28].

(ii) *Physiological relevance* was evaluated by comparing *in vitro* SMP digestion with that of an *in vivo* pig trial [29]. 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

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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 [70,71].
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[72]. 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[64] or human
722 gastric lipase if available[61].
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[73]. However, human gastric analogue
725 and phospholipases A2 (PLA_2) were added in this procedure. The degree of hydrolysis (%
726 TAG disappearance) ranged between 73 and 87 % (± 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)[74] to an almost complete
732 disappearance of triglycerides[75].
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[74] 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[76] 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 combined with densitometry or gas chromatography with a
743 flame ionization detector (GC-FID)[77] or HPLC coupled to a light scattering detector [78]. Free
744 fatty acids can also be quantified after solid phase extraction with GC-FID, using fatty acids

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(typically C11:0, C15:0, C17:0 or C23:0) as internal standards [72,79]. 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 24 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[42]; (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[42]; (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[80].

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

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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[81,82]. However, slight alterations of the digestion parameters suggested by the
796 original INFOGEST method[27] can drastically influence bioaccessibility. For instance,
797 increasing the amount of pancreatin and/or bile[83] 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 [84] and
802 polyphenols [85] and polymerisation of the latter [86]. It is recommended to flush samples with Ar
803 or N_2 for a few minutes prior to small intestinal digestion to remove oxygen [82,87] 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[88,89]. 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 [90]. 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[91], 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

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814 widely used method[31] 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 [92-94], 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 [88],
821 and may be absorbable in the colon, little is known for carotenoids, though a significant
822 fraction would be bioaccessible in the colon [95].

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[96] 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[6-10] account for some of these factors. A low-cost semi-dynamic method was recently
839 developed[49] and described in detail[50], 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[39,97,98] and can provide physiologically consistent
849 information[99]. However, to date BBM enzymes are not commercially available and are

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extracted from fresh animal intestines[100] 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

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

Steps 2 and 3, preparation of solutions: 2 hours

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

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

Steps 7 to 32, whole digestion experiment: 5 to 8 hours, 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

Steps 7 to 16, oral phase: 30 min

Steps 17 to 24, gastric phase: 3 hours

Steps 25 to 32, intestinal phase: 3 hours

TROUBLESHOOTING

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

Acknowledgments

COST action FA1005 INFOGEST[26] (http://www.cost-infogest.eu/) is acknowledged for providing funding for travel, meetings and conferences (2011-2015). The French National Institute for Agricultural Research (INRA, www.inra.fr) is acknowledged for their continuous support of the INFOGEST network by organising and co-funding the International Conference on Food Digestion and workgroup meetings. André Gonçalo Fernandes Lopes (Universidade de Lisboa, Portugal) is acknowledged for his help in the final preparation of the videos. The many other researchers mostly associated to the above COST action and

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subsequent events, which have contributed to the discussion on digestion parameters, are also acknowledged.

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Manuscript submitted in Word format to Nature Protocols November 28 2018; Figures and Box 1 were subsequently added to the document. Citation: Brodkorb, A., Egger, L., . . . Recio, I. (2019). INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nature Protocols*, doi:10.1038/s41596-018-0119-1 ; Full text version available here: https://rdcu.be/brEMd

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

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

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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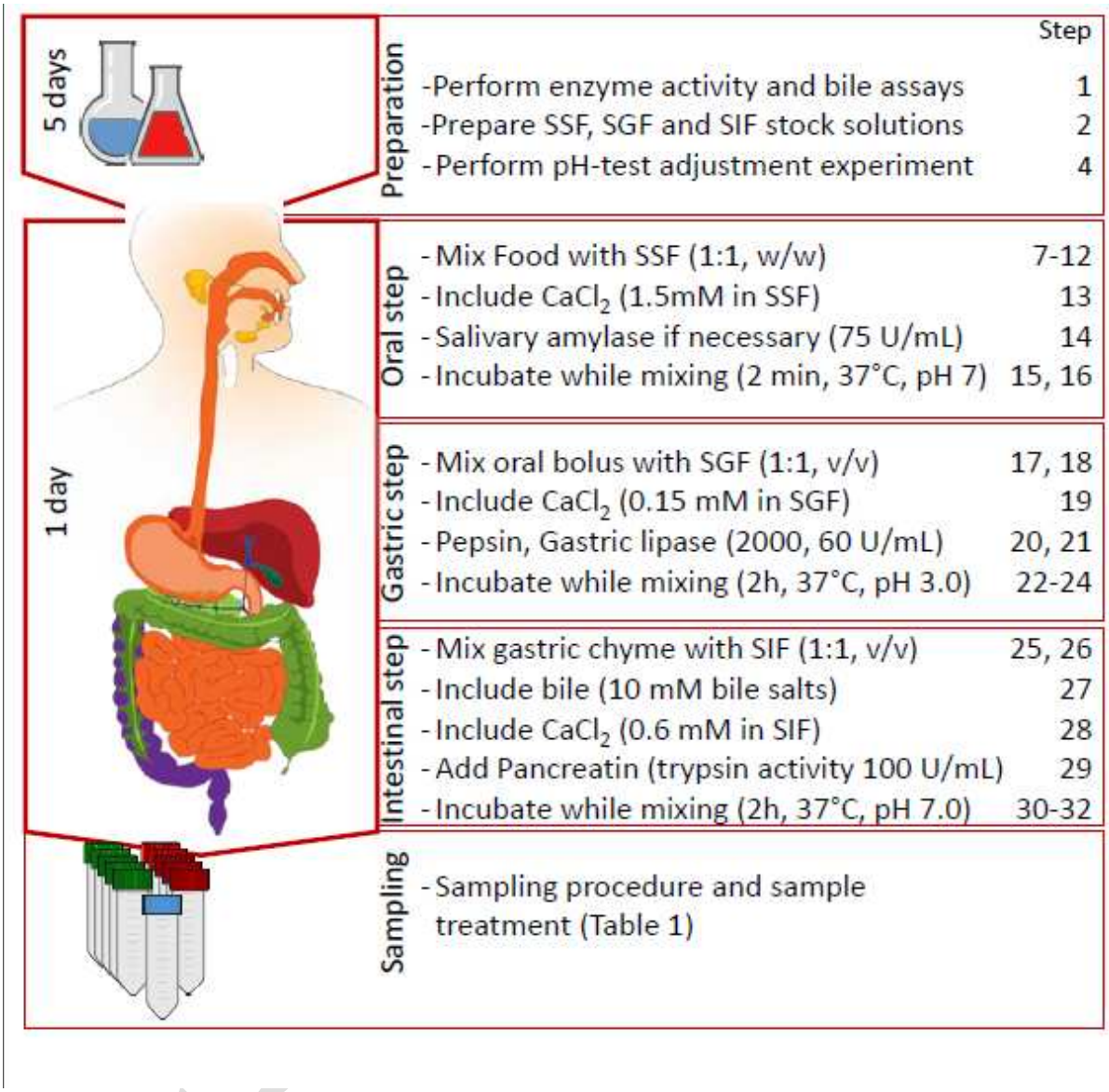
1007 # Figures



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

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Fig. 2

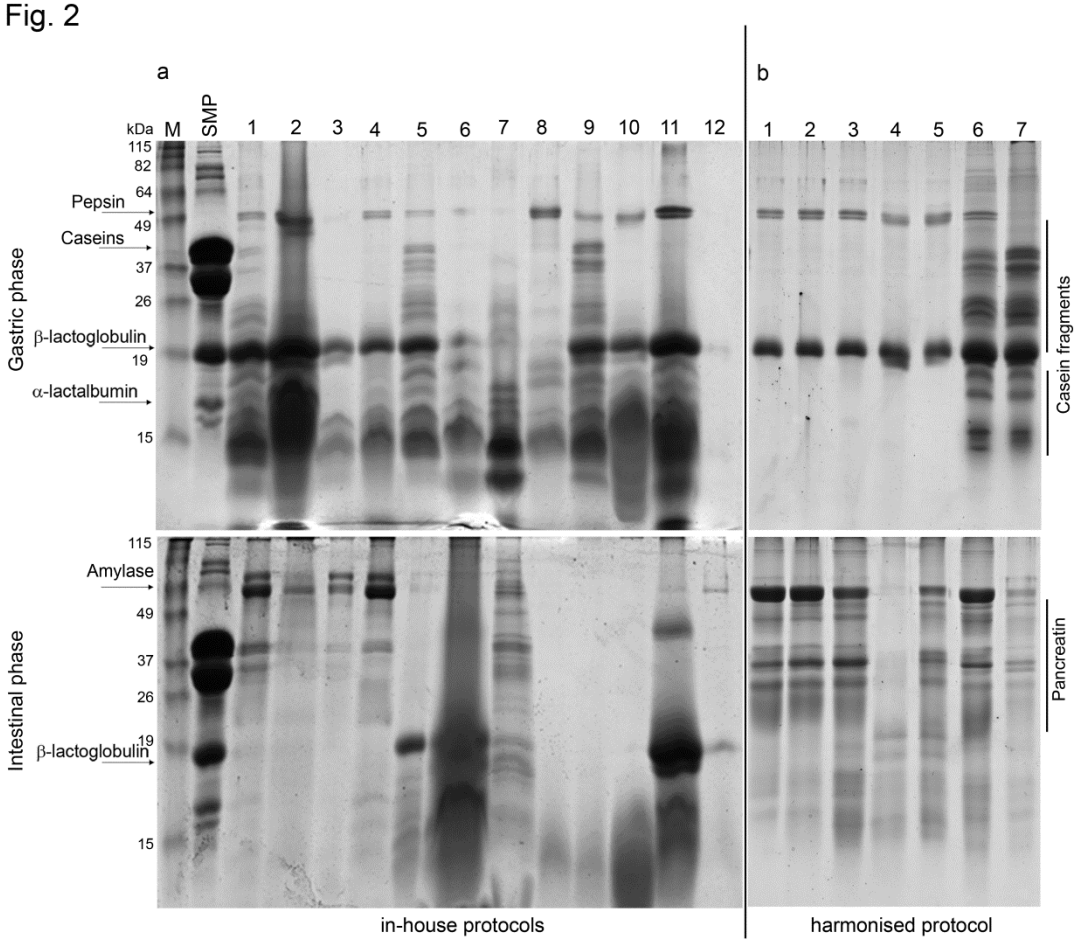

Figure 2: Protein separation by gel electrophoresis of *in vitro* digested skim milk powder (SMP)

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 a control, specific protein bands are highlighted with arrows: casein fragments, partly hydrolysed casein; pancreatin, bands originating from pancreatin. Figure adapted from Egger et al.[28]

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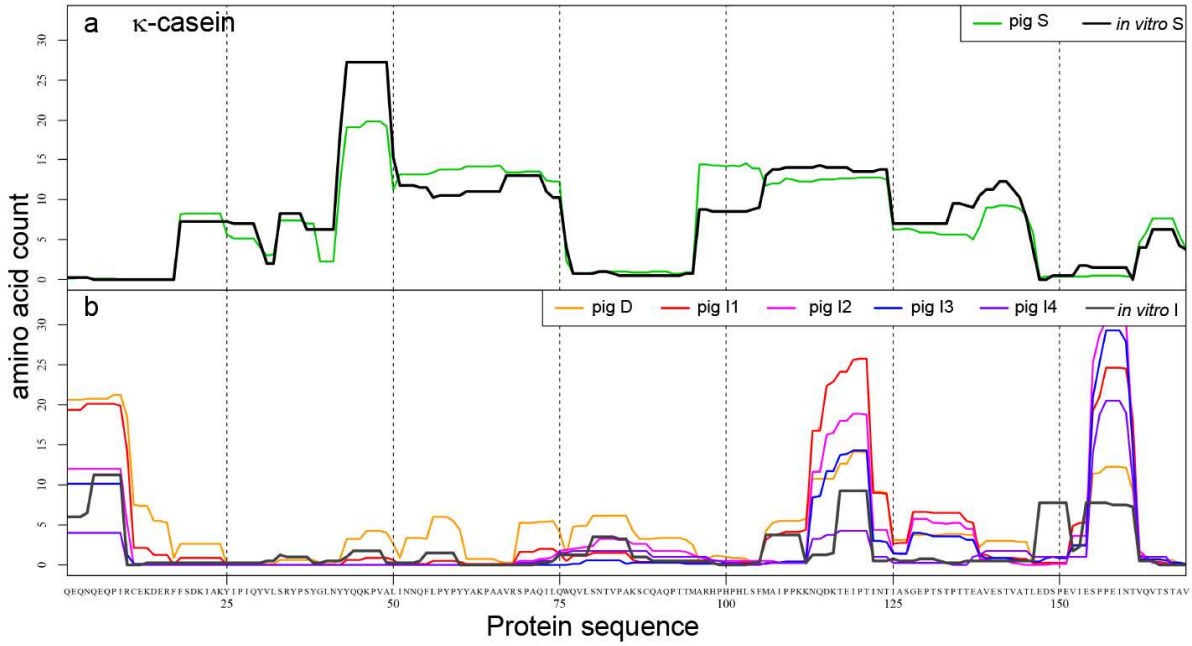

Figure 3: Comparison of *in vitro* digested skim milk powder (SMP) peptide patterns of κ-casein with *in vivo* (pig) digestion

(a) Gastric *in vitro* digestion samples (*in vitro* S) were compared to gastric pig samples (pig S, n = 8, as previously published by Egger et al.[29], approval number 2015_04_FR;26115). (b) Intestinal *in vitro* digestion samples were compared to pig sampling sections collected along the digestive tube from duodenum (D), proximal- (I1), median- (I2), distal jejunum (I3), and to ileum (I4)[29]. 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.

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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):
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1392 **Box 1**

Box 1 | Enzyme activity assays[1]

PEPSIN ACTIVITY ASSAY

Principle: Haemoglobin + H_2O $\xrightarrow{\text{Pepsin}}$ TCA soluble tyrosine peptides

Unit definition: One unit produces a ΔA280 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 µg/mL

Mix 500 µL of haemoglobin with 100 µL of each pepsin solution (5-30 µg/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 × *g* during 30 min and read the absorbance at 280 nm in quartz cuvettes.

LIPASE ACTIVITY ASSAY

Principle: Tributyrin + H_2O $\xrightarrow{\text{Lipase}}$ butyric acid + *sn-2* monobutyrin

Unit definition: One unit releases 1 µmol butyric acid per minute at 37°C at the pH of the assay

Substrate: Tributyrin purity ≥ 99%

Enzyme solution: Lipase 1 mg/mL in H_2O

Assay solution for gastric lipase: 2mM Sodium taurodeoxycholate, 150 mM NaCl, 1µM BSA

Assay solution for pancreatic lipase: 4mM Sodium taurodeoxycholate, 150 mM NaCl, 1.4mM µM$CaCl_2$

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 µL 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_2O $\xrightarrow{\text{Trypsin}}$ p-Toluene-Sulfonyl-L-Arginine + Methanol

Unit definition: One unit hydrolyses 1 µmol p-Toluene-Sulfonyl-L-arginine methyl ester (TAME) per minute at pH 8.1 and 25°C

Substrate: 10 mM TAME in H_2O

Enzyme solution: Trypsin in 1 mM HCl at concentrations ranging 10-20 µg/mL

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

AMYLASE ACTIVITY ASSAY

Principle: Starch+ H_2O $\xrightarrow{\alpha\text{-Amylase}}$ 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_2O

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-dinitrosalicyclic acid, 5.3 M sodium potassium tartrate). Complete enzyme volume with H_2O to 1 mL, cap the tube and boil it for 15 min. Add 9 mL of H_2O and read absorbance at 540 nm. Calculate the activity against a maltose standard curve.

[1]Detailed assays for all enzymes in Supplementary Information

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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).	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 $NaHCO_3$ 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® 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

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			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.	[103]
			Heat shock treatment	Sample treatment: 100°C, 5 min, but detrimental to food structure, especially protein and carbohydrate structures	[41]
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)	[104]
			Raise the pH to 8		[59]
		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).	[105]
			Addition of methanol:chloroform	Addition of methanol: chloroform mixture used for Folch extraction	[76]

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Breakdown of nutrients: Carbohydrates	Starch hydrolysis	Stop amylase activity (4 options)	Addition of $NaCO_3$	Dilute digesta in 2 volumes of 0.3 M $NaCO_3$	106
			Heat shock treatment	100°C for 5 min	
			TCA precipitation	Add 700 µL of 100% TCA to 5 mL digesta	107
			Ethanol	Add sample to equal volume of ethanol	108
Breakdown of oxygen sensitive phytochemicals	Degradation of polyphenols and carotenoids	Prevent contact with Oxygen	Flushing with Ar or N_2, pyrogallol addition (carotenoids) prior to small intestinal digestion	Flush sample 1 minute with Ar or N_2	87
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 *Stop intestinal digestion*	28
			Use of dialysis membranes/ centrifugation tubes having		109

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			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_2O).	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

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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_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

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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**	
$CaCl_2$ (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) 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_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	#Rabbit gastric extract (RGE) contains gastric lipase and pepsin, i.e. the pepsin content needs to be accounted for in the total pepsin activity			

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	ratio in the static digestion method and may need to be further diluted with water prior the oral phase, see **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. [27]), the enzyme assays for gastric lipase and pepsin are described in the supplemental materials of this manuscript

**Total volume of SIF (1.25×): 16 mL including pancreatin and bile, both of which are 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 *in vivo* digestion of cheese [29] 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

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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.

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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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Supplementary information

The Supplementary Information (SI) consists of:

1. Supplementary Figure 1
2. Supplementary Methods: protocols of enzyme assays
3. Supplementary videos
4. Supplementary spreadsheets in Excel format

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[116-123]

Supplementary Methods

Protocols of enzyme activity assays (summarised in **Box 1**) for α-amylase (EC 3.2.1.1), pepsin (EC 3.4.23.1), gastric lipase (EC 3.1.1.3), 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)

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 activity assay

Supplementary Video 4
Pepsin activity assay

Supplementary Video 5
Lipase activity assay (both gastric and pancreatic)

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Supplementary Video 6

Trypsin activity assay

Supplementary Video 7

Chymotrypsin activity assay

Supplementary spreadsheets

Supplementary spreadsheets 1

Excel spreadsheets to calculate the enzyme activities of all digestive enzymes.

Supplementary spreadsheets 2

Excel spreadsheets to calculate all volumes of simulated digestive fluids, enzyme and bile solutions based on the initial amount of digested food.

In addition, the corresponding online spreadsheets and videos of the enzyme assays and digestion procedures are available here: www.proteomics.ch/IVD and on the INFOGEST website https://www.cost-infogest.eu/.

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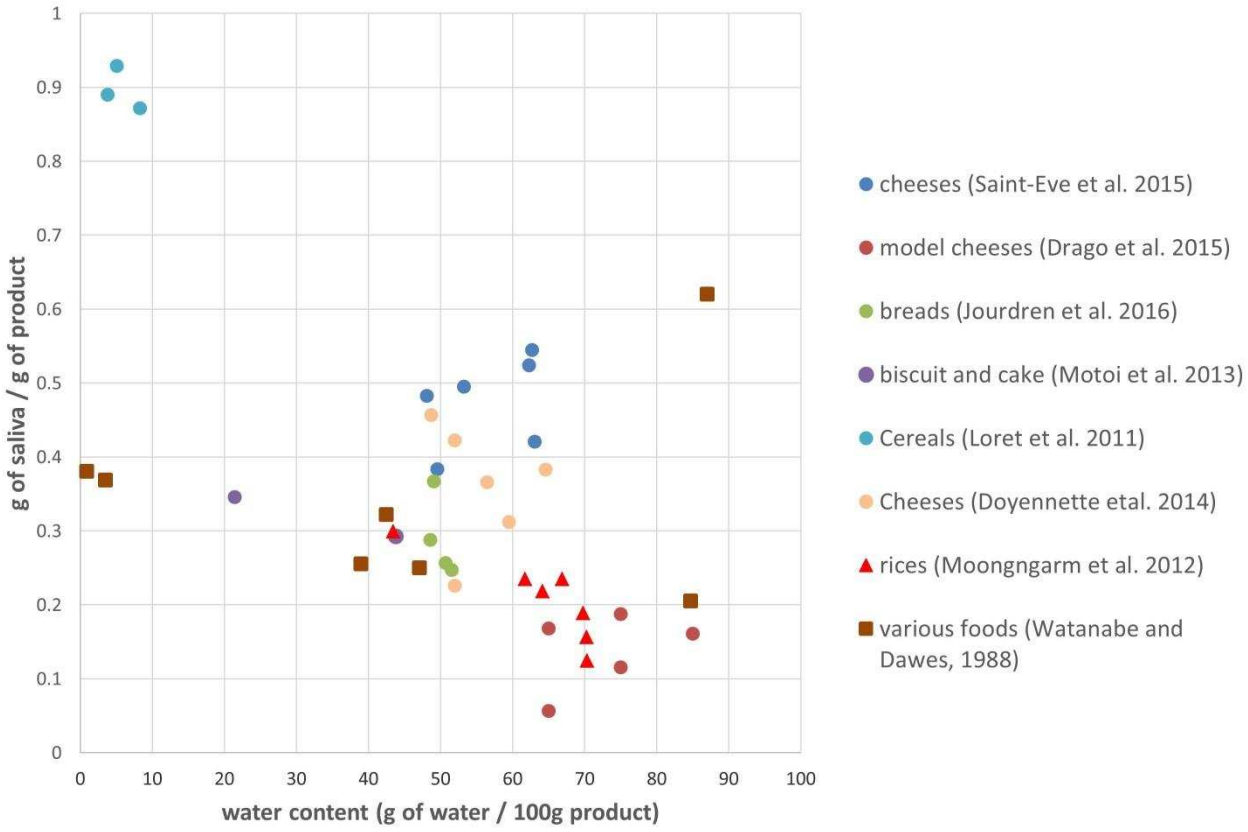

Supplementary Figure 1

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[1-8]

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Supplementary Methods - Enzyme assays

Enzyme and bile assays are adapted from Minekus et al.[1], 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 3.1.13) and bile salts (according to supplier's protocol). The assay for gastric lipase has been adapted from Carrière et al.[2] and merged with that for pancreatic lipase.

α-Amylase Activity Assay (EC 3.2.1.1)

References: according to Bernfeld[3]

Method: Spectrophotometric Stop Reaction

Principle:

Starch + H_2O $\xrightarrow{\alpha\text{-Amylase}}$ Reducing Groups (Maltose)

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

Conditions: T = 20°C, pH = 6.9, A_{540nm}, light path = 1 cm

Procedure

Preparation of reagents

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

Preparation of substrate solution:

Prepare 100 mL of a 20 mM sodium phosphate buffer containing 6.7 mM NaCl. Adjust the pH to 6.9 at 20°C with 1 M NaOH. Dissolve 0.25 g soluble potato starch (ref S2630 Sigma-Aldrich) in 20 mL of the sodium phosphate buffer, pH 6.9. Heat the covered beaker while stirring and maintain the solution just below boiling temperature for 15 minutes. Cool to room temperature and complete the starch solution to the appropriate volume (25 mL) by addition of H_2O.

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Standard Curve: Prepare 10 mL of 0.2 % w/v maltose standard (M5885 Sigma-Aldrich).

Enzyme: Shortly before the assay, prepare an enzyme solution of an estimated activity of 1 unit/mL of α-amylase in purified H_2O

Assay solution: Colour reagent solution 3,5-dinitrosalicylic acid (DNS)

Prepare a 5.3 M sodium potassium tartrate solution in 2 M NaOH by dissolving 0.8 g NaOH in 10 mL H_2O and heating the solution at a temperature ranging between 50 to 70°C. Add 12.0 g of sodium potassium tartrate tetrahydrate (in 8.0 mL of warm 2 M NaOH solution, maintain the temperature constant while stirring to dissolve the tartrate but do not boil it.

Prepare a 96 mM DNS solution by dissolving 438 mg of DNS in 20 mL of H_2O. Heat the solution at a temperature between 50 to 70°C. Maintain at this temperature while constant stirring to dissolve DNS but do not boil it.

Heat 12 mL of purified water to 60°C and add slowly 8 mL of the 5.3 M the sodium potassium tartrate solution. Add 20 mL of the 96 mM 3,5-dinitrosalicylic acid solution and stir until complete dissolution. The solution can be stored in an amber flask at room temperature for one month.

Assay:

Set the spectrophotometer at 540 nm and 20°C. Set a bench top shaking incubator fitted with a sample holder at 20°C, a heating bath or block at 100°C to stop the reaction, and an ice-bath to cool the sample.

Test: Pipette 1 mL of substrate solution (potato starch) into cap covered tubes (15 mL), mix and incubate at 20°C for 5 min to achieve temperature. Add 0.5 – 1 mL of enzyme solution (according to the scheme below), mix and incubate at 20°C for exactly 3 minutes. Immediately thereafter, stop the reaction by addition of 1 mL of DNS solution. Complete the enzyme volume added to 1 mL, cap the tube, place it at 100°C (heating bath or block) and boil it for exactly 15 minutes. Cool the tube for a few minutes on ice and add 9 mL of H_2O. Mix the reaction and pipette 3 mL in a cuvette and record the absorbance at 540 nm.

Blank: For blank tests, follow the same procedure but no enzyme is added before the 3 minutes incubation time.

Pipetting scheme for three different enzyme concentrations:

Volumes in mL	1st enzyme	2nd enzyme	3rd enzyme	Blank

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	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
2nd addition of enzyme	0.50	0.30	-	1.00
H_2O	9.00	9.00	9.00	9.00

Standard Curve with maltose:

Dilute the maltose solution (0.2% w/v) according to the scheme in H_2O

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_2O	1.95	1.80	1.60	1.40	1.20	1.00	-	2.00

1mL DNS reagent solution is added to each maltose standard, thereafter the tubes are boiled for 15 minutes, cooled on ice to room temperature and 9mL of H_2O are added.

Calculations

Standard Curve:

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

Plot the ΔA_{540}nm of the Standards versus the quantity of maltose [mg] and establish a linear regression:

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

Enzyme activity:

$$\Delta A_{540}\text{Sample} = \Delta A_{540}0\text{Test} - \Delta A_{540}\text{Test Blank}$$

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

a: slope of the linear regression for standards ΔA_{540}nm *vs* the quantity of maltose (mg).

b: intercept of the linear regression for standards ΔA_{540}nm *vs* the quantity of maltose (mg).

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

Pepsin Activity Assay (EC 3.4.23.1)

References: adapted from Anson *et al.* [4,5]

Method: Spectrophotometric Stop Reaction

Principle:

Haemoglobin + $H_2O \xrightarrow{pepsin}$ > TCA soluble tyrosine containing peptides

Unit definition: One unit will produce a ΔA_{280} of 0.001 per minute at pH 2.0 and 37°C, measured as TCA-soluble products. These units are often referred to "Sigma" or "Anson" pepsin units.

Conditions: T = 37°C, pH = 2.0, A_{280nm}, light path = 1 cm

Procedure:

Preparation of reagents

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

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

Assay:

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

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

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

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

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

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

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

Calculations:

$$\text{Units/mg} = \frac{[\,\text{A280 Test} - \text{A280 Blank}] \times 1{,}000}{(\Delta t \times \text{X} \times 0.001\,)}$$

Δt: duration of the reaction, i.e. 10 minutes

X = amount of pepsin powder (µg) in 1mL in the assay solution (i.e., 5, 10, 15, 20, 25, 30 µg)

1,000 = dilution factor to convert µg to mg

0.001 = ΔA_{280} per unit of pepsin

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

Gastric and pancreatic lipase activity assay (EC 3.1.1.3)

References: Gargouri et al.[6]; Moreau et al.[7]; Carrière et al. [2,8], Erlanson and Borgström [9]

Method: pH titration

Principle:

$$\text{Tributyrin} + H_2O \xrightarrow{\text{lipase}} \text{butyric acid} + \text{glycerol}$$

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

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

Unit definition: One unit releases 1 µmol of butyric acid per minute at 37°C at the pH of the assay: 6.0 for Human Gastric Lipase, 5.5 for Rabbit Gastric Lipase and 8 for Pancreatic Lipase. These units are often referred to International Units. Both, purified Human and Rabbit Gastric Lipases show a specific activity of approx. 1,200 U/mg protein on tributyrin[7,10] and human Pancreatic Lipase has a specific activity of ca. 8,000 U/mg of protein on tributyrin [2]

Procedure:

Preparation of reagents:

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

	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 µM)	20	-	-
$CaCl_2$	-	-	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	

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

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

Substrate: Use tributyrin of purity grade (≥99%; ref T8626 Sigma-Aldrich)

Assay:

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

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

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

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

Calculations:

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

R(NaOH): Rate of NaOH delivery in µmol NaOH per minute, *i.e.*, µmol free fatty acid titrated per minute

v: volume [µL] of enzyme solution added in the pH-stat vessel

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

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

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

Trypsin Activity Assay (EC 3.4.21.4)

References: adapted from Hummel[11] and following recommendations from the Worthington laboratory

Method: Kinetic spectrophotometric rate determination

Principle:

TAME + $H_2O \xrightarrow{\text{trypsin}}$ p-Toluene-Sulfonyl-L - Arginine + Methanol

Unit definition: One unit hydrolyses 1 µmol of p-toluene-sulfonyl-L-arginine methyl ester (TAME) per minute at 25°C and pH 8.1

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

Conditions: T = 25°C, pH = 8.1, A_{247}nm, Light path = 1 cm

Preparation of reagents

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

Enzyme: Prepare at least 2 concentrations of trypsin (porcine trypsin, ref. T0303 Sigma-Aldrich) ranging between 10-20 µg/mL in 1 mM HCl.

Assay solution: 46 mM Tris/HCl buffer, containing 11.5 mM $CaCl_2$ at pH at 8.1 and 25°C.

Assay:

Set the spectrophotometer at 247 nm and 25°C.

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

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

Blank: For blank assays, follow the same protocol by replacing the enzyme with buffer (equilibration is usually reached faster, 5 min). The blank slope, ΔA_{247}, should be close to zero.

Calculations:

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

$$\text{Units/mg} = \frac{[\,\Delta\text{A247 Test} - \Delta\text{A247 Blank}] \times 1000 \times 3}{(540 \times \text{X})}$$

ΔA_{247}: slope of the initial linear portion of the curve, [unit absorbance/minute] for the Test (with enzyme) and ΔA_{247} Blank without enzyme

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

3: Volume (in millilitres) of reaction mix

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

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

Chymotrypsin activity assay (EC 3.4.21.1)

References: adapted from Hummel[11] and Rick[12]

Method: Kinetic spectrophotometric rate determination

Principle:

$$\text{BTEE} + H_2O \xrightarrow{\text{chymotrypsin}} \text{N - Benzoyl - L - Tyrosine} + \text{Ethanol}$$

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

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

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Preparation of reagents:

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

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

Assay solution: 80 mM Tris/HCl buffer, containing 100 mM $CaCl_2$ at pH at 7.8 and 25°C.

Assay:

Set the spectrophotometer at 256 nm and 25°C.

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

Blank: For blank assays, follow the same protocol by replacing the enzyme with buffer only (equilibration is usually reached faster, 5 min). The blank slope ΔA_{256} Blank should be close to zero.

Calculations:

The slopes Δ_{A256} [unit absorbance/minute] are established for both the blank and the test reactions by using the maximum linear rate over at least 5 minutes:

$$\text{Units/mg} = \frac{[\,\Delta\text{A256 Test} - \text{A256 Blank}] \times 1000 \times 3}{(964 \times \text{X})}$$

ΔA_{256}: slope of the initial linear portion of the curve, [unit absorbance/minute] for the Test (with enzyme) and ΔA_{256} Blank without enzyme

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

3: Volume (in millilitres) of reaction mix

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

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

Pancreatin

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

Bile salts in bile

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

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