

Chapter 2

InfoGest Consensus Method

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Abstract This section describes the consensus static digestion method developed within the COST Action InfoGest. Simulated gastro-intestinal digestion is widely employed in many fields of food and nutritional research. Various different digestion models have been proposed, which often impedes the possibility of comparing results across research teams. For example, a large variety of enzymes from different sources such as porcine, rabbit or human have been used and these differ in their activity and characterization. Differences in pH, mineral composition and digestion time that alter enzyme activity and other phenomena may also significantly alter results. Other parameters such as the presence of phospholipids, specific enzymes such as gastric lipase and digestive emulsifiers, etc. have also been discussed at length. In this section, a general standardised and practical static digestion method is given, based on physiologically relevant conditions that can be applied for various endpoints. A framework of parameters for the oral, gastric and small intestinal digestion is outlined and their relevance discussed in relation to available in vivo data and enzymes. Detailed, line-by-line guidance recommendations and justifications are given but also limitations of the proposed model. This harmonised static, in vitro digestion method for food should aid the production of more comparable data in the future.

Keywords In vitro • Digestion • Oral • Gastric • Small intestinal

2.1 Introduction

The static protocol for simulating digestion in the upper GI tract published by InfoGest and led by Andre Brodkorb was the result of more than 2 years' work involving extensive discussion among scientists from a wide range of relevant disciplines (Minekus et al. 2014). The final consensus recommendation is relatively simple, based on physiological parameters that have been cited and is widely

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supported by those undertaking *in vitro* digestions, especially in food research. In keeping with the requirement for simplicity but not oversimplification discussed in the general introduction to this chapter, this is a static model using values of pH, ionic composition endogenous surfactants and enzyme activity that are fixed at the start of the experiment. All aspects of digestion in the upper GI tract were considered in the development of the method and the reasons for the inclusion or exclusion of specific features will be discussed below. The method comprises up to three stages that mimic the oral, gastric and small intestinal phases of digestion *in vivo*. At each stage the duration and physical and biochemical environment are described and the reasons for their selection given. The enzymes recommended for inclusion are described using their IUBMB Enzyme Nomenclature and the method has been written in such a way as to allow the sourcing of material from any suitable supplier. The method is outlined in the flow diagram given in Fig. 2.1. All enzyme activities and other concentrations are given per mL of digesta as they will finally be used.

2.2 The Oral Phase

The oral phase of digestion is where solid foods are physically broken down through the process of chewing. Residence time is short, especially for liquid or semi-solid foods, and solids are mixed with saliva to form a bolus with a paste-like consistency before swallowing. In addition to processing there is a great deal of sensing, including taste, texture, aroma, etc. However, most of these functions do not affect digestion in any tangible way and so for the purposes of the method they have been ignored. The exception to this is the texture, which *in vivo* is continually assessed and generally only when particles of food have been reduced to 2 mm or smaller will the bolus be swallowed (Peyron et al. 2004). Before the oral phase is started a decision needs to be made about what kind of processing is to be included as shown in Fig. 2.1. On the face of it this seems simple as liquid samples don't need to be chewed and so can simply be mixed with simulated salivary or gastric fluid and passed to the gastric phase while solid samples go through the full oral phase as outlined below. However, the user needs to decide where the boundary between solid and liquid lies and whether the addition of salivary amylase is important for their sample.

In addition to chewing the other important factor for solid food is the addition of saliva, which contains a broad range of ions, proteins and peptides, only some of which are directly relevant to digestion (Humphrey and Williamson 2001). Saliva also contains the enzyme α -amylase (EC 3.2.1.1) but not lingual lipase as is often quoted. There is general interest in the importance of mucin in saliva (Sarkar et al. 2009) and much debate about whether it is important to add it or not. There are two types of mucin secreted into saliva MUC5B and MUC7 although there is none in parotid saliva. Mucin represents less than 20 % of the total protein in whole saliva, which is normally around 0.7 mg/mL (Lee et al. 2007). At such low levels as 0.15 mg/mL, other surface active proteins are more likely to be important than mucin for the behaviour of saliva. Also the availability of reliable sources of such

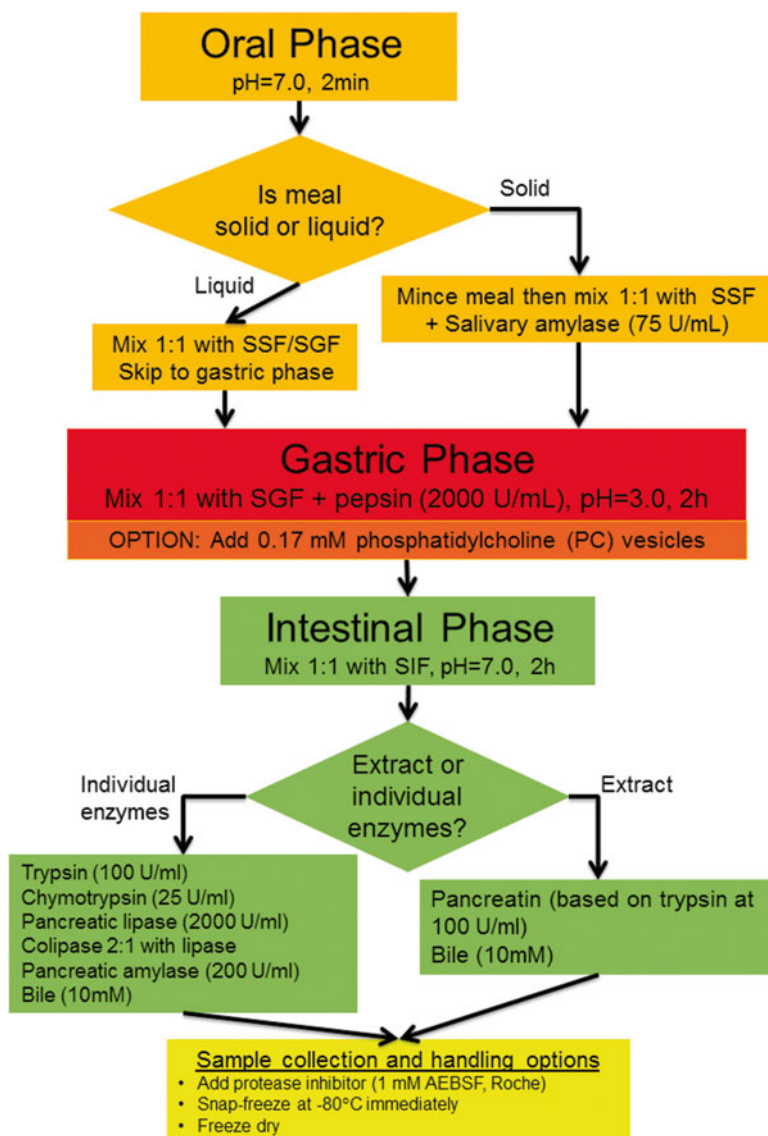


Fig. 2.1 A flow diagram describing the InfoGest digestion method involving simulated salivary fluid (SSF), simulated gastric fluid (SGF) and simulated intestinal fluid (SIF)

salivary mucin would make inclusion difficult under normal circumstances. Thus the method uses a simulated salivary fluid (SSF) containing the ion composition given in Table 2.1 at pH 7.0 and α -amylase at 150 units per mL of SSF (Bornhorst et al. 2014; Hoebler et al. 1998) but no mucin or other proteins. Here, 1 unit is defined as liberating 1.0 mg of maltose from starch in 3 min at pH 6.9 at 20 °C and the activity

Table 2.1 The ionic composition of simulated digestion fluids

Constituent	SSF (pH 7) (mmol/L)	SGF (pH 3) (mmol/L)	SIF (pH 7) (mmol/L)
K ⁺	18.8	7.8	7.6
Na ⁺	13.6	72.2	123.4
Cl ⁻	19.5	70.2	55.5
H ₂ PO ₄ ⁻	3.7	0.9	0.8
HCO ₃ ⁻ , CO ₃ ²⁻	13.7	25.5	85
Mg ²⁺	0.15	0.1	0.33
NH ₄ ⁺	0.12	1.0	–
Ca ²⁺	1.5	0.15	0.6

should be determined using the method of Bernfeld (1955). We now have our saliva but how do we simulate mixing and chewing of the food in a standardised way? After much discussion it was decided to recommend the use of what is known in the UK as a mincer, which is commonly used in kitchens to mince raw or cooked meat. Having chewed the food, how much saliva do we need to add? On average, unstimulated flow rate is 0.3 mL/min but stimulated flow rate is, at maximum, 7 mL/min (Humphrey and Williamson 2001). Stimulated saliva is reported to contribute as much as 80–90 % of the average daily salivary production. Thus based on stimulated flow, the amount of saliva to add is a 1:1 v/w ratio with the food i.e. 5 g of food + 5 mL SSF. The only remaining question is how long should we expose the food to the SSF? Although a value of 0.5 min might be close to the situation in vivo, the practicalities of handling suggest that in order to be confident of reproducing the oral phase in a consistent manner, including mixing of saliva, 2 min would be more appropriate. The temperature at which the amylase containing SSF is mixed with the “chewed” food should of course be 37 °C and the 2 min is the contact time between the food and SSF.

In a typical example: 5 g of solid or 5 mL of liquid food is mixed with 3.5 mL of SSF electrolyte stock solution, either during or after mincing, if necessary. Next, 0.5 mL salivary α -amylase solution of 1,500 U/mL made up in SSF electrolyte stock solution (α -amylase from human saliva Type IX-A, 1,000–3,000 U/mg protein, Sigma) is added followed by 25 μ L of 0.3 M CaCl₂ and 975 μ L of water and thoroughly mixed.

2.3 The Gastric Phase

Following whatever oral processing has been undertaken there needs to be a gastric phase of digestion. Commonly held beliefs about the stomach are that the pH is very low (1–2) and that there is a lot of mixing. Neither of these is a useful idea as the pH is generally only very acidic in the fasted state and there is only mixing in the small region close to the exit of the stomach known as the antrum. The pH in the gastric compartment is rather dynamic and is highly dependent of the buffering capacity of

the food (Carrière et al. 1991; Dressman et al. 1990; Kalantzi et al. 2006). However, as this is a static method a specific value needs to be recommended and this was chosen in conjunction with a decision about the duration of the gastric phase. Given that the method needs to be broadly applicable, the method recommends 2 h. This time represents the half emptying of a moderately nutritious and semi-solid meal (Dressman et al. 1990) and in order to match the 2 h recommendation for the duration of the gastric phase the pH we recommend must represent a mean value for a general meal over that time. Thus we recommend the use of a static value of pH 3 combined with the ionic composition outlined in Table 2.1. In this static model the physical environment of the gastric compartment will not be reproduced but some mixing is required and this can be supplied either by shaking or stirring the sample at 37 °C.

The only proteolytic enzyme present in the stomach is pepsin, which is secreted in the form of the precursor pepsinogen. Large variations in pepsin activities are reported in the literature due to the use of different assays and calculations (Ulleberg et al. 2011; Armand et al. 1995). Based on the literature the recommended activity of porcine pepsin (EC 3.4.23.1) is 2,000 U/mL of gastric contents where one unit will produce a ΔA_{280} of 0.001 per minute at pH 2.0 and 37 °C, measured as TCA-soluble products using haemoglobin as a substrate, adapted from Anson (1938) and Anson and Mirsky (1932). The use of lipolytic enzymes is always more difficult and whilst the potential importance of human gastric lipase (HGL) is acknowledged it has not been included for the following reasons. Firstly, because of the relatively low pH, lipid interfaces tend to become saturated and thus gastric lipolysis is generally limited. Secondly, there is no lipase currently widely available, affordable and that has the correct pH and site specificity. The final recommended option is to include phosphatidylcholine (PC) at 0.17 mM in vesicular form (Macierzanka et al. 2009; Mandalari et al. 2009).

In a typical example: 10 mL of liquid sample or oral bolus is mixed with 7.5 mL of SGF electrolyte stock solution, 2.0 mL porcine pepsin solution of 20,000 U/mL made up in SGF electrolyte stock solution (pepsin from porcine gastric mucosa 3,200–4,500 U/mg protein, Sigma), 5 μ L of 0.3 M CaCl_2 , 0.2 mL of 1 M HCl to reach pH 3.0 and 0.295 mL of water.

2.4 The Small Intestinal Phase

The final and most complex phase is the small intestinal phase in which the pH is again raised to 7 and the gastric effluent is exposed to a broader range of enzymes and surfactants (Kalantzi et al. 2006; Kopf-Bolanaz et al. 2012; Versantvoort et al. 2005). As with the gastric compartment the intestinal phase duration is 2 h. This is again a compromise but is based on normal transit times in the human gut but also on the fact that because there is no product removal, inhibition may become a problem at extended times, especially if there is a significant amount of lipid present. The suggested ionic composition for the SIF is again given in Table 2.1. There are

two possible approaches that can be used with regard to the enzymes used. Firstly, for reasons of simplicity and cost one can use a pancreatic extract (pancreatin) containing all the relevant enzymes but in a fixed ratio or secondly if preferred one can use the individual enzymes (proteases, lipases and amylase). If pancreatin is used then the amount to add must be based on a specific enzyme activity and we suggest that it is based on the trypsin activity and should be added in sufficient quantity to provide 100 U/mL of intestinal phase content. The activity should be based on the TAME assay. The pancreatin should also be assayed for its chymotrypsin, lipase and amylase activities. Where individual enzymes are to be used the following activities should be added per mL of intestinal content. Bovine trypsin (EC 3.4.21.4) at 100 U/mL where one unit hydrolyses 1 μ mole of *p*-toluene-sulfonyl-L-arginine methyl ester (TAME) per minute at 25 °C, pH 8.1, in the presence of 10 mM calcium ions (Walsh and Wilcox 1970); bovine chymotrypsin (EC 3.4.21.1) at 25 U/mL *N*-Benzoyl-L-Tyrosine Ethyl Ester (BTEE) units where one unit will hydrolyse 1.0 μ mole of BTEE per minute at pH 7.8 at 25 °C (Bergmeyer et al. 1974); porcine pancreatic amylase (EC 3.2.1.1) at 200 U/mL where one unit will liberate 1.0 mg of maltose from corn starch in 3 min at pH 6.9 at 20 °C (Bernfeld 1955); porcine pancreatic lipase (EC 3.1.1.3) at 2,000 U/mL where 1 unit will release 1 μ mole of free fatty acid per minute from a substrate of tributyrin at 37 °C, pH 8.0, in the presence of 2 mM calcium ions and 4 mM sodium taurodeoxycholate and excess colipase at a 2:1 molar excess, which is approximately a mass ratio of 1:2 colipase/lipase.

In addition to the enzymes there are also a range of endogenous surfactants including bile salts and phospholipids. These are important in the digestion of both protein and lipid and in the case of the latter they are involved in product removal to the gut epithelium. They can conveniently be added as a bile extract or as frozen porcine or bovine bile. Sufficient should be added to provide 10 mM bile in the final intestinal content (Kalantzi et al. 2006). This can be assayed using a number of different kits such as the bile assay kit 1 2212 99 90 313 from Diagnostic Systems GmbH in Germany (Collins et al. 1984). Of course none of the animal bile will be a really close match for human bile and it is currently unclear what impact that is likely to have on the digestion process. For information, the typical composition of human, bovine and porcine bile are given in Table 2.2. Surprisingly, the bovine bile is a closer match to human than porcine bile, at least in terms of tauro- and glycocholate. The bile will also contain phospholipids and cholesterol in sufficient quantity for the digestion.

In a typical example of intestinal simulation, 20 mL of gastric chyme is mixed with 11 mL of SIF electrolyte stock solution, 5.0 mL of a pancreatin solution 800 U/mL made up in SIF electrolyte stock solution based on trypsin activity, 2.5 mL fresh bile (based on 160 mM fresh bile), 40 μ L of 0.3 M CaCl₂, 0.15 mL of 1 M NaOH to reach pH 7.0 and 1.31 mL of water. Verification of the pH is recommended to determine the amount of NaOH/HCl required in a test experiment prior to digestion. In this way base/acid can be added more rapidly and followed by final verification of the pH.

Table 2.2 The approximate bile acid composition of human, bovine and porcine bile

Bile acid	Human bile (%)	Bovine bile (%)	Porcine bile (%)
Taurohyodeoxycholate	0	0	37
Glycohyodeoxycholate	0	0	34
Taurocholate	11	31	0
Glycocholate	26	46	0
Taurochenodeoxycholate	13	2	2
Glycochenodeoxycholate	25	3	26
Taurodeoxycholate	5	8	0
Glycodeoxycholate	11	10	0
Other	9	0	0

Table 2.3 Preparation of stock solutions of simulated digestion fluids

Salt	Stock conc.		SSF	pH 7	SGF	pH 3	SIF	pH 7
			Vol of stock	Conc. in SSF	Vol of stock	Conc. in SSF	Vol of stock	Conc. in SSF
	g/L	mol/L	mL	mmol/L	mL	mmol/L	mL	mmol/L
KCl	37.3	0.5	15.1	15.1	6.9	6.9	6.8	6.8
KH ₂ PO 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 ₂ O) ₆	30.5	0.15	0.5	0.15	0.4	0.12	1.1	0.33
(NH ₄) ₂ CO ₃	48	0.5	0.06	0.06	0.5	0.5	–	–
For pH adjustment								
NaOH		1	–	–	–	–	–	–
HCl		6	0.09	1.1	1.3	15.6	0.7	8.4
CaCl ₂ (H ₂ O) ₂ is not added to the simulated digestion fluids, see legend								
CaCl ₂ (H ₂ O) ₂	44.1	0.3		1.5 (0.75) ^a		0.15 (0.075) ^a		0.6 (0.3) ^a

^aIn brackets is the corresponding Ca²⁺ concentration in the final digestion mix

2.5 Practicalities

The outline method given above gives the general approach that should be used in terms of enzymes, their activities, ionic composition and endogenous surfactants. However, there are some practicalities that need to be taken into account when executing the method. For example the simulated digestion fluids (SSF, SGF and SIF) are made up using the electrolyte stock solutions given in Table 2.3, enzymes, bile, CaCl₂ and water. The volumes are calculated for a final volume of 500 mL for each simulated fluid. However, we recommend making up the stock solution with distilled water to 400 mL, i.e. 1.25 times concentrated, for storage at –20 °C. The addition of enzymes, bile, Ca²⁺ solution etc. and water will result in the correct electrolyte

concentration in the final digestion mixture. CaCl_2 is not added to the electrolyte stock solutions as precipitation may occur. Instead, it is added to the final mixture of simulated digestion fluid and food.

2.6 Sampling

The way that sampling should be done depends on the nature of the study and should be carefully considered for each study. For example, it may be advisable to have individual sample tubes for each time point rather than withdrawing samples from the reaction vessel. Also, it may be important to sample at multiple time points through both gastric and intestinal phases or it may only be necessary at the end of digestion. Regardless of such questions, the way in which the reactions are stopped will depend on what the samples will be subsequently required for. The following are some recommendations to inhibit further enzyme action in the digesta samples:

- Snap freezing of samples is recommended in liquid nitrogen immediately after the reaction for further analysis. It should be born in mind that enzymes will continue to act, even in frozen samples albeit slowly. Therefore the colder the sample is stored the better.
- If samples are sent to other labs, i.e. by courier or by post, the digestion should be stopped completely and for this, the following procedures are recommended:
 - Neutralize the pH in the gastric phase by adding 0.5 M sodium bicarbonate. This will inactivate the pepsin before snap-freezing in liquid nitrogen and subsequent storage and/or freeze drying.
 - Addition of protease inhibitor (e.g. 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride [AEBSF], Roche or similar), snap freezing in liquid nitrogen and subsequent freeze drying of samples.

2.7 Conclusions

The InfoGest harmonised static digestion simulation method has been endorsed by a wide range of international experts. We have endeavoured to make it as physiologically relevant as a static model can be but readers should bear in mind that it is still only a simplified model. The main limitations of such a simplified model are the fixed pH and duration of the various phases. However, this can also be seen as an advantage in terms of consistency and comparability. The other potentially problematic issue is the lack of an adsorption step but there are now a number of cellular systems where this aspect can be investigated. It is hoped that this simple model will be widely adopted, allowing faster progress when developing healthier foods and more effective bioactives.

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