Universitat de les Illes Balears

In vitro digestion behavior of complex formulations for clinical nutrition applications based on model systems

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1. ABSTRACT

In vitro digestion methods simulating digestion processes are widely used to study the gastro-intestinal behavior of food or pharmaceuticals. *In vitro* digestion methods typically include the oral, gastric, and small intestinal phases, and occasionally the large intestinal phase. These methods try to mimic physiological conditions *in vivo*, taking into account the presence of digestive enzymes and their concentrations, pH, digestion time, and temperature, among other factors.

In vitro digestion methods have been used to address such diverse scientific questions as the digestibility and bio-accessibility of pharmaceuticals, mycotoxins, and macronutrients such as proteins, carbohydrates, and lipids. Bio-accessibility provides an indication for the maximum of bioavailability via the oral route and is an important parameter. In this master's thesis two different methods were studied:

- *Static digestion method:* Static *in vitro* digestion models use sequential exposure to simulate digestion in different compartments (mouth, stomach, and intestine). During each step, the substrate is incubated for a specific time with the appropriate simulated digestive fluids. The pH is generally maintained at a fixed value by using a pH-stat or a buffer.

- *Dynamic digestion method:* Dynamic *in vitro* digestion models reproduce the gradual transit of ingested compounds through the gastrointestinal tract more. The system reproduces the temperature, pH changes, gastric emptying, addition of simulated fluids and dialysis of digestion end products.
To carry out this thesis three different carbohydrate sources were selected, Maltodextrin DE 11 – 16, Tapioca Dextrin and Modified starch, and all of them are starch derivatives. To carry out different studies like the study of digestibility, bio-accessibility, volume effect, matrix effect, etc. the static and dynamic digestion methods were used.

The obtained results show that Maltodextrin DE 11 – 16 liberates more amount of glucose than Tapioca Dextrin and Modified starch and the results also show the dependency between length chain and digestibility. The comparison of the static and dynamic digestion method show that there are no big differences between the recovery obtained from each method. The obtained results of the study of volume effect suggest that is possible use smaller volumes with static digestion method, which is important to save resources. And the results obtained with the study of matrix effect indicate that the matrixes used do not affect the digestibility of Tapioca Dextrin and the pre-treatment of the meal favors the release of glucose.

The objectives of this thesis are the following:

- *Study of digestibility and bio-accessibility of three different carbohydrate sources:* this is of interest because with this study the amount of released glucose from the different sources (in a time-dependent manner) can be obtained. The released glucose represents available glucose for intestinal absorption. *In vivo*, this glucose levels would impact on the blood glucose levels and is of special interest for products intended for patients with diabetes.

- *Study matrix effect:* clinical nutrition products for enteral root are rarely including only polysaccharides, but also contain macro- and micronutrients. In those complex mixtures it is most likely that matrix could affect digestibility of the contained polysaccharides. For this reason, the effect of different matrixes was also studied.

- *Comparison between two different digestion methods:* this part of the thesis should reveal advantages and drawbacks of each method, and, those results should serve as the basis of decision for the application of each system in future.

- *Study volume effect:* this part of the study is focused on the used static digestion method and intents to determine the impact of the used reaction volume and to explore the potential to save resources.

2. INTRODUCTION

Human digestion is a complex process essential for health wherein ingested food is broken into nutrients that can be used by the body for growth, cell maintenance, and fuel. During human digestion, two main processes occur simultaneously:

- Mechanical transformations that reduce the size of the food particles.
- Enzymatic transformations where macromolecules are hydrolyzed into smaller constituents that are absorbed into the bloodstream.

Food disintegration mainly occurs in the mouth and stomach, whereas enzymatic digestion and absorption of nutrients and water take place mainly in the small and large intestine.

The digestive system is central to numerous questions raised by researchers and industrials in various fields such as nutrition, toxicology, pharmacology and microbiology[1].

2.1. Human digestion

An overview on the main parts of the human gastrointestinal tract is depicted in Figure 1.

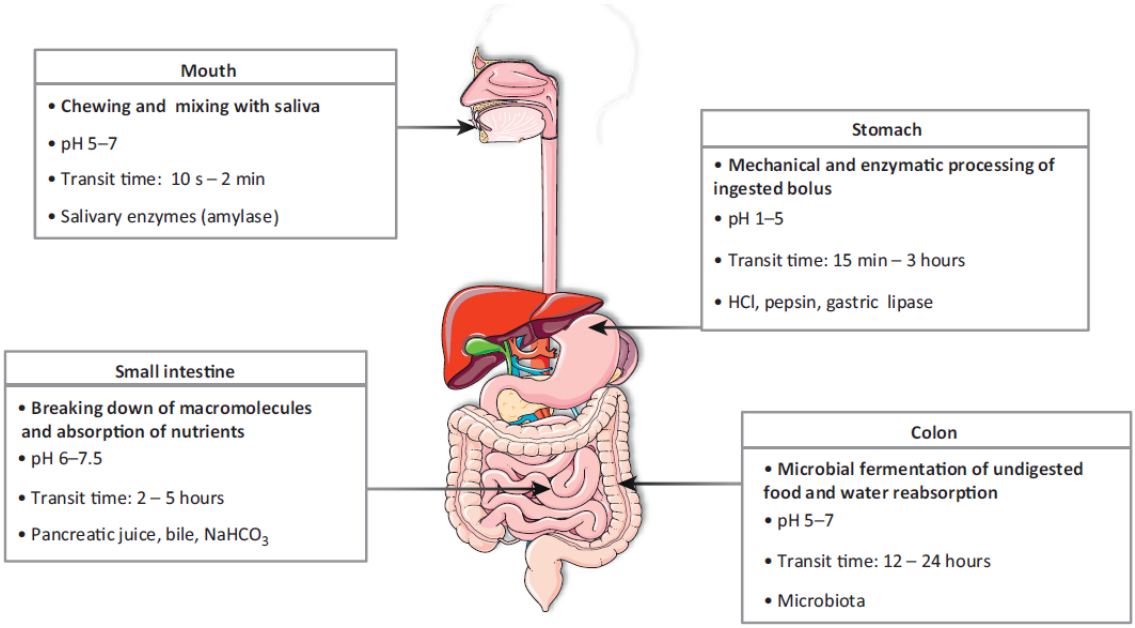


Figure 1. Main parts of the human gastrointestinal tract[1]

Digestion starts with chewing food in the mouth. Mastication is a short but important step with a significant influence on the overall digestive process and particularly on the gastric emptying rate. The food bolus, resulting from mechanical and enzymatic degradations in the mouth, is transported through the esophagus to the stomach by the mechanism of peristalsis.

The gastric digestion is a regionalized dynamic step (Figure 2). In the proximal part of the stomach, fundus and stomach body act as a reservoir for food and initiate the contact between bolus and gastric juice.

The latter is mostly composed of pepsin and lipase enzymes responsible for protein and lipid digestion, hydrochloric acid leading to a regular fall in pH (approximately from pH 6-5 to 1.5) promoting protein hydrolysis, and mucus that protects mucosal surfaces.

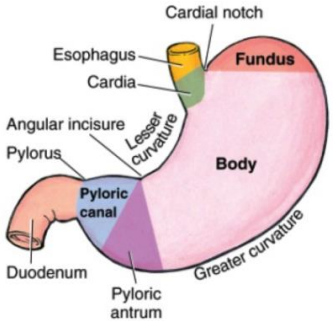


Figure 2. Parts of stomach[2]

In the distal part, peristaltic waves in the antrum help to break down large particles by grinding and mixing gastric contents. The stomach ends at the pylorus, which acts as a sieve and a pump for the selective emptying of small particles (chyme) to the duodenum, whereas the larger particles are maintained in the stomach, by a mechanism of retropulsion, and to be further degraded. Gastric emptying is a crucial parameter of digestion that is influenced by many factors such as food composition or structure and biological factors.

The acidic chyme from the stomach is then delivered to the small intestine. This compartment is divided into three parts: a short section receiving digestive secretions from the pancreas and liver, the duodenum, and two longer ones, the jejunum and ileum (Figure 3).

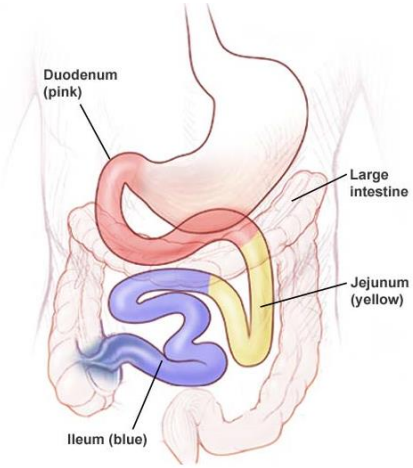


Figure 3. Parts of small intestine[3]

The morphology of the small intestine is highly adapted to its two main roles in digestion, that are, breakdown of macromolecules and absorption of water and nutrients. After entering the duodenum, the acidic chyme is neutralized with sodium bicarbonate to give an appropriate pH for optimal enzyme activities of the digestive enzymes. Pancreatic enzymes (a complex mixture of proteases, amylases, and lipases) and other digestive enzymes produced by the inner wall of the small intestine act together in the breakdown of food constituents. Bile (produced by the liver) plays a specific role in lipid digestion by emulsifying dietary fats into small droplets promoting pancreatic lipase activity. Most bile salts are actively reabsorbed and reused in the bile through enterohepatic cycling.

The complex topology of the inner lining of the small intestine gives it a huge absorptive surface area (Figure 4). Water and nutrients are absorbed by villus enterocytes via simple diffusion, facilitated diffusion, or active transport. This prevents accumulation of digestion products in the lumen of the small intestine, which could inhibit enzyme activities. Mechanical digestion includes segmentation movements, allowing chyme to mix with digestive enzymes, and peristalsis activity, which propels the digesta through the small intestine. Small intestinal regions harbor distinct microbial populations that may play a role in human health and diseases.

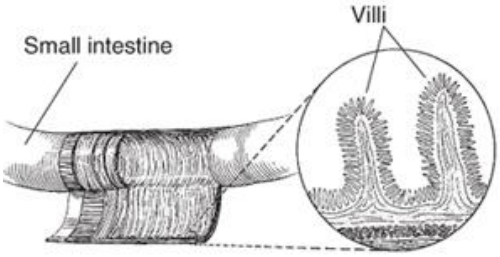


Figure 4. Small intestine villi[4]

Non-absorbed material travels further down to the large intestine (colon). The main functions of the colon are absorption of water and electrolytes, fermentation of undigested polysaccharides and proteins by colonic microbiota, reabsorption of bile salts, and formation, storage, and elimination of feces.

The main digestive processes, such as gastric emptying intestinal transit, secretion of digestive fluids and mucus, and motility are closely controlled by hormonal and neural regulation mechanisms. Digestive hormones may enhance or inhibit the secretory activity of glandular organs and the contractions of smooth muscles. Both the autonomic and enteric nervous systems are involved in the regulation of digestion processes.

Glucose is a major source of energy in our body, but unfortunately, free glucose is relatively rare in natural human typical diet. Instead, glucose is locked up in many larger forms, including lactose and sucrose, where two small sugars are connected together and long chains of glucose like starches and glycogen. One of the major tasks of digestion is to break these chains into their individual glucose units, which are then delivered by the blood to hungry cells throughout your body.

2.2. In vitro digestion

Testing foods and new developed products requires on the availability of digestion models that accurately simulate the complex physicochemical and physiological events that occur in the human gastrointestinal tract.

In vivo feeding methods, using animals or humans, usually provide the most accurate results, but they are time consuming, costly and imply ethical restrains, which is why much effort has been devoted to the development *in vitro* procedures[5]. In principle, *in vitro* digestion models provide a useful alternative to animal and human models by rapidly screening food ingredients. The ideal *in vitro* digestion method would provide accurate results in a short time[6] and could thus serve as a tool for rapid of screening foods or food delivery systems with different compositions and structures. In practice, any *in vitro* method is inevitably going to fail to match the accuracy that can be achieved by actually studying a food *in vivo* due to the inherent complexity of the process[6,7]. Consequently, some compromise is needed between accuracy and ease of utilization of any *in vitro* digestion model.

During the past few years, the scientists have utilized a number of *in vitro* digestion models to test the structural and chemical changes that occur in different foods under simulated gastrointestinal conditions[8].

In general, *in vitro* experiments are less expensive, are easy to perform, and, are not limited by ethical constrains. They are not hampered by biological variation among subjects, they allow studies with toxic compounds or doses, and they permit the variation of experimental conditions[9].

Several factors, such as sample characteristics, enzyme activity, ionic composition, applied mechanical stresses, and digestion times, have significant influences on the results of *in vitro* digestion methods. Therefore, *in vivo* conditions can never be completely simulated under *in vitro* conditions[5].

In the last 10 years there have been many studies related to *in vitro* digestion models for foods. There were important differences in these studies, which depended on the specific food component being analyzed, the nature of the food matrix, and the sophistication of the *in vitro* digestion model used. The survey[8] (*Hur et al*) found that the most predominant food samples tested using *in vitro* digestion models were: plant-based foods, such as starch, tea, rice, or bread (45%); meats (18%); dairy foods (9%); marine foods (9%); and emulsions (9%).

Table 1 gives an overview on differences in between used methods dealing with starch digestion.

Table 1. In vitro digestion studies with starch

Study	***Measurement parameters***	***Enzymes or chemicals***	***Digestion times***	***References***
Starch digestibility	Hydrolysis, kinetics of starch digestion	Alpha-amylase	0 – 180 min	*10*
Starch digestion	Digestion rate of starch, concentration of starch	Pepsin, alpha-amylase, amyloglucosidase	0 – 15 h	*11*
Digestion of starch	Digestion coefficients and characteristics, digestion of horse beans	Pepsin, enzyme cocktail (pancreatin and amyloglucosidase)	30 min 0 – 6 h	*12*
Alpha-amylase digestion of starches	Transmission electron microscopy, size-exclusion chromatograms	Pancreatic amylase	2h	*13*
In vitro digestion of starch	Percent digestion of starch	Alpha-amylase	0 – 24 h	*14*
Develop a model stomach system and to investigate the kinetics of food disintegration	Food disintegration and stomach emptying, disintegration and texture change, kinetic parameters	Alpha-amylase, mucin, pepsin	30 sec 2 h	*15*

The *in vitro* digestion models surveyed also differed from one another in their operation:

- The number and type of steps included in the digestion sequence, e.g., mouth, stomach, small intestine, large intestine.
- The composition of the digestive fluids used in each step, e.g., enzymes, salts, buffers, biological polymers, and surface-active components.
- The mechanical stresses and fluid flows utilized in each step in the digestion sequence, e.g., magnitude and direction of applied stresses, flow geometries, and flow profiles.

The characteristics of foods, enzyme type, and enzyme concentrations are key factors that control the digestion of foods during *in vitro* digestion. *In vitro* digestion characteristics such as digestion time, enzyme contents or enzyme composition must be adjusted according to sample characteristics.

In vitro digestion models do not usually take the large intestine into account, because the human digestion (and absorption) of compounds mainly takes place in the small intestine[16].

Several researchers have used *in vitro* digestion methods to analyze structural changes, bioavailability, and digestibility of foods, indicating that *in vitro* digestion systems are common and useful tools for analyses of foods and drugs[8].

The most common parameters measured in *in vitro* digestion studies were:

digestibility /degradation > bio-accessibility > sample stability > structural changes

Bio-accessibility as an indicator of bioavailability via the oral ingestion route

- *Bio-accessibility:* fraction of ingested component released from food matrix and available for intestinal absorption (typically based on in vitro procedures) (Figure 5).
- *Bioavailability:* fraction of ingested component available for utilization in normal physiological functions that reaches the target organ or systemic circulation (determined by in vivo assays) (Figure 5).

Bioavailability of ingested components is an extremely important area of food and pharmaceutical research. The main issue is to determine which fraction of e.g. ingested nutrients, food pollutants, or drugs can be really used by the organism to exert their health or deleterious effects.

Bioavailability of ingested components is under the dependence of numerous factors such as dosage form, food matrix or food processing, and gastrointestinal parameters. *In vivo* approaches (blood sampling) provide direct data on bioavailability, but present major drawbacks of cost and variability in individual physical states. Animal studies are often limited by differences in digestion and absorption capacity between animals and humans. *In vitro* gastric and small intestinal models provide a suitable alternative to *in vivo* assay by determining the bio-accessibility of an ingested substance, that is, the amount available for absorption in the gut[1].

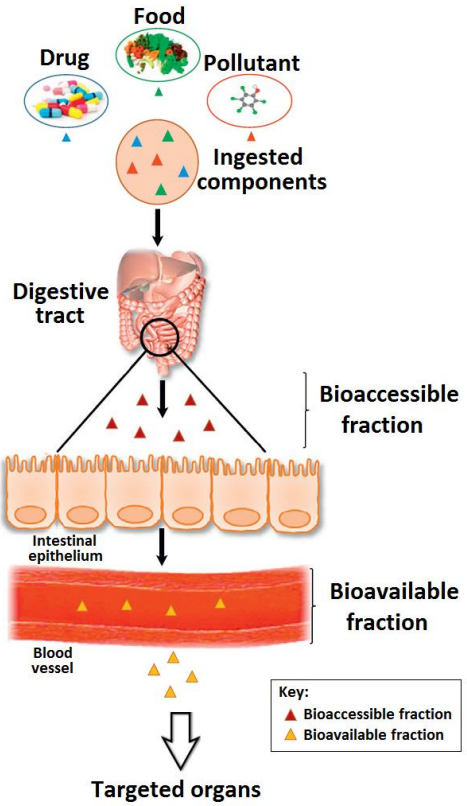


Figure 5. Differences between bio-accessibility and bioavailability[1]

Gastric and small intestinal models as relevant tools for nutrition and health studies

- *Nutrient bio-accessibility*

In vitro digestion tools have been extensively used to determine the bio-accessibility of ingested nutrients. Reported studies range from macronutrients to micronutrients such as vitamins, minerals, or phytoconstituents. Such approaches are particularly relevant to assess the effects of the chemical form of the nutrient, food structure, interactions with other food components, thermal, physical, or biological processing, and transit time. In addition, *in vitro* models have emerged as relevant tools for global analysis of nutritious components released during digestion, also known as 'nutriomic' analysis.

Compared with static systems, dynamic models include specific parameters, such as stomach emptying rate or gastrointestinal transit time, which may greatly influence the bio-accessibility of ingested substances by affecting their release from the food matrix, their solubility and their stability in the digestive lumen. Knowledge of the various factors influencing nutrient bio-accessibility would be helpful when designing functional foods or establishing process conditions that maximize the health benefits of bioactive compounds.

- *Safety assessment of food constituents*

Food intake supplies nutrients essentials for life to the body, but is also a source of substances that can have adverse effects on health, such as pollutants (e.g., heavy metals, polychlorinated biphenyls, and polycyclic aromatic hydrocarbons) or allergens (proteins mainly found in milk, eggs, peanuts, or fish). Both substance classes are considered as major concerns of food safety.

Among *in vitro* gastric and small intestinal models, the static mono-compartmental model is specifically dedicated to the evaluation of food pollutants bio-accessibility. Most studies have been carried out on soil samples because their oral ingestion is a major route of human exposure, especially in young children. The fraction of contaminant mobilized during digestion depends crucially on soil parameters and particle size. Bio-accessibility of food pollutants is also affected by many other parameters: food matrix and food structure, chemical form and concentration of pollutant, and digestive parameters such as pH or bile salts. Multi-compartmental systems offer the possibility of gaining further insight into the metabolism of pollutants by following their fate through the digestive tract.

- *Drug development and testing*

The oral route is the one most frequently used for the administration of drugs in humans due to its safety, reduced cost, and high degree of patient compliance, but it is also the most complex way for an active pharmaceutical ingredient (API) to enter the body. USP (United States Pharmacopeia) standard methods are routinely applied for disintegration, dissolution, and drug release studies, but involve a static, closed environment, remote from *in vivo* complexity. To investigate further some aspects of drug pharmacokinetics, this approach was improved by adding mechanical forces or combining dissolution and absorption models.

However, these models do not simulate the continuous changes in luminal conditions that widely impact drug bioavailability. To predict better *in vivo* performance of oral formulations, dynamic *in vitro* models have been used to investigate the effect of dosage form, feeding state, drug interaction, and transit time on API bio-accessibility. Bi- or multi-compartmental models are particularly relevant when following drug precipitation in the intestine or assessing the performance of controlled-release formulations.

Although multi-compartmental systems remain more complex to use and more costly than established compendial dissolution, they generate better *in vitro*/*in vivo* correlations (IVIVCs). Their potential has been fully exploited in the development of innovative drug delivery systems based on genetically engineered microorganisms.

In vitro models

Most of the *in vitro* models developed until 1995 have been dedicated to a single application and include a limited number of simulated parameters. As has been proposed by Longland[17], the following five aspects should be taken into account when devising new *in vitro* models of the gastrointestinal tract:

- Sequential use of enzymes in physiological amounts.
- Appropriate pH for the enzymes and addition of relevant co-factors such as bile salts and co-enzymes.
- Removal of the products of digestion.
- Appropriate mixing at each stage of digestion.
- Physiological transit times for each step of digestion.

Many attempts to model the human stomach and small intestine have been made in the past two decades. Most of these *in vitro* tools are static, include a limited number of simulated parameters, and are dedicated to a particular application.
However, to simulate the complex physiological and physicochemical events occurring within the upper human digestive tract, it is crucial to expose a meal to each step of digestion with realistic transit time, pH and enzymatic conditions.

Consequently, a few dynamic bi-compartmental or multi-compartmental models have been developed and applied in a large number of studies. Despite their complexity, the gastric and small intestinal models described so far remain simplified compared to the *in vivo* situation: they do not include feedback mechanisms, resident microbiota, immune system, or specific hormonal controls. Further efforts and technological innovations are therefore needed to improve *in vitro* models and keep up with the growing interest of industry researchers.

A wide range of gastric and small intestinal systems has been designed to study the fate of orally ingested substances, from single static bioreactors to multi-compartmental and dynamic systems.

- *Static mono-compartmental models (same reaction vessel)*

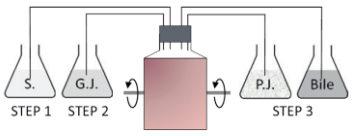
Static models are the most widespread digestive systems. The gastric phase is reproduced by pepsin hydrolysis of homogenized food, under fixed pH and temperature, for a set period of time (e.g. pH 1–2, 37°C, 1–3 h). This step may be followed, in the same bioreactor, by an intestinal phase involving pancreatic enzymes with or without bile (pH 6–7). Most of these models have been developed for specific applications and are cheap high-throughput tools, particularly relevant for large prescreening approaches. Several models were designed to assess the digestibility of protein, starch, and the bio-accessibility of carotenoids and pollutants.

The United States Pharmacopeia (USP) apparatus dissolution testes also provides a static, closed environment, widely used to assess dosage form disintegration and dissolution in single medium reproducing digestive conditions, both gastric (Simulated Gastric Fluid) and intestinal (Simulated Intestinal Fluid). However, in this approach, large volumes of media are often deployed and the mechanical forces (continuous stirring) are not representative of complex peristaltic movements. Other static mono-compartmental models include additional parameters such as mechanical forces or removal of digestion end products.

However, none of these static models reproduce the dynamic processes occurring during human digestion such as gastric emptying or continuous changes in pH and secretion flow rates.

Table 2 gives an overview of an example of static digestion model.

Table 2. Static mono-compartmental model[1]

System	Schematic representation	Body temperature	Fall of gastric pH	Control of intestinal pH	Gastric emptying	Intestinal transit	Digestive secretions	Chyme mixing	Intestinal microbiota	Intestinal absorption
Static mono-compartmental										
Oomen		+	-	-	-	-	Saliva Gastric juice Pancreatic juice Bile	Head-over-heels rotator	-	-

Oomen is an experimental digestive model developed by Oomen et. al. (2001) [18] to investigate the effects of a soil matrix on oral bioavailability.

Various digestion models have been proposed, often impeding the possibility to compare results across research teams. In 2014 consensus method was presented from a scientist and industrial network (Minekus et al. (*2014*)[19]) proposing a general standardized and practical static digestion method based on physiologically relevant conditions that can be applied for various endpoints, which may be amended to accommodate further specific requirements. A frameset of parameters including the oral, gastric and small intestinal digestion are outlined and their relevance discussed in relation to available *in vivo* data and enzymes.

To agreement with the requirement for simplicity but not oversimplification, the consensus static model uses ionic composition endogenous surfactants and enzyme activity that are fixed at the start of the experiment. The method comprises up to three stages that mimic the oral, gastric and small intestinal phases of human digestion *in vivo*.

- *Dynamic mono-compartmental models (one organ)*

To overcome these limitations, several dynamic gastric models have been developed. One model developed by Hoebler et. al. (2002)[20] reproduces, based on *in vivo* data, the progressive acidification of gastric content by HCl addition, the time course of pepsin flow rate and gastric emptying.

The Dynamic Gastric Model (DGM) was designed to take into account the region specificity of the stomach. It is composed of two successive compartments:

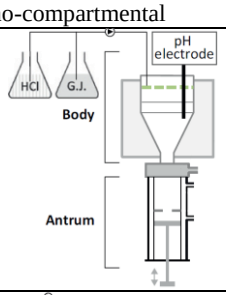
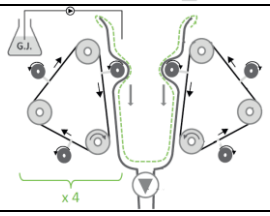
- The ‘body’ where gastric secretions are mixed with food.
- The ‘antrum’ where shear forces and stomach grinding are reproduced.

The gastric emptying is regulated by a valve that allows the smallest particles to leave the stomach, whereas the bigger ones are refluxed into the top chamber to be further digested. Despite its complexity, the DGM does not accurately reproduce the *in vivo* peristaltic forces.

The Human Gastric Simulator (HGS), composed of a latex chamber surrounded by a mechanical driving system, more effectively emulates the peristaltic movements of the stomach in amplitude, intensity, and frequency.

An overview of mono-compartmental systems is given in Table 3.

Table 3. Dynamic mono-compartmental model[1]

System	Schematic representation	Body temperature	Fall of gastric pH	Control of intestinal pH	Gastric emptying	Intestinal transit	Digestive secretions	Chyme mixing	Intestinal microbiota	Intestinal absorption
Dynamic mono-compartmental										
DGM		+	+	NA	+	NA	Gastric juice HCl	Water pressure Piston/Barrel	NA	NA
HGS		+	+	NA	+	NA	Saliva (mixed with food) Gastric juice (HCl)	Mechanical driving device	NA	NA

Although these systems are particularly relevant for gastric digestion studies, they only provide a partial insight into gastric and small intestinal digestion. *In vivo*, the stomach and small intestine form separate compartments dedicated to a specific function in digestion, such as mixing, degradation of macromolecules, or nutrient absorption. To study accurately the fate of a digested compound, it is therefore particularly important to expose the meal to each step of digestion, with a realistic transit time. In response, bi- and multi-compartmental dynamic systems were developed.

- *Dynamic bi- and multi-compartmental models*

Based on *in vivo* data, computer-controlled bi-and/or multi-compartmental systems reproduce the temperature, pH changes in the gastric and duodenal compartments, gastric emptying, addition of pepsin, pancreatic juice and bile, and dialysis of digestion end products. These systems have been mostly standardized and validated for specific applications, such as the study of alkaline activity or the survival of probiotics.

To date, the TIM-1 (TNO Gastro-Intestinal Model 1) is the only gastric and small intestinal system characterized as ‘full’, that is, combining multi-compartmentalization and dynamism, and that is commercially available.

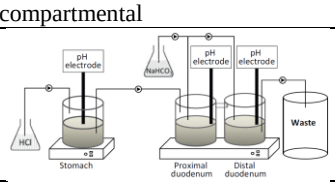
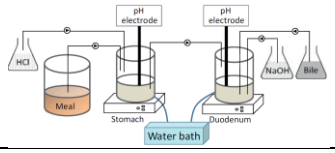
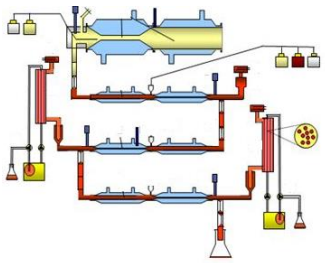
This model is composed of stomach and three parts of the small intestine, the duodenum, jejunum, and ileum. It integrates key parameters of human digestion: temperature, kinetics of gastric and intestinal pH, gastric and ileal deliveries, transit time, peristaltic mixing and transport, sequential addition of digestive secretions, and passive absorption of water and small molecules through a dialysis system. TIM-1 is so far the system that allows the closest simulation of *in vivo* dynamic events occurring throughout the human gastric and small intestinal lumen. It has been applied in a large number of nutritional, toxicological, pharmaceutical, and microbiological studies.

In this multi-compartmental dynamic system, bio-accessibility is determined by measuring the fraction of a compound that has passed the dialysis or filtration membrane.

The effect of variability of a specific condition on digestion within a population can be tested by changing only this specific condition in the digestive protocol. The reproducible conditions allow comparison of different compounds under the same conditions and do not need as many replicates as are necessary to obtain sufficient statistical power for *in vivo* studies.

An overview of dynamic bi and multi-compartmental systems is given in Table 4.

Table 4. Dynamic bi and multi-compartmental models

System	Schematic representation	Body temperature	Fall of gastric pH	Control of intestinal pH	Gastric emptying	Intestinal transit	Digestive secretions	Chyme mixing	Intestinal microbiota	Intestinal absorption
Dynamic bi-compartmental										
Vatier		+	-	+	+	+/-	HCl $NaHCO_3$	Magnetic stirrers	-	-
Mainville		+	+	+	+	-	Bile HCl	Magnetic stirrers	-	-
Dynamic multi-compartmental										
TIM		+	+	+	+	+	Saliva (mixed with food) Gastric juice Pancreatic juice Bile Electrolytes HCl $NaHCO_3$	Water pressure	-	+

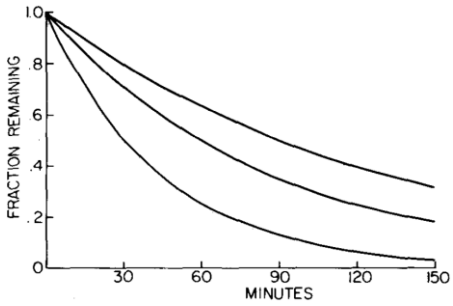
Computer program and mathematical modelling (TIM-1, TNO Gastro-Intestinal Model 1)

TIM-1 computer program has been designed to accept parameters and data obtained from *in vivo* studies in animals or human volunteers, such as the quantity and duration of a meal, the pH curves for the stomach and duodenum, secretion rates into the different compartments, water absorption from the small intestine and gastric and ileal delivery into the duodenum and colon. To control the transit of chyme, a power exponential formula for gastric and ileal delivery is used, as described by Elashoff et al.[21]:

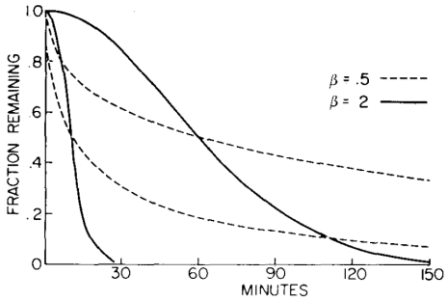
$$f = 1 - 2^{-\left(\frac{t}{t^{1/2}}\right)^{\beta}} \qquad f' = 2^{-\left(\frac{t}{t^{1/2}}\right)^{\beta}}$$

Equation 1. Power exponential formula for gastric and ileal delivery

Where t is the time, f' represents the fraction of the meal remaining in the stomach at time t, f represents the fraction of meal delivered, $t_{1/2}$ is the time from the start of the meal until 50% of the meal has emptied and β determines the shape of the curve. For β = 1, the power exponential is the same as the restricted simple exponential. For a curve with an initial lag in emptying, β is > 1 and this type of curve is often seen for solid meals, where the initial lag phase may represent the time to grind the food into smaller particles. A value of β < 1 describes a curve with a very rapid initial emptying followed by a s slower emptying phase.



(a) Simple exponential emptying, $f' = 2^{-\left(\frac{t}{t^{1/2}}\right)}$, *with* $t_{1/2}$ *= 30, 60, 90 min*



(b) Power exponential emptying, $f' = 2^{-\left(\frac{t}{t^{1/2}}\right)^{\beta}}$, *with* $t_{1/2}$ *= 10, 60 min,* β *= 0.5, 2*

Graph 1. Stomach delivery representations (a) $\beta = 1$, for subjects who have undergone ulcer operations, (b) $\beta < 1$, for liquid meals and $\beta > 1$, for solid meals

This formula offers a method for controlling the gastric and ileal delivery in the model, with only two parameters to describe the curve.

Digestion time

An important factor influencing the digestion time is the nature of the sample being tested. It is known that large food particles move through the stomach more slowly than smaller ones. Particles need to be small enough (<1mm) to pass through the pylorus valve separating the stomach and small intestine. A swallowed food containing large particles therefore requires a longer incubation time in the stomach.

The digestion time for each step (e.g., mouth, stomach, and small intestine) is an important factor to establish when designing an appropriate *in vitro* digestion model. *In vivo*, the digestion time depends upon individual characteristics (age, sex, health status, mental state, time of day) and food properties (total amount, composition, particle size, viscosity), and may vary quite considerably[22]. Lipids in the gastrointestinal tract delay the gastric emptying. Therefore, in the case of testing high-lipid food samples, enzymes (lipase or pancreatin) and bile salt/phospholipid amounts and digestion time should be increased in an *in vitro* digestion system. The transit time or digestion time must be considered according to the food characteristics[8].

In vitro-In vivo correlation

In vitro-in vivo correlations in digestion models are extremely important[23]. Validation of the developed *in vitro* digestion models for consumer products is difficult, because human *in vivo* data from consumer products with contaminants are scarce[16]. It was reported that the *in vitro* solubilization data correlated well with the *in vivo* data for lipid-based drug samples. However, several studies showed that *in vivo* feeding studies demonstrated large differences in the microstructure of emulsions as they pass through the gastrointestinal tract depending on emulsifier type[8].

- *Static models in relation to in vivo conditions*

Static models use a relatively dilute digestive mixture that is well homogenized using a stirrer, shaker or impeller. Although this does not reflect the mixing of gastric content *in vivo*, it exposes all substrates to the set point pH and related enzyme activities, and allows representative samples to be taken.

The complete meal with simulated gastric digestive fluid is exposed to a fixed pH during a fixed period. Generally, the gastric pH is maintained around 2, which may be the right value for the fasting state but does not reflect the pH after intake of a meal. Whether or not a static gastric digestion is adequate depends on the effect of each physiological parameter on the digestion and intended endpoint.

The omission of gastric lipase during the gastric step, might not be fully adequate for mimicking the complete process of gastrointestinal lipolysis as for example preliminary digestion of dietary triglycerides by gastric lipase is known to further trigger pancreatic lipase activity on lecithin-stabilized emulsions *in vitro*. In other cases, incubation at pH 2 during 1 h might lead to a complete peptic digestion, while this is not the case during a much milder exposure *in vivo*.

In the duodenum, the chyme that is gradually emptied from the stomach, is neutralized with bicarbonate, and mixed with bile and pancreatic juice. Bile is primarily important to emulsify fat and to form mixed micelles that solubilize and transport lipophilic products to the gut wall for absorption. During transit of approximately 3 h through the small intestine, substrates and enzyme to substrate ratios are changing due to the digestion and absorption of digestive products and water. The major drawback of small intestinal static models is that they do not include removal of digestive products during the digestion process, which may cause product inhibition of enzymes. This is generally overcome by using non physiological low substrate concentrations in a dilute system.

Limitations and challenges in modeling human gastric and small intestinal digestion

Despite their potential and broad applicability, gastric and small intestinal systems are hindered by their inability to mimic fully the overall processes occurring *in vivo*, particularly hormonal and nervous control, feedback mechanisms, mucosal cell activity, complexity of peristaltic movements, and involvement of the local immune system. Yet simulating the complexity of the human gastrointestinal tract remains a crucial challenge and scientific and technological efforts need to be joined to pursue improvement and validation of *in vitro* digestive tools.

The mayor limitations and challenges in modeling human digestion are given in Table 5.

Table 5. Major limitations and challenges in modeling human digestion[1]

Limitations	***Challenges***
No host response factors.	Combination of gastric and small intestinal models with human intestinal cells.
No accurate model of complex mechanical forces.	Improvement of peristalsis and gastrointestinal motility.
No accurate reproduction of complex gastric emptying pattern.	Differential gastric emptying of solids and liquids.
Absence of microbial ecosystem.	Integration of resident microbiota in the small intestinal compartments.
No simulation of the overall digestive process.	Serial combination of gastric and small intestinal models with masticatory and colonic systems.
Reproduction of healthy adults conditions by most of gastric and small intestinal models.	Development of age related or pathological gastric and small intestinal models.
Biological significance of *in vitro* experiments.	Crucial need for *in vitro-in vivo* correlations.

To simulate digestion process more accurately, a combinatorial approach involving *in vitro* models and intestinal microorganism in culture has been proposed. This approach, which integrates active and facilitated transport processes as well as brush border enzyme activities, has already been used for a more relevant prediction of nutrient, drug, and food pollutant bioavailability. Recent studies have also investigated the effect of *in vitro* digesta on intestinal cell proliferation[24] and inflammatory pathways to assess the potential anti-carcinogenic or anti-inflammatory properties of ingested substances.

So far, gastric and small intestinal systems have mainly mimicked physicochemical conditions of digestive lumen and gastrointestinal transit, and reproduce real forces in play during digestion less accurately. Physiological contractions are difficult to simulate due to high complexity in frequency and strength. In most of the current models, chyme is simply mixed with an impellor, a magnetic stirrer or a shaking bath, which does not allow any assessment of the importance of mechanical forces on food or dosage form disintegration[25]. Hence, one of the major challenges in *in vitro* digestion is to simulate as accurately as possible the peristalsis and realistic shape and motility of gastric and small intestinal compartments.

Interestingly, newly developed gastric and small intestinal models should also reproduce the biphasic gastric emptying curves observed *in vivo*, where emptying of solid food components presents a linear pattern starting after a lag phase, whereas emptying of liquids begins immediately in an exponential manner.

In addition, gastric and small intestinal models are currently devoid of intestinal microorganisms. With the growing interest in assessing interactions between ingested compounds and resident microbiota and the great potential of '-omics' technologies, another major advance would be to inoculate the resident microbiota in the small intestinal compartments of gastric and small intestinal models.

Although multi-compartmental systems have been developed, none of them includes all the steps from mouth to large intestine. The SHIME (Simulator of Human Intestinal Microbial Ecosystem) integrates the whole gastrointestinal tract from the stomach to colon but has been more specifically design to study the interactions of food components with human resident microbiota. Oral processing is particularly difficult to simulate, and most *in vitro* studies lack this step or use an oversimplified homogenization procedure. As bolus properties strongly impact the gastric phase and subsequent steps in digestion, it is critically important to use a bolus with relevant physicochemical and textural properties during *in vitro* digestions.

Therefore, in a more holistic view of the human digestion process, gastric and small intestinal systems should be used in combination with models mimicking the oral phase and large intestinal conditions. An ultimate approach would be to develop a model integrating all the stages of digestion.

A major concern for *in vitro* models is providing an accurate estimation of the *in vivo* situation. As a result of the great complexity of the human gastrointestinal tract, none of them can truly replace *in vivo* experiments and a careful interpretation of results is always required.

In vitro/*in vivo* correlations, which reliably associates *in vitro* and *in vivo* data, remains a high priority to validate *in vitro* results. In addition, comparison between *in vitro* systems is complicated and it is difficult to ascertain which of the current models provides the most accurate values in terms of the human situation. Hence, selection of the most appropriate model requires careful evaluation of the study objectives to assess the advantages and limitations afforded by each type of system, and a compromise between technical complexity and physiological relevance has often to be made[1].

2.3. Enzymes

The most frequently utilized enzymes and other biological molecules used within *in vitro* digestion models were pepsin, pancreatine, trypsin, chymotrypsin, peptidase, α-amylase and lipase[8].

The types of enzyme included within an *in vitro* digestion model tend to reflect the major food components being investigate. For example, to study the lipid digestion in oil-in-water emulsions, researches utilized only pancreatic lipase[26].

It should be noted that different enzymes are usually added sequentially, rather than all together, so as to stimulate the different steps of the digestive process. It should also be noted that enzymes often require additional components within the digestive fluids to operate efficiently, for example pancreatic lipase requires the presence of calcium and bile salts[5].

Finally, it should be noted that the activity of an enzyme preparation may decrease over time (enzymes when dissolved into solution are much less stable than in powder form and lose their activity), and so it is important to prepare them freshly for each study.

The concentration and composition of enzymes are also very important factors to consider when designing *in vitro* digestion models. The levels of enzymes depend on the mental state, age and health status, the time of day the food is consumed, and the type and amount of food consumed[8].

Therefore, several factors, such as concentration, temperature, pH, stability, activators, inhibitors, and incubation time, affect enzyme activities[5].

Digestion of the major foodstuffs is an orderly process involving the action of a large number of digestive enzymes. Some of these enzymes are found in the secretions of the salivary glands, the stomach and the exocrine portion of the pancreas. Other enzymes are found in the luminal membranes and the cytoplasm of the cells that line the small intestine. The action of some enzymes is aided by the hydrochloric acid secreted by the stomach and the bile secreted by the liver.

Table 6 gives an overview of the principal digestive enzymes.

Table 6. Principal digestive enzymes ((the corresponding pro-enzymes are shown in parentheses)[27]

Source	***Enzyme***	***Activator***	***Substrate***	***Catalytic function or products***
Salivary glands	Salivary α-amylase	Cl^-	Starch	Hydrolyzes 1,4α linkages, producing α-limit dextrins, maltotriose and maltose
Stomach	Pepsin (*pepsinogen*)	HCl	Proteins and polypeptides	Cleave peptide bonds adjacent to aromatic aminoacids

Source	*Enzyme*	*Activator*	*Substrate*	*Catalytic function or products*
Exocrine pancreas	Trypsin (*trypsinogen*)	Enteropeptidase	Proteins and polypeptides	Cleaves peptides bonds adjacents to arginine or lysine
	Chymotrypsins (*chymotrypsinogen*)	Trypsin	Proteins and polypeptides	Cleave peptide bonds adjacent to aromatic aminoacids
	Pancreatic lipase		Triglycerides	Monoglycerides and fatty acids
	Pancreatic α-amylase	Cl-	Starch	Hydrolyzes 1,4α linkages, producing α-limit dextrins, maltotriose and maltose
	Ribonuclease		RNA	Nucleotides
	Deoxyribonuclease		DNA	Nucleotides
	Phospholipase A (*prophospholipase A*)	Trypsin	Lecithin	Lysolecithin

Glucosidases

- *Alpha-amylase*

Amylase is present in the mouth and small intestine and is mainly responsible for the conversion of starches to oligosaccharides and monosaccharides (e.g. glucose). Amylase is routinely used for *in vitro* digestion studies of plant-based food samples.

Saliva plays a vital role in maintaining the health of the oral cavity and gastrointestinal tract by aiding in lubrication, inhibiting potentially harmful microbes, and promoting oral tissue healing. Whether saliva also plays an important role in the digestion and metabolism of food is currently unknown. The presence of high concentrations of the enzyme α-amylase has led to the hypothesis that saliva could be important for the digestion of complex carbohydrates[8].

Amylase is a digestive enzyme produced by the salivary glands and pancreas that cleaves the glycosidic linkages in starch molecules to produce smaller saccharides, such as maltotriose, maltose, and small amounts of glucose.

Figure 6 gives the mechanism of alpha-amylase with starch.

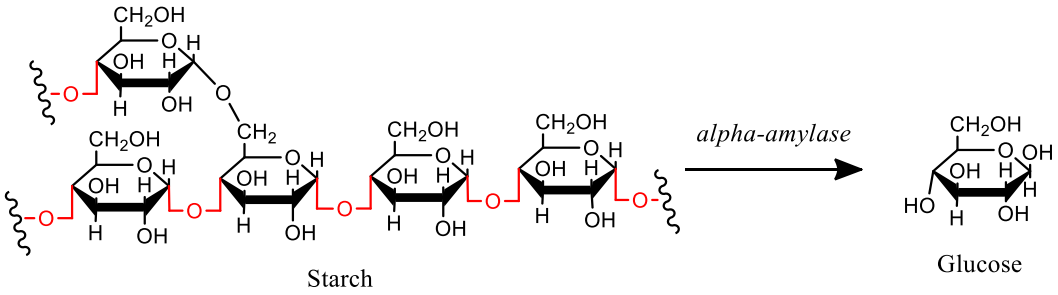


Figure 6. Reaction between alpha-amylase and starch

Salivary amylase can account for up to 50% of total salivary protein in some individuals, whereas others produce barely detectable concentrations. Such substantial variation in amylase production is due to both environmental (e.g., stress) and genetic factors, such as copy number variation in AMY1[40], the gene that codes for salivary amylase.

It is known that considerable starch hydrolysis occurs within s in the oral cavity and can also continue after swallowing, because partially digested starch protects salivary amylase from acid inactivation[28]. *In vivo* digestion studies demonstrate that delivery of starch directly into the small intestine, thereby skipping the oral digestion stage, results in substantially less starch digestion and glucose absorption. In addition, postprandial blood glucose concentrations following ingestion of starchy foods, such as rice and potatoes, are lower when the food is swallowed whole, rather than chewed first, mixed with saliva, and then swallowed[29].

Two similar types of amylase are made in human body, one is secreted in saliva, where it starts to break down starch grains when it chewed, and the other is secreted by the pancreas, where it finishes starch digestion. Then, these little pieces are broken into individual glucose units by a collection of enzymes that are tethered to the walls of the intestine.

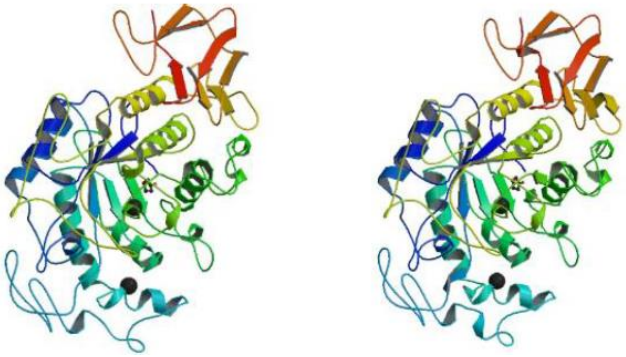


Figure 7. Structure of alpha-amylase (a) pancreatic (b) saliva

The active site of alpha-amylase contains a trio of acidic groups (colored white and red) which they are primarily responsible for the digestion of starch. In the amylase shown here (Figure 8) glutamate 233, aspartate 197, and aspartate 300 work together to cleave the connection between two sugars in a starch chain. This structure contains a short chain of five sugar units (colored yellow and orange) bound in the active site.

The site of cleavage is shown in pink. A calcium ion, shown as a large gray sphere, is found nearby where it stabilizes the structure of the enzyme. A chloride ion, shown as a green sphere, is bound underneath the active site in many amylases, where it may assist the reaction[30].

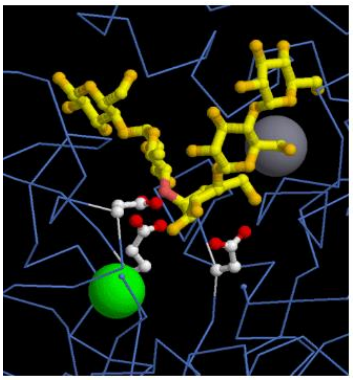


Figure 8. Active site of alpha-amylase[31]

The generally accepted catalytic mechanism of the α-amylase family is that of the α-retaining double displacement (each step passes through an oxocarbonium ion-like transition state). The mechanism of alpha-amylase involves two catalytic residues in the active site; a glutamic acid as acid/base catalyst and an aspartate as the nucleophile. It involves five steps:

- After the substrate has bound in the active site, the glutamic acid in the acid form donates a proton to the glycosidic bond oxygen, i.e. the oxygen between two glucose molecules at the subsites −1 and +1 and the nucleophilic aspartate attacks the C1 of glucose at subsite −1.
- An oxocarbonium ion-like transition state is formed followed by the formation of a covalent intermediate.
- The protonated glucose molecule at subsite +1 leaves the active site while a water molecule or a new glucose molecule moves into the active site and attacks the covalent bond between the glucose molecule at subsite −1 and the aspartate.
- An oxocarbonium ion-like transition state is formed again.
- The base catalyst glutamate accepts a hydrogen from an incoming water or the newly entered glucose molecule at subsite +1, the oxygen of the incoming water or the newly entered glucose molecule at subsite +1 replaces the oxocarbonium bond between the glucose molecule at subsite −1 and the aspartate forming a new hydroxyl group at the C1 position of the glucose at subsite −1 (hydrolysis) or a new glycosidic bond between the glucose at subsite −1 and +1 (transglycosylation).

Studies with cyclodextrin glycosyltransferase have shown that the intermediate indeed has a covalently linked bond with the enzyme[32].

Proteases

Proteases are mainly present in the stomach (pepsin) and small intestine (trypsin and chymotrypsin) where they are responsible for breaking down proteins/peptides into smaller peptides and amino acids. The daily pepsin secretion in adults is 20–30 kU of enzyme activity at 37°C, equivalent to around 10 mg (of pepsin), while a typical adult dietary intake of protein comprises around 75 g/24 h, giving a pepsin/protein ratio of 1:7500.

Abdel-Aal (*2008*)[33] found that the three-enzyme (trypsin, chymotrypsin, and peptidase) one-step digestion gave approximately 39–66% higher protein digestibility than that obtained by the two-enzyme (pepsin and pancreatin) two-step digestion method depending on the type of product and the method used for determining protein hydrolyzates. Therefore, Abdel-Aal (*2008*) suggested that the three-enzyme digestion method is more comparable to *in vivo* conditions. Thus, they assume that *in vitro* digestion methods that use complex enzymes (e.g., a mix of saliva, gastric juice, duodenal juice or bile juice) have the advantage of being more reproducible than those that use single enzymes. Therefore, enzyme composition and concentrations may be influenced by the characteristics of the sample. Several studies showed that the number and type of proteolytic enzymes, digestion conditions, and analysis of protein hydrolyzates employed in *in vitro* digestion produced different digestibility results (*Abdel-Aal, 2008*). An increase in dietary protein induces an increased secretion of pancreatic proteolytic enzymes, while an increase in starch or lipid intake induces may increase secretions of amylase and lipase, respectively.

- *Pepsin*

Pepsin is an enzyme belonging to the family of aspartic protease enzymes. All members of this class of enzymes have two aspartic acid residues within their structure that act as the active site.

For the most part, this class of enzymes is active at acid pH. In the case of pepsin, the pH of optimal activity is extremely acid, between 1 and 4. The specific reaction catalyzed by pepsin is the acid hydrolysis of the peptide bond. This reaction will break down proteins into smaller units to enable the digestive process.

Pepsin demonstrates an unusual property for an enzyme; it does not actually form chemical bonds with its substrate. The unique aspect of the pepsin mechanism is the ability of the two aspartic acids at the reaction site to simultaneously act as both an acid and a base.

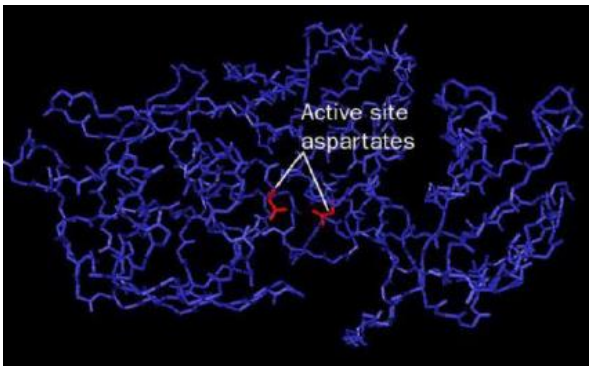


Figure 9. Active site of pepsin

The backbone structure above (Figure 9) has been adjusted to illustrate the location of Asp32 and Asp215. The general structure consists of one amino acid strand that folds itself into two, almost identical lobes.

Pepsinogen (Figure 10) is similar in composition to pepsin but it contains 44 additional amino acid residues that prohibit the enzyme reactive site from functioning. The acid conditions of the stomach cause the pepsinogen to alter its structure and become the activated enzyme, pepsin. The top space-filing model below represents pepsinogen. The 44 residue chain is colored in green. This effectively masks the active site of pepsin. Once the active site is cleared, the enzyme can begin its work on protein cleaving.

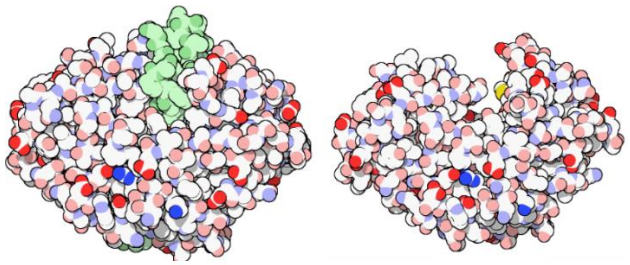


Figure 10. Structure of pepsinogen and pepsin

The reaction mechanism for the catalyzed hydrolysis of a peptide bond by pepsin:

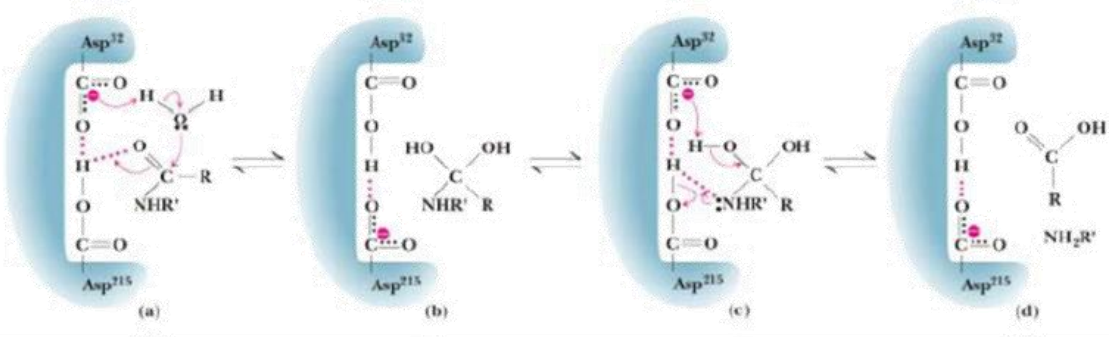


Figure 11. Mechanism of reaction of pepsin[34]

In Figure 11 (a), the carboxyl ends of the aspartic acid residues at 215 and 32. Even though they have the same formula, they have different pKa values. This is due to the different residues surrounding each aspartic acid.

In Figure 11 (a), the Asp32 is deprotenated while Asp215 still retains its proton. Water will nucleophillically attack the carbonyl carbon of the substrate while Asp32 accepts a proton from the water and Asp215 donates a proton to the carbonyl carbon of the substrate. The intermediate (Figure 11 (b)) that is formed is called an amide dehydrate. This intermediate accepts a proton from Asp215. This action cleaves the peptide bond in the substrate. The final step of the mechanism, not pictured in the diagram above is one in which the proton on Asp32 is transferred to Asp215[34].

- *Trypsin*

Trypsin is a proteolytic enzyme, important for the digestion of proteins. In humans, the protein is produced in its inactive form, trypsinogen, within the pancreas. Trypsinogen enters the small intestine, via the common bile duct, where it converted to active trypsin.

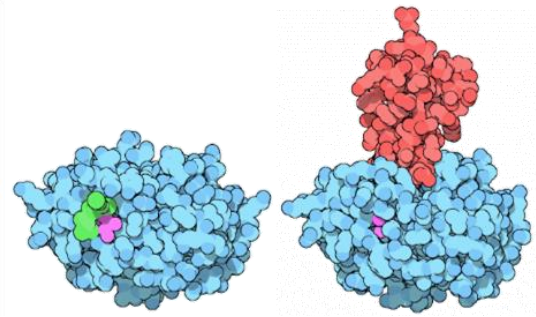


Figure 12. Structure of trypsinogen and trypsin with trypsin inhibitor (red)[35]

The digestion of proteins is a complex process. First it is created the enzyme in an inactive form (termed a zymogen), and it is activated once it is in the intestine. Trypsin is built with an extra piece of protein chain, colored in green in the structure on the left (Figure 12). This longer form of trypsin, called trypsinogen, is inactive and cannot cut protein chains. Then, when it enters the intestine, the enzyme entero-peptidase makes one cut in the trypsin chain.

The pancreas produces a small protein, trypsin inhibitor (shown in red), that binds to any traces of active trypsin that might be present before it is secreted into the intestine. It binds to the active site of trypsin, blocking its action but not itself being cut into tiny pieces.

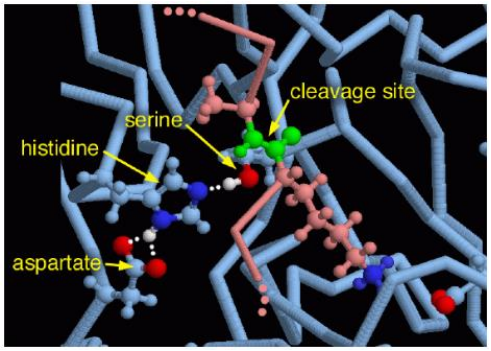


Figure 13. Active site of trypsin[35]

The active site (Figure 13) is formed by a serine amino acid that is activated by a histidine and an aspartate. Together, these three amino acids have been termed the charge relay system. The histidine and the aspartate assist in the removal of the hydrogen atom from the serine (colored white), which makes it more reactive when attacking the target protein chain. The site of cleavage in this inhibitor, colored green here, is held just far enough away that it is not cleaved the way most proteins would be in this location. Notice also the long lysine amino acid extending down to the lower right from the cleavage site, where it interacts with another aspartate in the enzyme (shown down in the lower right corner with red oxygens). Through this interaction, trypsin favors cutting at places next to lysine or arginine amino acids[36].

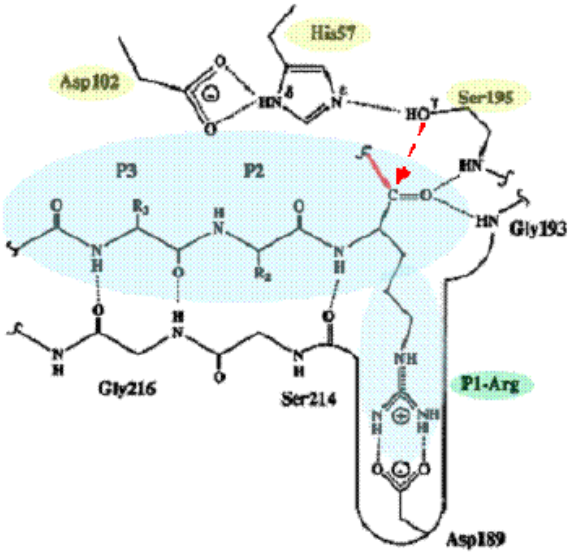


Figure 14. A schematic representation of trypsin interacting with a peptide substrate

The catalytic residues (His57, Asp 102 and Ser195, yellow) and the enzyme residues that contact substrate residues are shown (blue). The positively charged arginine side chain at position P1 of the substrate is attracted by the negatively charged aspartate 189. This interaction as well as five enzyme-substrate hydrogen bonds at positions P1 and P3 and glycine 193 help to position the scissile peptide bond (red) for the nucleophilic attack by the polarized hydroxyl group of Ser 195 (red arrow)[37].

Trypsin is a protein that degrades proteins, therefore it can degrade itself: a process called autolysis. Autolysis is important for the regulation of trypsin levels within living organisms. This regulation is assisted by Ca^{2+} ions, which bind to trypsin (at the Ca^{2+} binding loop) and protect the molecule against autolysis. In living organisms, autolysis is controlled and normally does not cause problems. However, when working with trypsin *in vitro*, the process of autolysis often poses some problems. For *in vitro* processes that require the use of trypsin, such as working with cell cultures or manufacturing insulin, trypsin's degradation can become expensive as active trypsin gets "used up". Developing mutant trypsin that does not auto-degrade could be of great use for researchers.

There are several sites on the trypsin molecule at where autolysis is known to occur. Research has been done to investigate these sites, because the inability of trypsin to self-degrade has been linked to human hereditary pancreatitis. This deadly disease is believed to occur due to inappropriate activation of trypsin within the pancreas. This results in the autodigestion of pancreatic tissue.

Trypsin cleaves a terminal hexapeptide from trypsinogen to yield a single-chain beta-trypsin. Subsequent autolysis produces other active forms having two or more peptide chains. The two predominant forms of trypsin are alpha-trypsin, which has two peptide chains bound by disulfide bonds, and beta-trypsin[38].

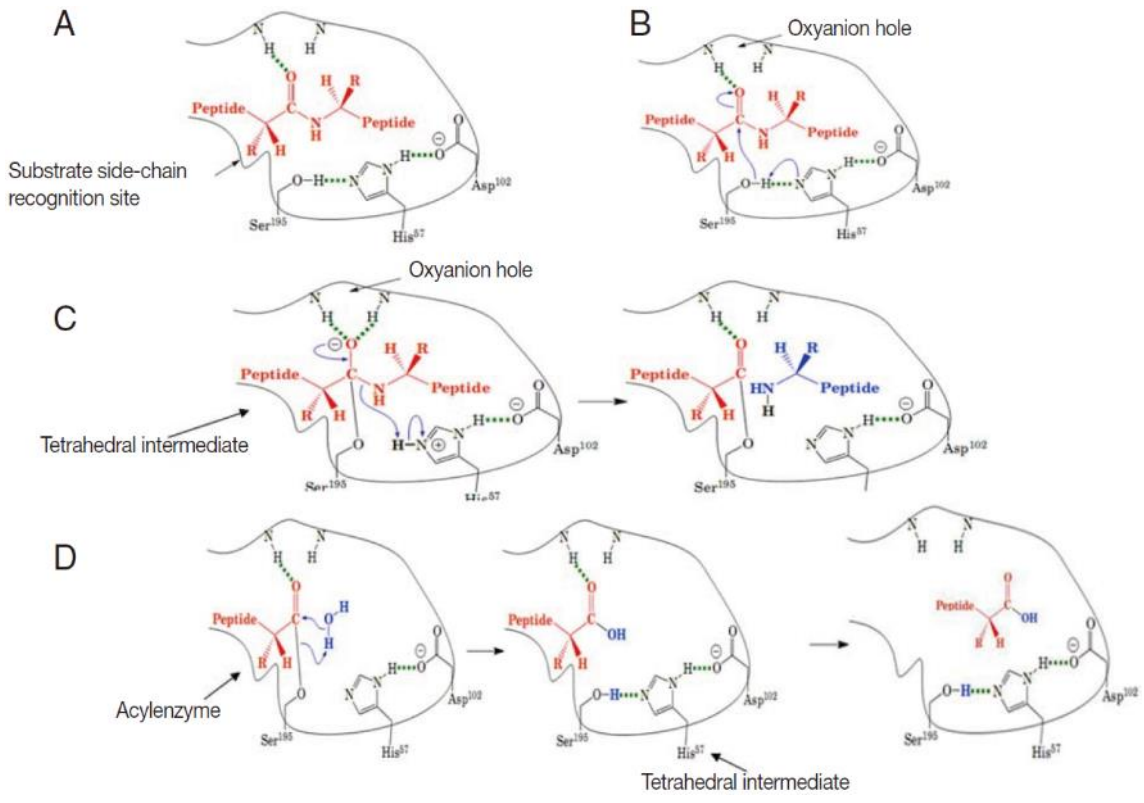


Figure 15. Mechanism of reaction of trypsin[39]

The above figure (Figure 15) is a schematic illustration of general catalytic mechanism for serine proteases (like trypsin).

(A) *Substrate binding:* substrate binds to the recognition site of the serine protease and exposes the carbonyl of the scissile amide bond.
(B) *Nucleophilic attack:* His 57 attracts the proton from the hydroxyl group of Ser 195 and the Ser 195 attacks the carbonyl of the peptide substrate.
(C) *Protonation:* The amide of peptide subtract accepts a proton from His 57 and dissociates.
(D) *Deacylation:* water molecule attacks the acyl-enzyme complex and catalytic triad is restored[39].

Lipases

Lipases are present in the stomach (gastric lipase) and pancreas (pancreatic lipase), where they absorb to the surfaces of emulsified lipids and convert tri-acylglycerols and di-acylglycerols to mono-acylglycerols and free fatty acids.

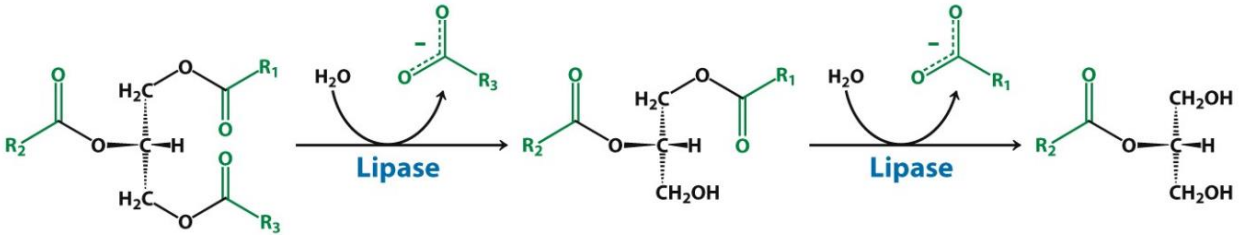


Figure 16. Reaction of lipase with triacylglycerol

These lipid digestion products are these solubilized within mixed micelles and vesicles that transport them to the epithelium cells through the mucous layer. The activity of pancreatic lipase depends on the presence of co-lipase, bile salts, and calcium. Pancreatic lipase has an absolute requirement for Ca^{2+}, which binds in a stoichiometric ratio of 1:1 to the lipid substrate and the enzyme. Calcium reacts with liberated free fatty acids by means of ionic complexation, thereby removing them from the surface of the lipid droplets and preventing them from inhibiting the lipase. It is also reported that when calcium is added at the start of the lipolysis, it results in a very fast initial lipolysis rate followed by a leveling out at longer times, which was attributed to product inhibition by free fatty acids and possibly precipitation of bile salts with calcium. It has therefore been proposed that it is better to add calcium continuously throughout the *in vitro* digestion process, rather than adding it all at the beginning.

The bile salts and phospholipids are surface active molecules that adsorb to droplet surfaces and displace any existing emulsifier molecules. This change in interfacial composition can facilitate the subsequent adsorption of the lipase-co-lipase complex to the lipid droplet surfaces.

Bile salts and phospholipids also form mixed micelles and vesicles in the aqueous phase, which are capable of incorporating lipid digestion products and removing them from the lipid droplet surfaces. It is therefore important to include the appropriate amounts of lipase, co-lipase, bile salts, phospholipids and calcium in an *in vitro* digestion model for lipid digestion. As a response to the intake of a meal, bile is secreted into the duodenum, and in the fed state, the mean bile salt concentrations in human duodenal and jejunal fluids are between 8 mM and 12 mM. The lipid hydrolysis rate is influenced by bile salt and lipase concentrations.

Digestive enzymes

The most appropriate composition and concentration of enzymes, such as lipase, pepsin, trypsin, and a-amylase, used within an *in vitro* digestion model must be considered for each specific food sample. As mentioned above, several studies have utilized enzymes collected from human subjects.

However, several studies have suggested that the replacement of human pancreatic lipase and co-lipase with porcine pancreatic lipase and co-lipase is generally acceptable. Thus, it may be very difficult to define which enzymes are better for *in vitro* digestion, and more research is needed in order to analyze the advantages and disadvantages of using enzymes from human subjects.

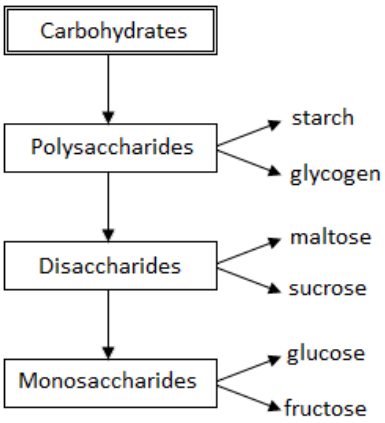
Various *in vitro* methods have been developed to predict the digestibility or physiological changes of food samples. However, predicting the bioavailability and digestion from the food matrix is very difficult, as it depends on many factors associated with food composition and structure. Usually, *in vitro* methods are based upon starch digestion by a-amylase, lipid digestion by lipase, and/or protein digestion by pepsin or trypsin. Gastric digestion is imitated using pepsin at pH around 2. The protease precursors – pepsinogens – produced by chief cells of the stomach, are optimally activated at a pH between 1.8 and 3.2 in the gastric lumen. This indicates that any elevation in the pH may result in a limitation of peptic degradation. Bile salt did not inhibit the lipolytic activity at pH 5.5. Moreover, the changes in the pH in the stomach and intestine can be influenced by the initial pH or amount of the samples tested. Thus, pH is also an important factor for *in vitro* digestion systems. Therefore, the choice of enzyme characteristics such as composition, concentration, and pH should be considered according to sample characteristics[8].

2.4. Starch digestion

Starch is the main source of digestible carbohydrate in the human diet and as such, is the major source of glucose that appears at relatively high concentrations in the blood circulation following intestinal digestion of a starch-containing meal. The first stage in the metabolism of starch is catalyzed by salivary alpha-amylase which progressively brings about hydrolysis of the polysaccharide resulting in the production of maltose, maltotriose and limit dextrins as the main products. Considerable differences, however, can occur in the postprandial blood glucose and the corresponding insulin response, to the ingestion of different foods containing identical amounts of starch. That such differences occur is evidence of large variations in the rate and extent of starch digestion in the gastrointestinal tract. Attenuating the fluctuations in postprandial glycaemia and insulinaemia is important in the prevention and treatment of life-style associated diseases, notably diabetes mellitus and cardiovascular disease, and also has implications for obesity management[40].

A considerable interest exists for foods containing slow release carbohydrate from the gastrointestinal tract because they contribute to improve diabetic control and reduce serum lipid levels[41].

The principal dietary carbohydrates (Flow chart 1) are polysaccharides, disaccharides, and monosaccharides. Starches (glucose polymers) and their derivatives are the polysaccharides that are digested to any degree in the human gastrointestinal tract. In glycogen, the glucose molecules are mostly in long chains (glucose molecules in α 1,4 linkage), but there is some chain branching (produced by α 1,6 linkages). Amylopectin, which constitutes 80-90% of dietary starch, is similar but less branched, whereas amylose is a straight chain with only α 1,4 linkages. Glycogen is found in animals, whereas amylose and amylopectin are of plant origin. The disaccharides lactose (milk sugar) and sucrose (table sugar) are also ingested, along with the monosaccharides fructose and glucose.



Flow chart 1. Types of carbohydrates

Deficiency of disaccharidases leads to diarrhea, bloating, and flatulence after ingestion of sugar. The diarrhea is due to the increased number of osmotically active oligosaccharide molecules that remain in the intestinal lumen, causing the volume of the intestinal contents to increase. The bloating and flatulence are due to the production of gas (CO_2 and H_2) from disaccharide residues in the lower small intestine and colon.

Studies on the digestion of carbohydrates and consequent glucose plasma levels are important for diabetic patients, obesity control and designing sport foods. As an alternative to expensive and time consuming human trials, a rapid *in vitro* method has been developed to predict the glycemic response after intake of carbohydrates (*Bellmann et al. 2010*)[42].

Absorption of carbohydrates

Hexoses (monosaccharide with six carbon atoms) and pentoses (monosaccharide with five carbon atoms) are rapidly absorbed across the wall of the small intestine. Essentially all of the hexoses are removed before the remains of a meal reach the terminal part of the ileum. The sugar molecules pass from the mucosal cells to the blood in the capillaries draining into the portal vein.

The transport of some sugars (monosaccharides such as glucose) is uniquely affected by the amount of Na^+ in the intestinal lumen; a high concentration of Na^+ on the mucosal surface of the cells facilities and a low concentration inhibits sugar influx into the epithelial cells. It now appears that glucose and Na^+ share the same carrier molecule. Intracellular Na^+ is low, and Na^+ moves into the cell along its concentration gradient. Glucose moves with the Na+ and is released in the cell.

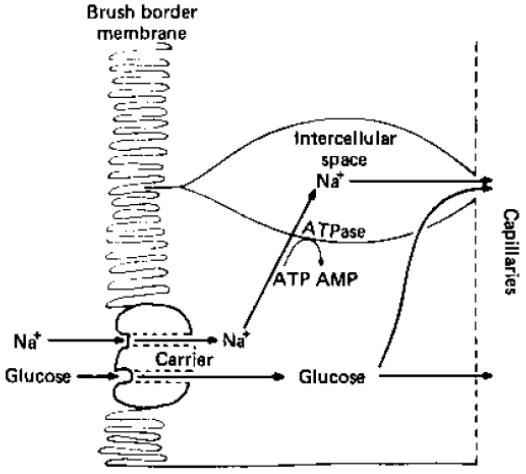


Figure 17. Mechanism for glucose transport across intestinal epithelium

The Na^+ is transported into the lateral intercellular spaces, and the glucose diffuses into the interstitium and hence to the capillaries. Thus, glucose transport is an example of sary active transport, the energy for glucose transport is provided indirectly, by the active transport of Na^+ out of the cell. This maintains the concentration gradient across the luminal border of the cell, so that more Na^+ and consequently more glucose can reach the intracellular. The glucose mechanism also transports galactose. Fructose utilizes a different carrier, and its absorption is independent of Na^+ or the transport of glucose and galactose; it is transported instead by facilitated diffusion. Some fructose is converted to glucose in the mucosal cells.
Pentoses are absorbed by simple diffusion[27].

Diabetes Mellitus

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action or both. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels.

Several pathogenic processes are involved in the development of diabetes. These range from autoimmune destruction of the β-cells of the pancreas with consequent insulin deficiency to abnormalities that result in resistance to insulin action. The basis of the abnormalities in carbohydrate, fat and protein metabolism in diabetes is deficient action of insulin on target tissues. Deficient insulin action results from inadequate insulin secretion and diminished tissue responses to insulin at one or more points in the complex pathways of hormone action.

Impairment of insulin secretion and defects in insulin action frequently coexist in the same patient, and it is often unclear which abnormality, if either alone, is the primary cause of the hyperglycemia.

The vast majority of cases of diabetes fall into two broad etiopathogenetic categories. In one category, type I diabetes, the cause is an absolute deficiency of insulin secretion. Individuals at increased risk of developing this type of diabetes can often be identified by serological evidence of an autoimmune pathologic process occurring in the pancreatic islets and by genetic markers. In the other, much more prevalent category, type II diabetes, the cause is a combination of resistance to insulin action and an inadequate compensatory insulin secretory response. In the latter category, a degree of hyperglycemia sufficient to cause pathologic and functional changes in various target tissues, but without clinical symptoms, may be present for a long period of time before diabetes is detected. During this asymptomatic period, it is possible to demonstrate an abnormality in carbohydrate metabolism by measurement of plasma glucose in the fasting state or after a challenge with an oral glucose load[43].

As it mentioned in section 1 of this thesis, one of the objectives is the study of glucose released from three different sources of carbohydrates, that they are used in some products of clinical nutrition. And in this section the importance of knowing the blood glucose levels is justified.

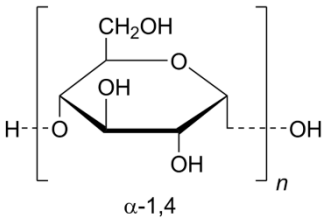
3. MATERIALS AND METHODS

Chemical products used were acquired commercially (Sigma Aldrich, Merck, Pfizer, Zoetis, …).

3.1. Preparation of model systems

Three carbohydrate sources were selected:

- Maltodextrin DE 11 – 16: is produced by enzymatic hydrolysis of maize-starch.

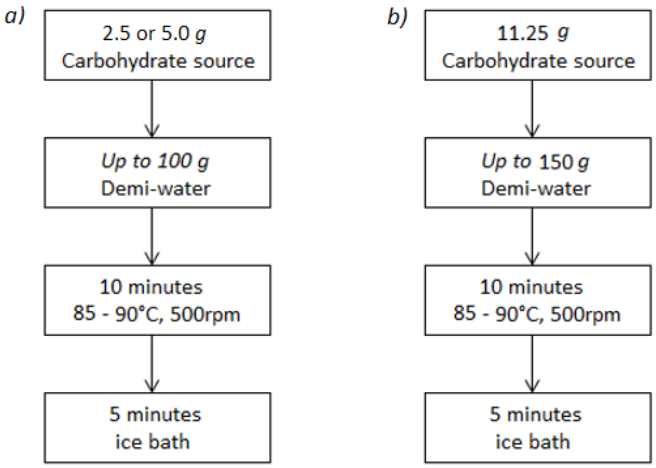


$11 < n < 16$

Figure 18. Structure of maltodextrin DE 11-16

- Tapioca Dextrin: is a high stability dextrin refined from tapioca starch (high hygroscopic).
- "Modified starch": chemically modified food starch refined from tapioca (E1442).

Samples were prepared using the following protocol:



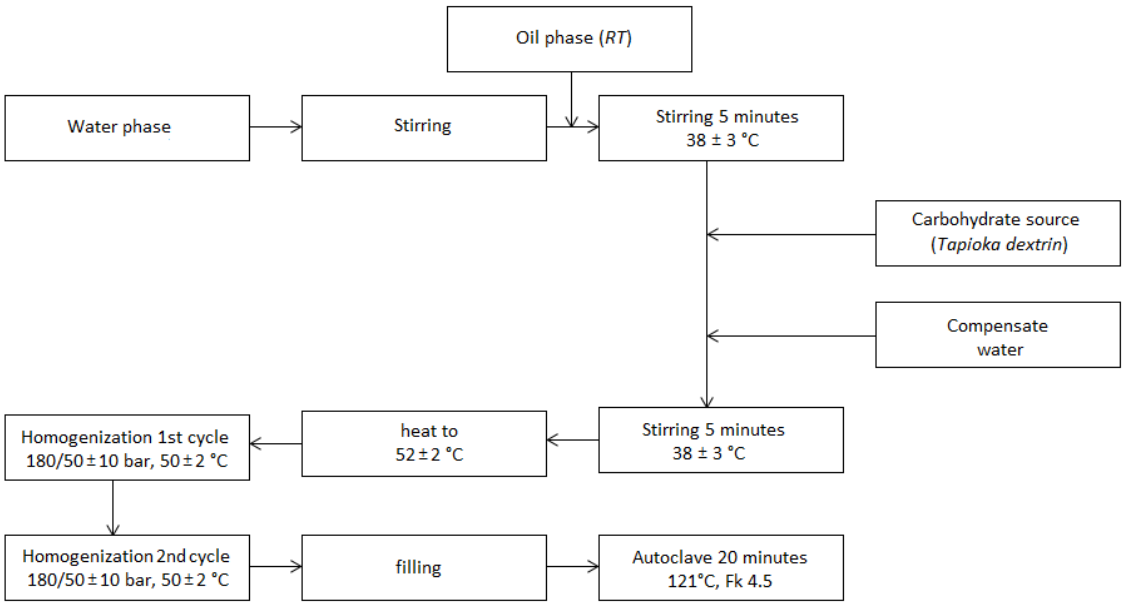
Flow chart 2. Sample preparation protocol (a) Static digestion method (b) Dynamic digestion method

The effect of volume of samples was also studied, for the static method. The same proportion were used, but with reduced final volume (1g of tapioca dextrin in 20mL of demi-water).

Besides these samples, three other samples were prepared. One of these samples was prepared in different conditions in order to compare how different preparation conditions affects. And the other two samples were prepared in different matrix (one with proteins, and the other with fat and proteins) in order to study how the matrix of the carbohydrate, affects starch digestibility.

Table 7. Description of different matrix/conditions

	Water phase	***Oil phase***	***Carbohydrate source***
Conditions 1 (CH)	925 g Demi-water Heating to 38°C		75 g Tapioca Dextrin
Conditions 2 (CH + protein)	882 g Demi-water 43 g Milk protein concentrate Stirring 20 min at 38°C		75 g Tapioca Dextrin
Conditions 3 (CH + protein + fat)	847.2 g Demi-water 43 g Milk protein concentrate Stirring 20 min at 38°C	34 g Rapeseed oil refined 0.8 g Lecithin Pre-solve at RT	75 g Tapioca Dextrin



Flow chart 3. Sample preparation protocol (effect of matrix)

3.2. Determination of enzyme activities[44]

The enzyme activities determination is essential to calculate the quantity of enzymes needed to achieve a certain activity during the *in vitro* digestion assays.

There are many methods to determine the enzyme activity, and each method uses different enzyme activity units (depending on the substrate, temperature, pH...). The protocol that Minekus et al (2014) proposed was used.

For each enzyme used in this protocol, the respective activity was measured and calculated as follows.

Trypsin Activity Assay (*EC 3.4.21.4*)

The principle of this assay is the following reaction:

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

Where one unit hydrolyses one µmole of p-toluene-sulfonyl-L-arginine methyl ester (TAME) per min at 25°C and pH 8.1.

A 46mM of TRIS/HCl buffer, containing 11.5mM $CaCl_2$ was prepared and adjusted its pH at 8.1 at 25°C. A solution of 10mM TAME (p-toluene-sulfonyl-L-arginine methyl ester) was prepared in purified water. Enzyme solutions with two concentrations ranging between 1-2 mg/mL were prepared in cold 1mM HCl.

A volume of 2.6mL of 46mM Tris/HCl buffer (pH 8.1) and 0.3mL of the substrate (10mM TAME) was added to a quartz cuvette, mixed by inversion and left for 3-4 min in the spectrophotometer at 25°C for temperature equilibration.

A volume of 100µL of enzyme solutions for each concentration (or 1mM HCL for the blank) was added to each cuvette (with Tris/HCl – TAME mix) and the kinetics was measured in the spectrophotometer at 247nm, 25°C during 10 min with measurements each 30 s.

The activity was then calculated using the slopes ΔA_{247} (unit absorbance/min) using the maximum linear rate and over at least 5 min.

$$\frac{units}{mg} = \frac{\left(\Delta A_{247}(test) - \Delta A_{247}(blank)\right) * 1000 * 3}{540 * X}$$

Equation 2. Trypsin activity (U/mg)

ΔA_{247}: Slope of the initial linear portion of the curve for the test and blank (unit absorbance/min).
540: Molar extinction coefficient of TAME at 247nm (L/(mol*cm)).
3: Volume of reaction mix (mL).
***X*:** Quantity of enzyme in the final reaction mixture, quartz cuvette (mg).
1000: Factor to equalize the units.

Pepsin Activity Assay (*EC 3.4.23.1*)

The principle of this assay is the following reaction:

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

Where one unit will produce a ΔA_{280} of 0.001 per min at pH 2.0 and 37°C, measured as TCA-soluble products (these units are often referred to "Sigma" or "Anson").

A 2% w/v hemoglobin (bovine blood hemoglobin, ref H2500 Sigma-Aldrich) solution was prepared by dispersing 0.5g hemoglobin in 20mL demi-water and then this solution was diluted in 5mL of 300mM HCl in order to get a solution at 2% w/v hemoglobin at pH 2.0.

Enzyme solution of 1.22mg/mL was prepared in 150mM NaCl (pH 6.5). Just before the assay, a range of 5 – 30 µg/mL was prepared in 10mM HCl.

A volume of 500µL of hemoglobin solution was added into 2mL Eppendorf, and incubated in shaking incubator at 37°C for 3-4 min to achieve temperature equilibration.
A volume of 100µL of pepsin solutions for each concentration was added to each Eppendorf, and incubated 10 min exactly. After that a volume of 1mL of 5% TCA (trichloroacetic acid) was added in each Eppendorf to stop the reaction (Charles Tanford proposed that TCA forces protein to precipitate by sequestering the protein-bound water[45]).

The tubes were then centrifuged at 6,000g for 30 min to get a clear soluble phase available for absorbance measurement. Finally, the supernatant was pipetted into quartz cuvettes and the absorbance was measured at 280nm. For blank test, the same procedure was followed but the enzyme was added after the addition of TCA.

The activity was then calculated using the following equation:

$$\frac{Units}{mg} = \frac{\left(A_{280}(test) - A_{280}(blank)\right) * 1000}{\Delta t * X}$$

Equation 3. Pepsin activity

Δt: Duration of the reaction (i. e. 10 min).
X: Concentration of enzyme powder in the final reaction mixture (quartz cuvettes, mg/mL).
1000: Factor to equalize the units.

α-Amylase Activity Assay (*EC 3.2.1.1*)

The type of alpha-amylase used during digestion experiments was a bacterial one, but Minekus et al (2014)[46] proposed using human alpha-amylase. The optimal temperature for bacterial and human alpha-amylase are different, for this reason, alpha-amylase activity was measured in different ways.

To know the factor between human and bacterial alpha-amylase, a commercial kit was used (KA1643, Abnova). But to determine the alpha-amylase activity in the correct units, it was used the protocol that Minekus et al (2014)[46] proposed.

The principle of this assay is the following reaction:

$$Starch + H_2O \xrightarrow{\alpha-amylase} Reducing\ groups\ (maltose)$$

One unit liberates 1.0mg of maltose from starch in 3 min at pH 6.9 at 20°C.
A potassium phosphate buffer was prepared by dissolving 0.7098g of KH_2PO_4 and 0.0979g of NaCl in 250mL demi-water and adjusted to pH 6.9.

A 1.0% soluble starch solution was prepared in the sodium phosphate buffer pH 6.9 by dissolving 0.25g soluble potato starch in an initial volume of 20mL of buffer, then it was covered the beaker to avoid water evaporation and brought to boil while stirring and was maintained just below boiling temperature for 15 min. After it was cooled to room temperature and completed the starch solution to the appropriate volume (25mL) by adding 5mL of demi-water.

After that, a color reagent solution was prepared. A 5.3M sodium potassium tartrate solution was prepared in 2M NaOH by dissolving 0.8g NaOH in 10mL demi-water, heated the solution to reach a temperature between 50 – 70°C. After that, 12g of sodium potassium tartrate tetrahydrate were dissolved in 8mL of this warm 2M NaOH solution. It was maintained at this temperature while constant stirring to dissolve but do not boil.

A 96mM 3,5-dinitrosalicylic acid solution was prepared by dissolving 438mg of acid in 20mL of demi-water, heated the solution to reach a temperature ranging between 50 – 70°C and it was maintained at this temperature while constant stirring to dissolve but do not boil.

Finally, 12mL of demi-water was heated to 60°C, added slowly 8mL of the 5.3M sodium potassium tartrate solution. Then 20mL of the 96mM 3,5-dinitrosalicylic acid solution was added and stirred until complete dissolution.

A solution of 0.2% (w/v) maltose was prepared by dissolving 2mg in 10mL of demi-water.

Just before use, a solution containing approximately 1 unit/mL of alpha-amylase in demi-water was prepared.

A volume of 1mL of substrate was pipetted into 15mL cap covered containers, mixed by swirling and incubated at 20°C for 3-4 min to achieve temperature equilibration. Then, 500µL to 1mL of enzyme solution was added, was mixed by swirling and incubated at 20°C for exactly 3 min. After 3 min, the reaction was stopped by adding 1mL of color reagent solution and was placed in the oil bath (100°C), the mixture was made up to 1 mL of enzyme solution. After that, the reaction mix was boiled for exactly 15 min and cooled on ice for a few min.

A volume of 9mL of demi-water was added, mixed by inversion and finally a volume of 3mL was pipetted in a cuvette of spectrophotometer and finally recorded the absorbance at 540nm.

For blank assays, the protocol was similar but no enzyme was added before the 3 min incubation. It was only after the addition of the color reagent and after putting the reaction vessel in the oil bath, that 1mL of enzyme solution was added.

Table 8. Protocol of alpha-amylase activity

	First concentration of enzyme	***S concentration of enzyme***	***Third concentration of enzyme***	***Test Blank***
Substrate: Potato starch solution (mL)	1.0	1.0	1.0	1.0
Enzyme solution (mL)	0.5	0.7	1.0	0.0
Color reagent (mL)	1.0	1.0	1.0	1.0
Enzyme solution (mL)	0.5	0.3	0.0	1.0
Demi-water (mL)	9.0	9.0	9.0	9.0

A standard curve was established by dilution the maltose solution (0.2% w/v) as followed in demi-water:

Table 9. Standard curve

	Maltose solution (mL)	***Demi-Water (mL)***
D1	0.05	1.95
D2	0.20	1.80
D3	0.40	1.60
D4	0.60	1.40
D5	0.80	1.20
D6	1.00	1.00
D7	2.00	0.00
Blank_Std	0.00	2.00

One milliliter of color reagent solution was added for each maltose concentration. After this addition the maltose solutions were boiled in the oil bath for 15 min, it was cooled on ice to room temperature and 9mL of demi-water was added. Solutions were mixed by inversion and the absorbance was recorded for the standards and standard blank.

The absorbance of maltose solutions was recorded, and corrected with standard blank:

$$\Delta A_{540(standard)} = A_{540}(maltose\ sol) - A_{540}(std\ blank)$$

Equation 4. Corrected absorbance (standard curve)

The absorbance (corrected) of standards was plotted versus quantity of maltose (mg), and the corresponding linear regression was established.

$$\Delta A_{540(standard)} = slope * [maltose(mg)] + intercept$$

Equation 5. Equation of standard curve

The absorbance of samples was also corrected, and was interpolated on the calibration curve:

$$\Delta A_{540(sample)} = A_{540}(sample) - A_{540}(test\ blank)$$

Equation 6. Corrected absorbance (sample)

$$\frac{Units}{mg\ powder} = \frac{\Delta A_{540(sample)} - intercept}{slope * X}$$

Equation 7. Alpha-amylase activity (U/mg)

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

Bile salts assays

The concentration of bile salts in the bile was tested using a commercial kit (bile acid kit, ref 1 2212 99 90 313, DiaSys Diagnostic System GmbH, Germany).

The principle of this assay is the following reactions:

$$3-\alpha-hydroxy\ bile\ acids + NAD^+ \xleftrightarrow{3-\alpha-HSD} oxidized\ bile\ acids + NADH + H^+$$

$$NADH + H^+ + NBT \xleftrightarrow{diaphorase} NAD^+ + formazan$$

In the presence of NAD+, the enzyme 3-α-hydroxysteroid dehydrogenase converts 3-α-hydroxy bile acids to 3-keto steroids and NADH. The NADH formed reacts with nitrotetrazolium blue to form a formazan dye in the presence of diaphorase enzyme. The dye formation is monitored by measuring absorbance at 540nm and is directly proportional to the bile acids concentration in the sample.

The composition of the reagents was:

- Reagent 1
  - Diaphorase (50 U/L)
  - NAD^+ (1 mM)
  - NBT (0.2 mM)
- Reagent 2
  - 3-α-HSD (60 U/L)
  - TRIS buffer (50 mM)
- Buffer
  - Phosphate buffer (100mM EDTA)
- Standard
  - 35 µM bile acids

A volume of 80µL of demi-water (for blank), sample or standard was mixed with 600µL of reagent 1. Then, it was mixed well and was incubated for 4 min at 37°C. The absorbance was read at 540nm (absorbance 1), after that a volume of 120µL of reagent 2 was added, mixed well and was incubated 5 min at 37°C, finally the absorbance was read (absorbance 2).

The concentration was then calculated using the following equations:

$$\Delta A = (A_2 - 0.85 * A_1)_{sample\ or\ standard} - (A_2 - 0.85 * A_1)_{blank}$$

Equation 8. Corrected absorbance

$$bile\ acids\ (\mu M) = \frac{\Delta A_{sample}}{\Delta A_{standard}} * Concentration\ standard\ (\mu M)$$

Equation 9. Bile acids concentration

Where A1 and A2 are first and s absorbance, respectevly. And the factor ***0.85*** compensates the decrease of the absorbance by addition of reagent 2. The factor was calculated as follows: ***(sample + R1)/Total volume***.

3.3. Static digestion method (according Mikenus et. al. (2004)[19])

The *in vitro* digestion protocol comprises three different phases: oral, gastric and intestinal.

Simulated Salivary Fluid (SSF), Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF) are made up of the corresponding electrolyte stock solutions, enzymes, calcium chloride and water.

The electrolyte stock solutions are 1.25 x Concentrated. For example, 4 parts of electrolyte stock solution + 1 part water give the correct ionic composition in the simulated digestion fluids.

Table 10. Recommended concentrations of electrolytes in simulated digestive fluids based on human *in vivo* data

	Simulated Salivary Fluid	***Simulated Gastric Fluid***	***Simulated Intestinal Fluid***
Constituent	*Concentration (mM)*	*Concentration (mM)*	*Concentration (mM)*
K^+	18.80	7.80	7.60
Na^+	13.60	72.20	123.40
Cl^-	19.50	70.20	55.50
$H_2PO_4^-$	3.70	0.90	0.80
HCO_3^-, CO_3^{2-}	13.70	25.50	85.00
Mg^{2+}	0.15	0.10	0.33
NH_4^+	0.12	1.00	0.00
Ca^{2+}	1.50	0.15	0.60

Simulated salivary fluid, simulated gastric fluid and simulated intestinal fluid were prepared as described in the following table:

Table 11. Simulated digestive fluids protocol

		Simulated salivary fluid (pH 7)		***Simulated gastric fluid (pH 3)***		***Simulated intestinal fluid (pH 7)***	
Constituent	*Stock conc. (M)*	*Vol. of stock (mL)*	*Final conc. (mM)*	*Vol. of stock (mL)*	*Final conc. (mM)*	*Vol. of stock (mL)*	*Final conc. (mM)*
KCl	0.50	7.55	15.10	6.90	6.90	13.60	6.80
KH_2PO_4	0.50	1.85	3.70	0.90	0.90	1.60	0.80
$NaHCO_3$	1.00	3.40	13.60	12.50	25.00	85.00	85.00
NaCl	2.00	0.00	0.00	11.80	47.20	19.20	38.40
$MgCl_2(H_2O)_6$	0.15	0.25	0.15	0.40	0.10	2.20	0.33
$(NH_4)_2CO_3$	0.50	0.03	0.06	0.50	0.50	0.00	0.00
**** For pH adjustment***							
HCl	1.00	0.27	1.10	7.80	15.60	8.40	8.40

The addition of enzymes, bile salts, Ca^{2+} solution etc. and water will result in the correct electrolyte concentration in the final digestion mixture. $CaCl_2(H_2O)_2$ was not added to the electrolyte stock solutions as precipitation may occur. Instead, it was added to the final mixture of simulated digestion fluid and food.

Oral phase

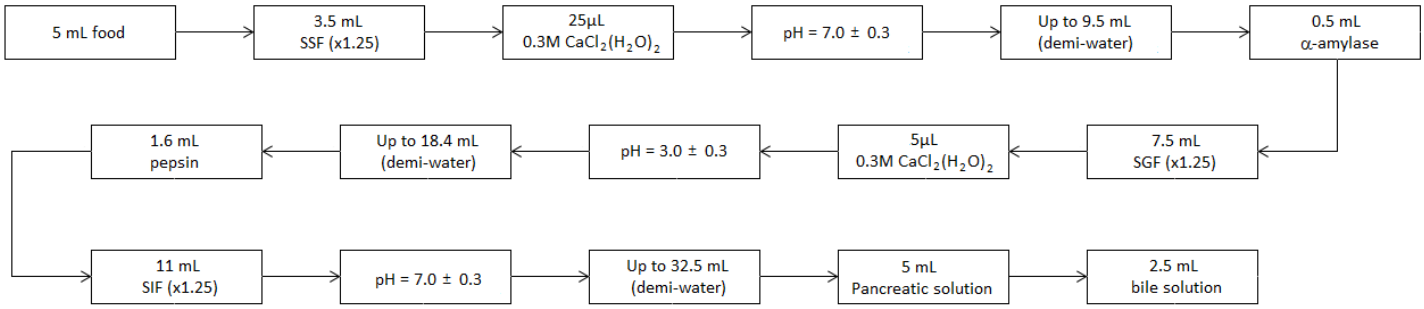
A volume of 5 mL of meal was mixed with 3.5 mL of simulated salivary fluid electrolyte stock solution (a final ratio of food to SSF of 50 : 50 was targeted) and 25µL of 0.3M $CaCl_2(H_2O)_2$ (to achieve 0.75 mM in the final mixture). Then the pH was adjusted to 7.0 ± 0.3 with 0.5M HCl, after that a volume of demi-water was added up to 9.5 mL and finally 0.5 mL of alpha-amylase was added to the mixture (bacterial alpha-amylase was added to achieve 75 U/mL). The oral phase was incubated 2 min at 37°C and 450rpm.

Gastric phase

Five parts of oral bolus was mixed with 4 parts of simulated gastric fluid to obtain a final ratio of oral bolus to simulated gastric fluid 50 : 50 in the final mixture (a volume of 7.5 mL of simulated gastric fluid was mixed with oral bolus). A volume of 5µL of 0.3M $CaCl_2(H_2O)_2$ was added to the mixture (*to achieve 0.075 mM in the final digestion mixture*) and the pH was adjusted to 3.0 ± 0.3 with 1M HCl, after that a volume of demi-water was added up to 18.4 mL and finally 1.6 mL of pepsin was added to the mixture (porcine pepsin was added to achieve 2000 U/mL). Gastric phase was incubated 2 h at 37°C and 450rpm.

Intestinal phase

Five parts of gastric chyme was mixed with 4 parts of simulated intestinal fluid to obtain a final ratio of gastric chyme to SIF of 50 : 50 in the final digestion mixture (a volume of 11mL of simulated intestinal fluid was mixed with gastric chyme). The pH was adjusted at 7.0 ± 0.3 and a volume of demi-water was added up to 32.5 mL. Then 5 mL of pancreatic solution (the amount of pancreatin added was based on the alpha-amylase activity, 200 U/mL in the final mixture) and 2.5 mL of bile solution (bile salts were added to give a final concentration of 10mM in the final mixture) were added to the mixture. Intestinal phase was incubated 2 h at 37°C and 450rpm.



Flow chart 4. Protocol of static digestion method

Sampling during digestion

Sample conservation depends on the study focus (food structure, bio-accessibility, enzymatic digestion product, etc.), and should be carefully considered for each study. In the current study, individual sample tubes for each time point were used to avoid withdrawing samples from the reaction vessel.

Directly after stopping the reaction (sampling point), the sample was ultra-filtrated to remove the enzyme (stop enzyme activity). Finally, the sample was frozen until glucose determination.

3.4. Dynamic digestion method

The *in vitro* dynamic model used for this study (TIM-1) consists of four successive compartments simulating the stomach, duodenum, jejunum and ileum. Each compartment is formed by two connected basic units consisting of a glass jacket with a flexible wall inside.

Water is pumped from a water bath into the glass jackets around the flexible walls to control the temperature inside the units and the pressure on the flexible walls. Changes in water pressure are achieved by computer-activated rotatory pumps. This enables mixing of the chyme by alternate compression and relaxation of the flexible walls.

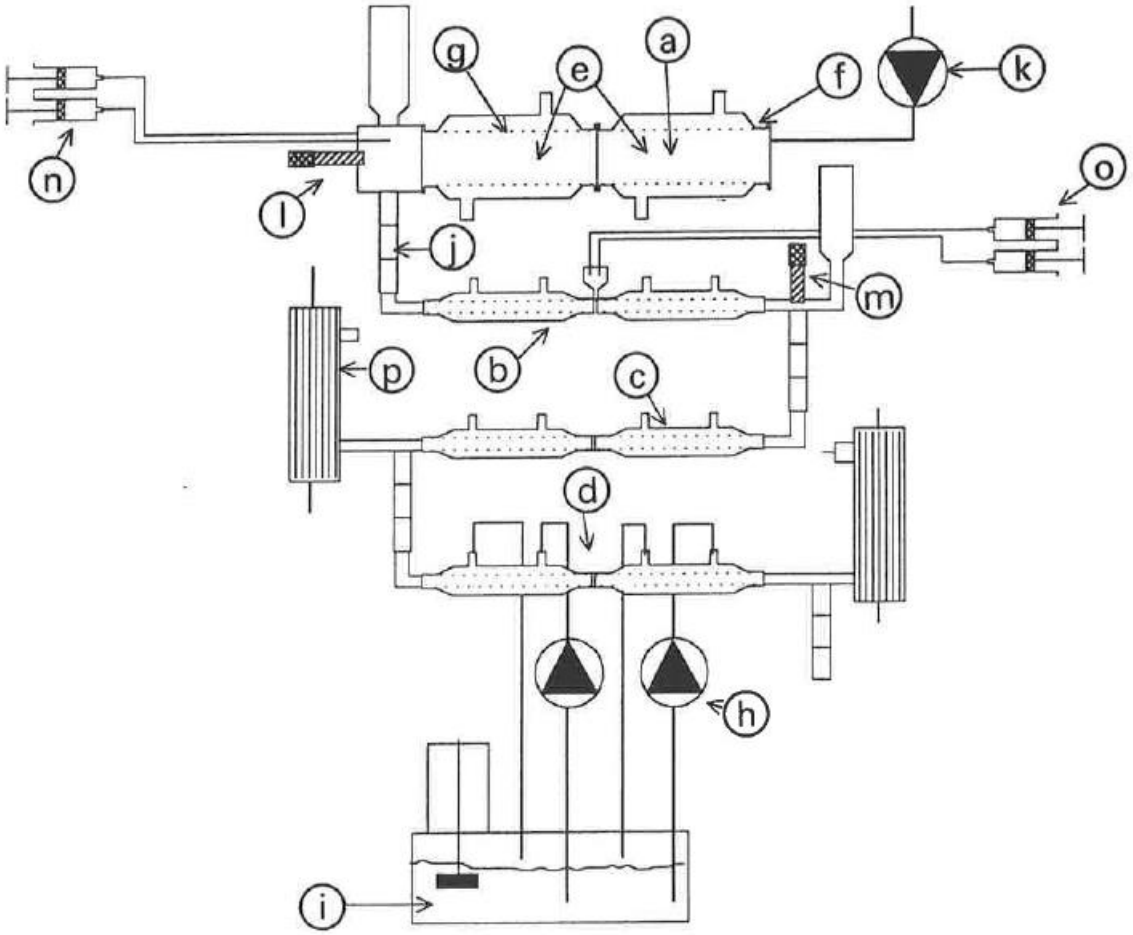


Figure 19. Dynamic model. (a) gastric compartment (b) duodenal compartment (c) jejunal compartment (d) ileal compartment (e) basic unit (f) glass jacket (g) flexible wall (h) rotary pump (i) water bath (j) peristaltic valve-pump (k) peristaltic pump (l,m) pH electrodes (n,o) syringe pumps (p) hollow-fibre device

The compartments are connected by peristaltic valve-pumps consisting of three connected T-tubes, each with a separate tube-like flexible wall inside. If pressure is applied to the outside of the flexible wall, the valve is closed, leaving minimal dead space inside. In the open position, the flexible walls facilitate unhindered passage of the chyme through the valves. Peristaltic pumping is achieved by regulating the sequence of opening and closing of the three parts of the valve-pump. During each peristaltic cycle, a constant volume of chyme is transferred.

The frequency of peristaltic cycles is dictated by computer, allowing the flow rate of the chyme to be controlled. The volume in each compartment (except for stomach) is monitored with a level sensor connected to the computer.

A predetermined quantity of the meal is introduced into the gastric compartment within a pre-set period of time, with a peristaltic pump. All the compartments are equipped with pH electrodes. The pH values are controlled via the computer by screening either water or 1M HCl into the stomach, or by screening either water or 1M $NaHCO_3$ into the small intestine via syringe pumps. To avoid the irregular pH values in the stomach due to incomplete mixing, HCl is secreted through a perforated tube.

Secretions of gastric electrolytes and enzymes, bile and pancreatic juices are regulated by using computer-controlled syringe pumps. The jejunal and ileal compartments are connected with hollow-fibre devices to absorb digestion products and water from the chyme and to modify electrolyte and bile salt concentrations in the chyme.

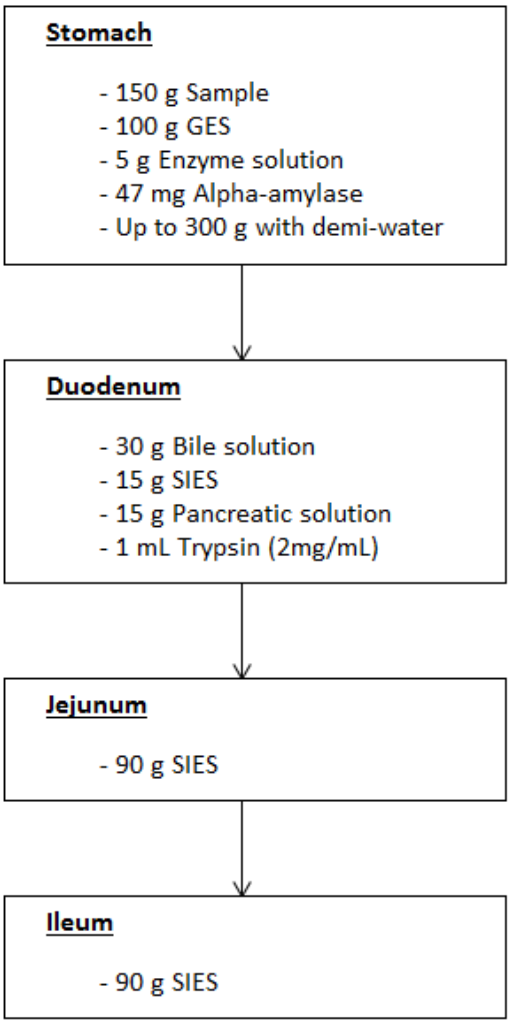
Procedure

The system is loaded with various reagents, mixed in specified ratios as described below. Some were derived from concentrated stock solutions, whereas all working solutions were prepared on the day of use. All solutions were made with deionized water unless specified.

Table 12. Concentrations of stock solutions

Constituent		***Concentration***	***Compartment***
$NaHCO_3$		1 M	Small intestine
HCl		1 M	Stomach
CH_3COONa		1 M (*pH 5.0*)	Stomach
Gastric electrolyte solution (*GES*)	NaCl	106.10 mM	Stomach
	KCl	29.51 mM	
	$CaCl_2(H_2O)_2$	2.04 mM	
Small intestine electrolyte solution (*SIES*)	NaCl	85.56 mM	Small intestine
	KCl	8.05 mM	
	$CaCl_2(H_2O)_2$	2.04 mM	
Pancreatic solution		65.4 mg/mL	Duodenum
Enzyme solution *(pH 5.0)*	Lipase	37.5 U/mL (*GES*)	Stomach
	Pepsin	600 U/mL (*GES*)	
Bile solution		84.33 mM	Duodenum

For an experiment, the system was filled with the following solutions:



Flow chart 5. Start residues

For each experimental run the secretion products (e.g. gastric juice with enzymes, electrolytes, dialysis liquids, bile, pancreatic juice) are freshly prepared, the pH electrodes calibrated and the filtration units replaced.

Sampling during digestion

Samples were collected every 30 min during the first 3 h, and every h the next two h, from two locations (jejunum and ileal dialysates, sugars that would enter the systemic circulation) and every 150 min (2.5 h) from one additional location (outlet of the ileal compartment, sugars that would enter the colon and assumed not to be available for absorption).

At the end of the experiment, the device protocol was stopped and all residues from Stomach to Ileum were collected. All the samples were stored at -20°C.

3.5. Determination of glucose concentration

Concentration of glucose were measured using a commercial kit.

The kit used to determine the glucose concentration was KA1648 (Abnova). First, a standard curve was prepared:

Table 13. Standard curve (glucose)

Nº	*Standard (3000 ppm)*	*Demi-water*	*Final concentration (glucose)*
1	150 µL	0 µL	3000 ppm
2	100 µL	50 µL	2000 ppm
3	50 µL	100 µL	1000 ppm
4	25 µL	125 µL	500 ppm
5	0 µL	150 µL	0 ppm

For the samples: 12 µL of sample or standard were mixed with 1200 µL of reagent. The mixture was incubated 8 min in a boiling water bath, followed by 4 min an ice bath. Finally, the absorbance was measured at 630 nm.

The absorbance of standard solutions was recorded, and corrected with standard blank:

$$\Delta A_{630(standard)} = A_{630}(glucose\ sol) - A_{630}(std\ blank)$$

Equation 10. Corrected absorbance

The absorbance (corrected) of standards was plotted versus quantity of glucose (mg/L), and the corresponding linear regression was established.

$$\Delta A_{630(standard)} = slope * [glucose(mg/L)] + intercept$$

Equation 11. Equation of standard curve

The amount of glucose was then calculated using the following equation:

$$mg\ of\ glucose = \frac{\left(\frac{A_{630}(sample) - A_{630}(std\ blank) - intercept}{slope}\right) * Amount\ of\ sample\ (mL)}{1000}$$

Equation 12. Amount of glucose (mg)

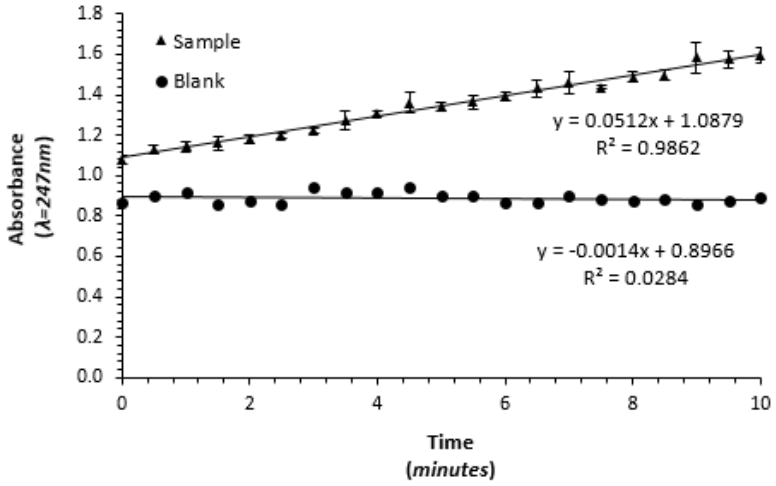
4. RESULTS AND DISCUSSION

4.1. Determination of enzyme activities[47]

The enzyme activities determination is essential to calculate the quantity of enzymes needed to achieve a certain activity during the *in vitro* digestion assays.

Trypsin Activity Assay (*EC 3.4.21.4*)

Trypsin activity of a pancreatic solution (1.37 mg/mL 1mM HCl) was measured. The activity was calculated using the slopes ΔA_{247} (unit absorbance/min):



Graph 2. Results of trypsin activity

$$\frac{units}{mg} = \frac{\left(\Delta A_{247}(test) - \Delta A_{247}(blank)\right) * 1000 * 3}{540 * X}$$

$$\frac{units}{mg} = \frac{(0.0512 - (-0.0014)) * 1000 * 3}{540 * 0.137} = 2.13 \ \ U/mg \ \rightarrow \ \ 2133.01 \ \ U/g$$

Equation 13. Trypsin activity

Where one unit hydrolyses one µmole of TAME per min at 25°C and pH 8.1.

Pepsin Activity Assay (*EC 3.4.23.1*)

Pepsin activity of a pepsin solution (1.22 mg/mL 150mM NaCl buffer) was measured. The activity was calculated using the following equation:

$$\frac{Units}{mg} = \frac{\left(A_{280}(test) - A_{280}(blank)\right) * 1000}{\Delta t * X}$$

Equation 14. Pepsin activity (U/mg)

Δt: Duration of the reaction (i. e. 10 min).
X: Concentration of enzyme powder in the final reaction mixture (quartz cuvettes, mg/mL).
1000: Factor to equalize the units.

Table 14. Pepsin activity results

Incubation time (min)	*Enzyme concentration (mg/mL)*	*Absorbance (λ = 280 nm)*		*Pepsin activity (U/mg)*
		Blank	*Sample*	
10	0.0004	0.210	0.223	3321.05
	0.0008	0.240	0.265	3322.61
	0.0011	0.282	0.320	3325.81
	0.0015	0.295	0.346	3317.95
	0.0019	0.300	0.363	3319.80
	0.0023	0.315	0.391	3323.90

Therefore, the pepsin activity of the powder is 3321.83 ± 2.85 (0.1) U/mg (one unit will produce a ΔA_{280} of 0.001 per min at pH 2.0 and 37°C, measured as TCA-soluble products).

α-Amylase Activity Assay (*EC 3.2.1.1*)

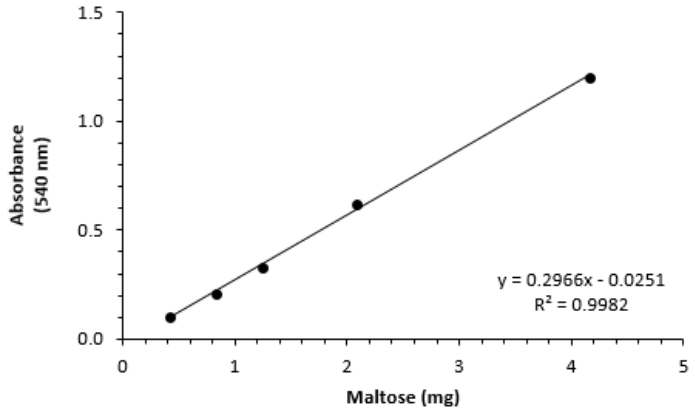
Alpha-amylase activity of an alpha-amylase solution (0.0086 mg/mL demi-water) was measured.

The absorbance of maltose solutions was recorded, and corrected with standard blank:

$$\Delta A_{540(standard)} = A_{540}(maltose\ sol) - A_{540}(std\ blank)$$

Equation 15. Corrected absorbance

The absorbance (corrected) of standards was plotted versus quantity of maltose (mg), and the corresponding linear regression was established.



Graph 3. Standard curve for alpha-amylase activity

The absorbance of samples was also corrected, and was interpolated on the calibration curve:

$$\Delta A_{540(sample)} = A_{540}(sample) - A_{540}(test\ blank) \rightarrow \frac{Units}{mg\ powder} = \frac{\Delta A_{540(sample)} - intercept}{slope * mg_{\alpha-amylase}}$$

Table 15. Results of alpha-amylase activity (One unit liberates 1.0mg of maltose from starch in 3 min at pH 6.9 at 20°C)

Amount of alpha-amylase (mg)	***Absorbance (λ = 540 nm)***		***Alpha-amylase activity (U/mg)***
	Blank	*Sample*	
0.0011		0.634	1361.2
0.0015	0.223	0.819	1384.8
0.0022		1.004	1258.1

Therefore, the alpha-amylase activity of the powder is 1334.7 ± 67.4 (5) U/mg. According product specification the activity of this product is 1333 U/mg (using the same unit definition).

The focus of these experiments is the study of digestion of carbohydrates, and the enzyme that cleaves glycosidic bond of starch is alpha-amylase. For this reason, alpha-amylase activity of pancrex powder was also measured (to know how to prepare pancreatic solution). The alpha-amylase activity of pancrex powder is 71.92 U/mg.

Bile salts assays

The concentration of bile salts in the bile was tested using a commercial kit (bile acid kit, ref 1 2212 99 90 313, DiaSys Diagnostic System GmbH, Germany). The concentration was then calculated using the following equations:

$$\Delta A = (A_2 - 0.85 * A_1)_{sample\ or\ standard} - (A_2 - 0.85 * A_1)_{blank}$$

Equation 16. Corrected absorbance

$$bile\ acids\ (\mu M) = \frac{\Delta A_{sample}}{\Delta A_{standard}} * Concentration\ standard\ (\mu M)$$

Equation 17. Bile acids concentration

Table 16. Bile acids concentration

	Absorbance 1	***Absorbance 2***	***ΔA***	***Bile acids (μM)***
Blank	0.084	0.075	---	---
Standard	0.075	0.160	0.074	35
Sample 1	0.133	0.250	0.114	54.34
Sample 2	0.128	0.243	0.112	53.03

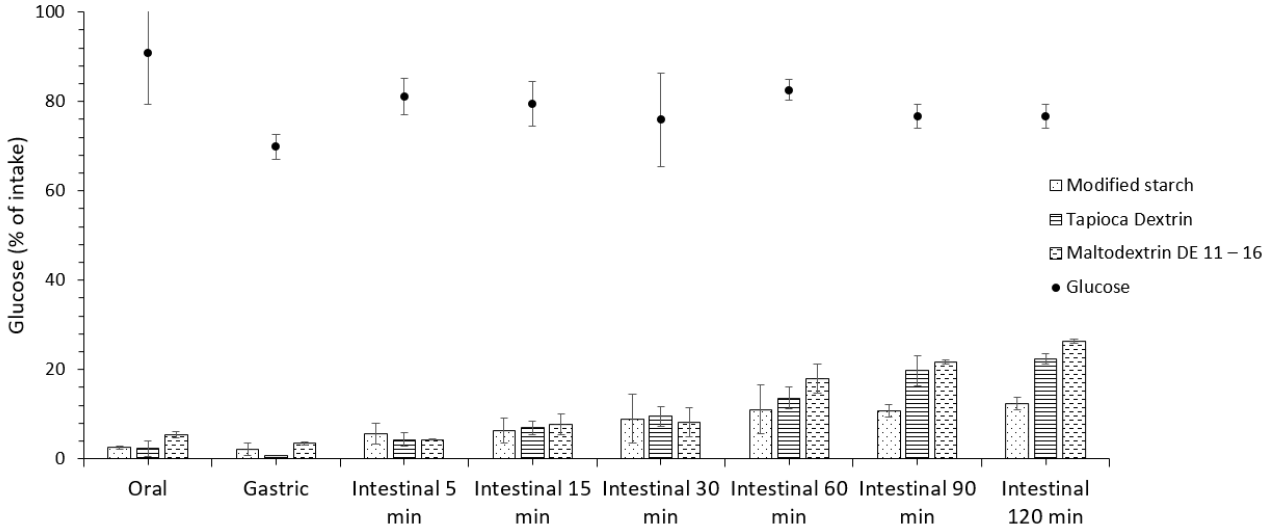
The bile solution was prepared with 12.2mg in 10mL of demi-water, therefore molecular weight of our bile powder was 454.54 ± 7.8 (2) g/mol.

4.2. Static Digestion method

To study the behavior of different samples during *in vitro* digestion 13 experiments were carried out, three for each sample, two with demi-water (negative control) and two with glucose (positive control).

Results of the experiments with demi-water (negative control) revealed that secretions fluids do not contain glucose (results not shown as below the detection limit of the used method of detection).

In Graph *4* the released glucose is depicted for the separated phases of digestion and in dependence of carbohydrate source (Maltodextrin DE 11 – 16, Tapioca Dextrin, "Modified starch").



Graph 4. Results of Static digestion method (glucose, %) (number of repetitions (polysaccharides)= 3, number of repetitions (glucose) = 2)

In the above graph the results are represented as % of intake, and this percentage is calculated with the following equation:

$$\%\ of\ intake = \frac{mg\ glucose\ for\ specific\ sample}{mg\ carbohydrate\ source} * 100$$

Equation 18. Calculation the % of intake

For glucose as the positive control an almost value between 80 – 100 % was observed without significant differences between the digestion phases. A slight increased value is observed for the oral phase, which may be because during the oral phase carbohydrate source is only mixed with simulated salivary fluid, and during the other phases there are a bigger background.

The results for different carbohydrate sources are very similar showing lower levels for oral and gastric phases slightly elevated levels for the first three intestinal samples (5, 15 and 30 min) and increased levels for the las three intestinal samples. However, the values are distinctly lower than the levels of glucose (representing the same carbohydrate concentration but as free glucose).
As expected the positive control (glucose) shows similar values for all phases of *in vitro* digestion as the monosaccharides is already present as glucose at time 0. In this study a complete sample is taken per sample point, so that each sample contains the entire amount of starting material (carbohydrate source; in this case the positive control 125mg of glucose).

For the studied carbohydrate sources, it was observed that only low levels of glucose were released during the static *in vitro* digestion (max. of ~30%), supposing that the carbohydrate sources are not very well digestible.

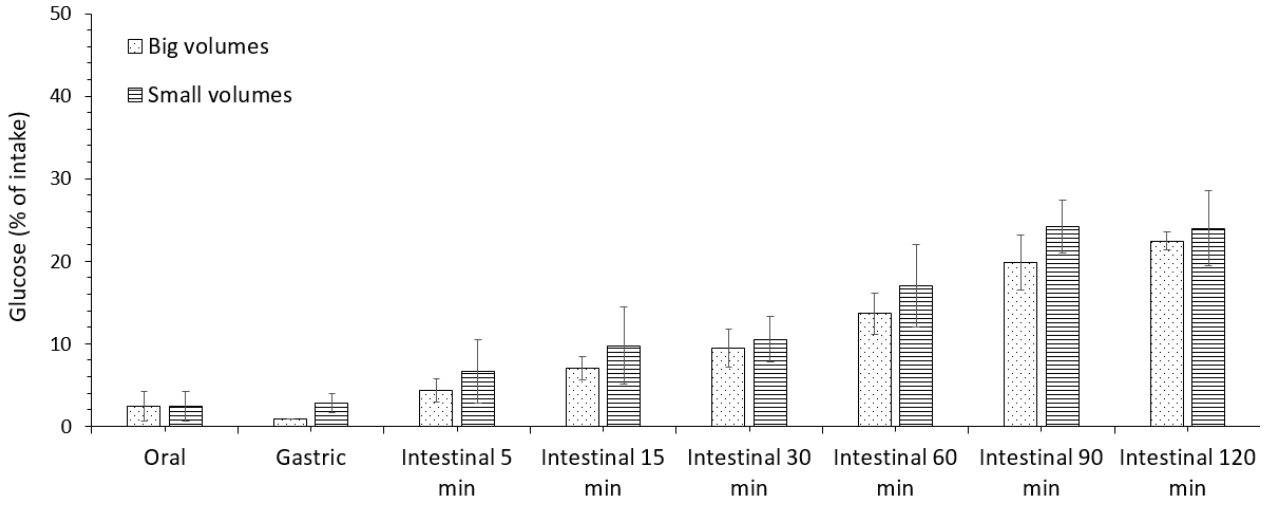
That however, can be explained with the fact that the monosaccharide glucose is the last product of starch digestion. Before reaching free glucose monomers the starch chains are broken into oligosaccharides that are consequently broken into tri- di- and monosaccharides. For an estimation of the digestibility of the carbohydrate sources other starch-digestion products need to be take into consideration.
Graph *4* shows that during the experiments was not lost sample (% of glucose is around 100% in almost all samples). It also shows that samples do not release big amount of glucose, and between them, Maltodextrin DE 11-16 release more amount of glucose than Tapioca Dextrin and "Modified starch". These results are as expected because Maltodextrin DE 11-16 has the shortest chain, and "Modified starch" the biggest one.

Volume effect

Besides studying digestibility of some carbohydrate sources, the effect of volume was also studied. These trials are useful to know if the protocol can be performed it with less volume of secretions/samples, because is more easy to handle and cost effective because it needs less amount of reagents.

Two experiments were carried out with Tapioca Dextrin and one with demi-water (as blank). Results of the experiments with demi-water (negative control) are bellow of the detection limit (for glucose results).

In Graph 5 the released glucose is shown for separated phases of digestion to check the possible differences between when big and small volumes are used during static digestion method.



Graph 5. Comparison between to use big (final volume: 40mL) and small volumes (final volume: 8mL), sample: Tapioca Dextrin (number of repetitions = 2)

It seems that with small volumes, results are higher than with big volumes, but if it takes account standard deviation it can affirm that there are not significant differences. For this reason, it can affirm that is more useful to use small volumes than big volumes.

Different conditions

In reality the studied carbohydrate sources are rarely eaten or formulated in food products alone, they are normally eaten with other proteins, fats, etc. In this section it was studied how affect proteins, fats or pre-treatment in the release of glucose from Tapioca Dextrin.

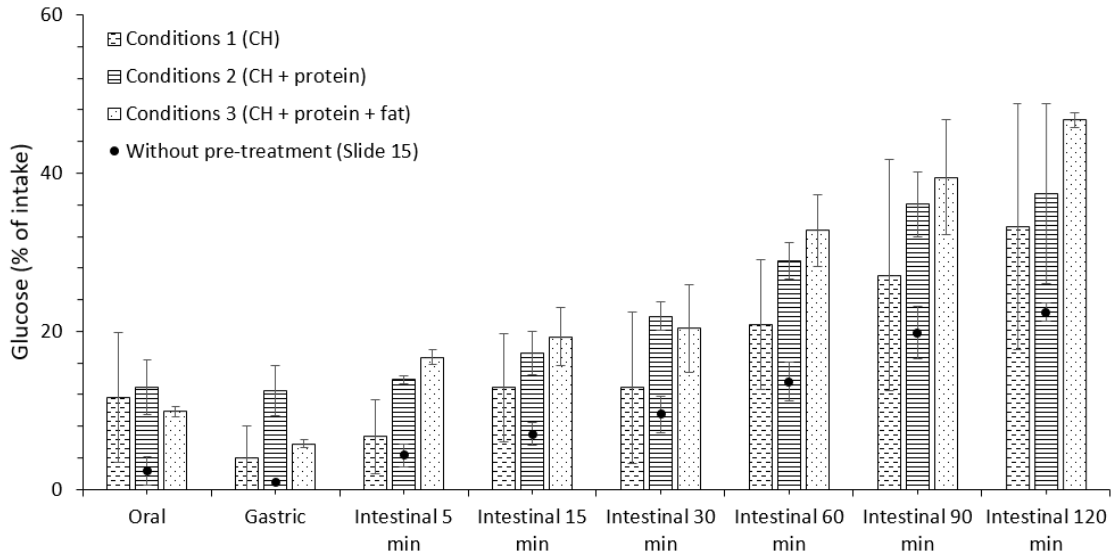
To carried out this section of the study, Tapioca Dextrin was subjected to different conditions and with presence of other macronutrients.

- *Conditions 1:* Sample was prepared like a real product for clinical nutrition (autoclave, 121°C, 20 min). This experiment is useful to know how affect the pre-treatment in the release of glucose, because a real product for clinical nutrition has to be sterilized with autoclave.

- *Conditions 2:* Sample was prepared with proteins and sterilized with autoclave (same protocol as conditions 1). This experiment is useful to know how affect the presence of proteins in the release of glucose, because normally a real product for clinical nutrition is a complex formulation (not only carbohydrates).

- *Conditions 3:* Sample was prepared with proteins, fats and sterilized with autoclave (same protocol as conditions 1). The objective of this experiment is the same than conditions 2, but more complex.

In Graph 6 the released glucose is depicted for the separated stages of digestion and in dependence of the different matrixes (only Tapioca Dextrin with pre-treatment, Tapioca Dextrin with protein, Tapioca Dextrin with protein and fat, Tapioca Dextrin without pre-treatment).



Graph 6. Effect of different conditions in the release of glucose (% of intake) (number of repetitions = 3)

With these results it can affirm that the pre-treatment favors the release of glucose from carbohydrate source, and this is maybe for the solubility of sample that is higher when the temperature of preparation is higher (with autoclave 121°C, without pre-treatment 85-90°C).

In the comparison between conditions 1, 2 and 3 it seems that with fat-protein the release of glucose is higher than with protein or alone, however there are no significant differences (standard deviation).

A difference of glucose released between different matrixes was expected, but the obtained results do not present significant differences. The conclusions that can be extracted with these results are that background does not affect to the glucose release, or that the limitations of method (absence of microbial ecosystem for example) could affect to the glucose release.

4.3. Dynamic Digestion method

To study the behavior of samples during their digestion 5 experiments were done, one for each sample, one with demi-water (negative control) and one with glucose (positive control).

Table 17. Digestion experiments

Meal	***Amount***	***Conditions***
Demi-water	150 g (100%)	
Maltodextrin DE 11 – 16 Tapioca Dextrin "Modified starch" Glucose	11.25 g (7.5%)	10 min 85 - 90°C 500rpm

In the following graphs, the results on the percentage of intake are all corrected with the amount of glucose were obtained for the negative control (originating from secretion fluids).

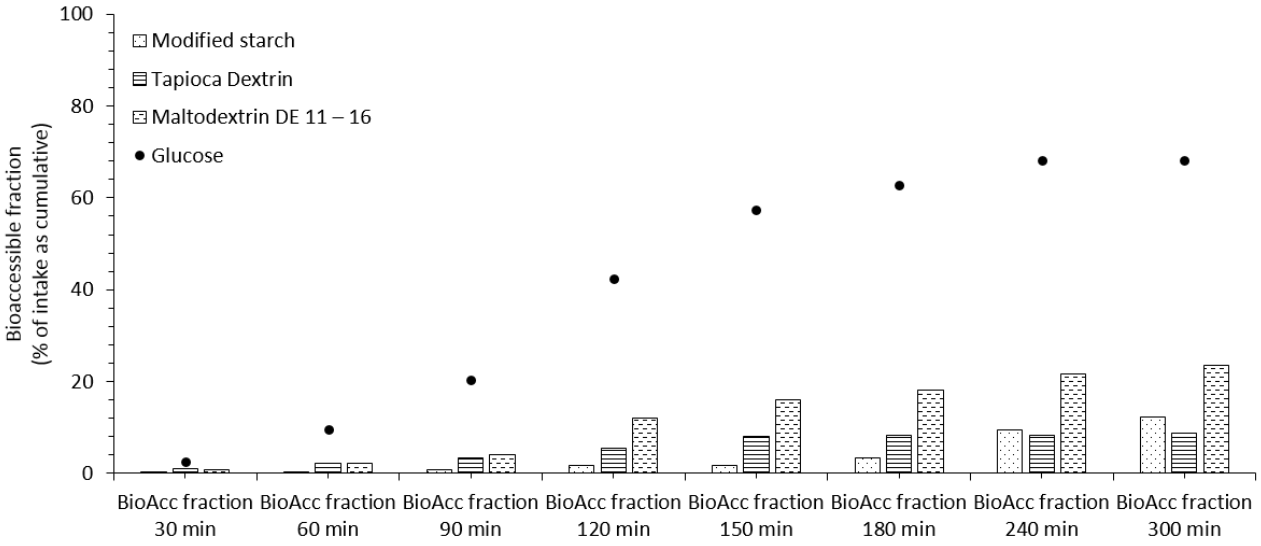
Bio-accessibility is the fraction of test compound that has become available for intestinal absorption. In TIM-1 studies, bio-accessibility is determined by measuring the fraction of a compound that has passed the dialysis or filtration membrane.

$$Bioaccessible\ fraction = Jejunum_{BioAcc\ fraction} + Ileum_{BioAcc\ fraction}$$

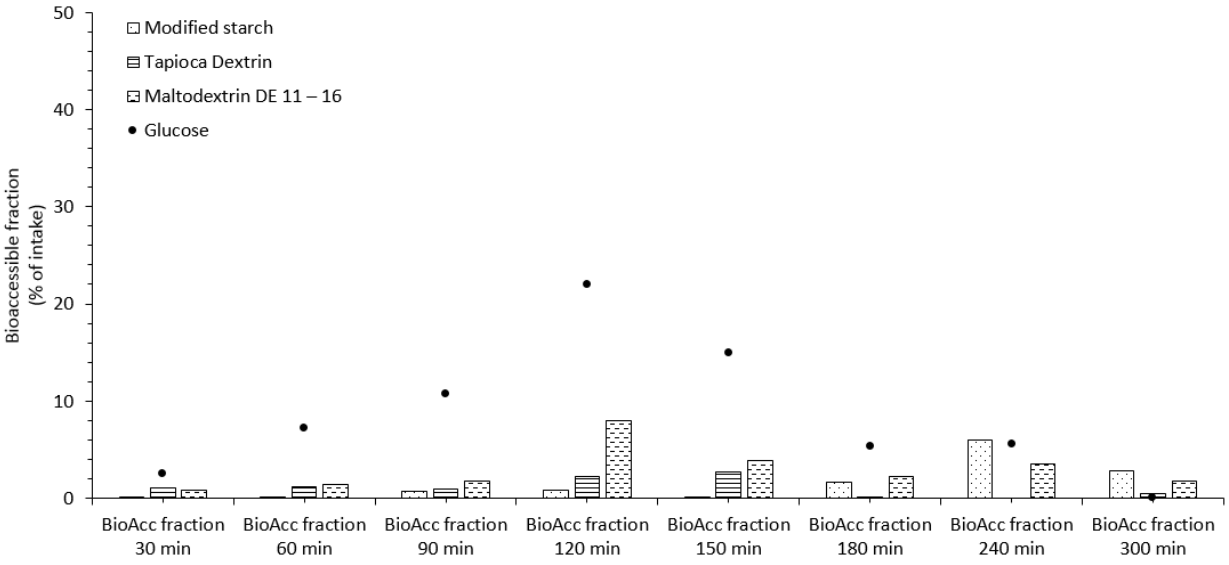
Equation 19. Bio-accessible fraction

In this case, the results for the experiment with demi-water are higher than detection limit and the results depicted in the following graphs are corrected with the negative control (glucose originating from secretion fluids).

In Graph 7 and Graph 8 the released glucose from the studied different carbohydrate sources (Maltodextrin DE 11 – 16, Tapioca Dextrin, "Modified starch") is depicted.



Graph 7. Bio-accessible fraction of glucose (% of intake as cumulative)



Graph 8. Bio-accessible fraction of glucose (% of intake)

In the previous graphs it has represented bio-accessible fraction of glucose in different ways, Graph 7 shows bio-accessible fraction as cumulative and in Graph 8 it has represented the same values of Graph 7 but in this case the graph show % of intake in each sampling time. So that in the first graph it can know the total amount of bio-accessible fraction in each sampling time and in the s graph it can know bio-accessible fraction in each moment during digestion.

Graph 7 shows that only ~70% of the meal is present in the bio-accessible fraction (experiment with glucose). Graph 8 shows that the maximum of bio-accessible fraction of glucose is found after 2 h.

The results of the experiment with Maltodextrin DE 11-16 suggest that the amount of glucose available for absorption grows as it goes digesting food until ~25% of intake. The growth profile is more or less linear and it seems that the amount of glucose does not reach stationary state (Graph 7).

The maximum of bio-accessible fraction of glucose for Maltodextrin DE 11-16 it's found at 120 min (time comparable with the one of the maximum bio-accessible fraction of pure glucose).

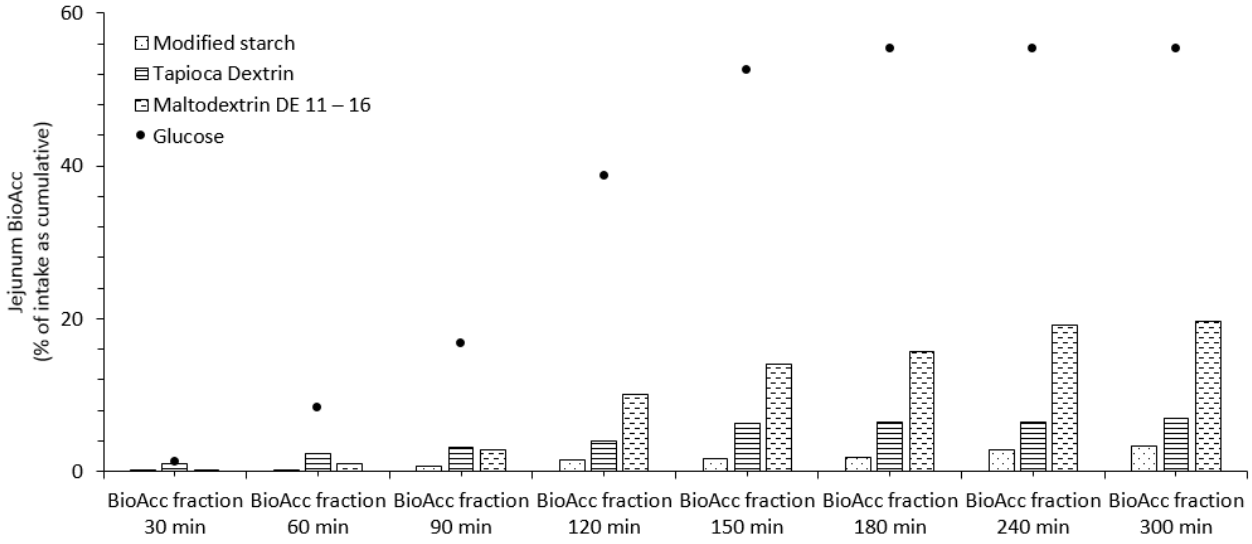
The results of the experiment with Tapioca Dextrin suggest that the amount of bio-accessible fraction of glucose increases significantly until 150 min. Then it reaches a constant value of ~10% of intake (30 min after positive control and Maltodextrin DE 11-16).

The results of the experiment with "Modified starch" suggest that the amount of glucose bio-accessible grows as it goes digesting food until ~15% of intake. It seems that in this case occurs something like with Maltodextrin DE 11-16; the amount of glucose doesn't arrive stationary state. The maximum of bio-accessible fraction of glucose for "Modified starch" it's found at 240 min (120 min after positive control and Maltodextrin DE 11-16, and 90 min after Tapioca Dextrin).

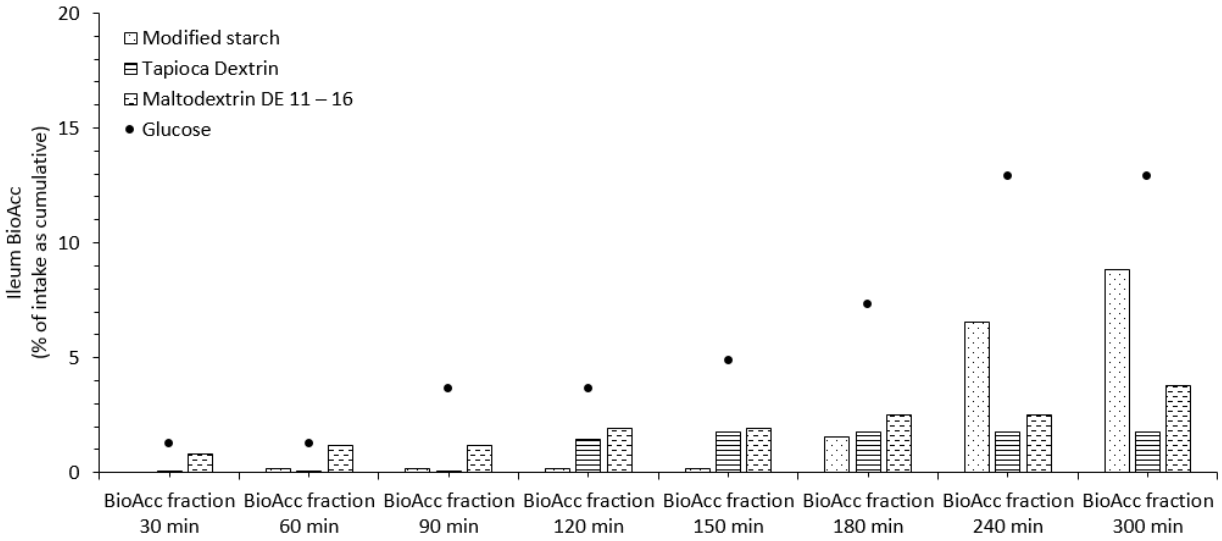
These results are more or less as expected. Maltodextrin DE 11-16 is digested more easily because it has the shortest chain. Short chain polysaccharide releases glucose more rapidly than longer chain polysaccharide. Therefore, it is normal that Maltodextrin DE 11-16 present the maximum of bio-accessible fraction earlier than Tapioca Dextrin and "Modified starch".

For this reason, is a bit strange that the maximum of digested sample is higher for "Modified starch" than for Tapioca Dextrin. Nevertheless, the differences between both sample are small, and it has to take account experimental error. This question could be resolved by carrying out more experiments with these samples, and calculate the standard deviation.

InGraph 9 and Graph 10 the released glucose is shown for each sampling point and in dependence of the carbohydrate source.



Graph 9. Bio-accessible fraction of glucose in jejunum (% of intake as cumulative)



Graph 10. Bio-accessible fraction of glucose in ileum (% of intake as cumulative)

As it can be observed on both graphs, the major part of bio-accessible fraction is in jejunum.

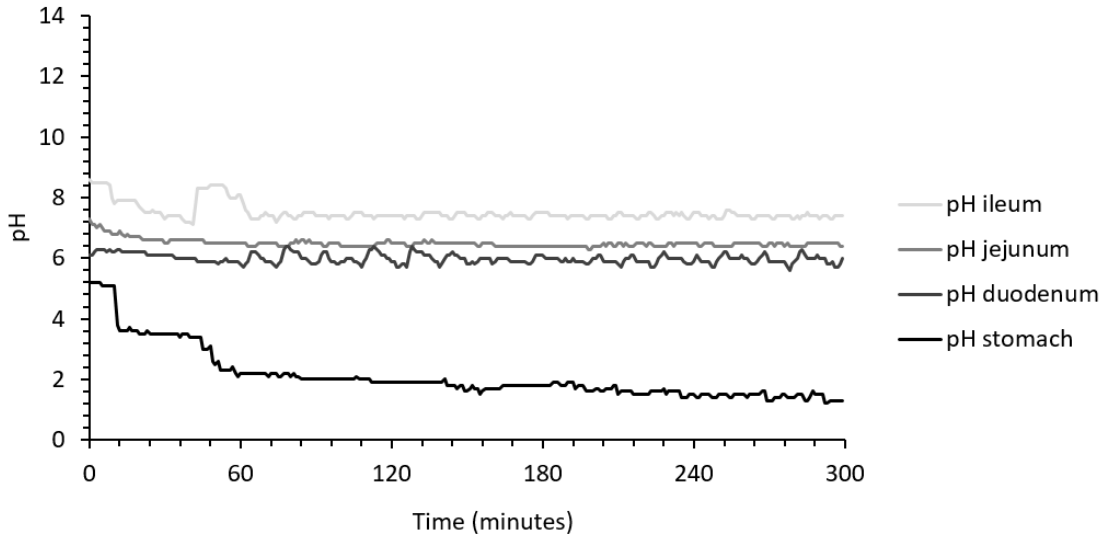
Graph 10 shows a very interesting thing for "Modified starch". The amount of glucose released during the last two h is very high compared with the other carbohydrate sources.

As we already mentioned before, the "Modified starch" is the polysaccharide with the longest chain, i.e. the one with the slowest digestion. Consequently, it's not surprising that the released glucose from "Modified starch" is absorbed in the ileum after three h of digestion.

4.4. Comparison between static and dynamic digestion method

pH profile

The following graph (Graph 11) shows the evolution of the pH values in the curse of time, in different compartments of TIM-1.



Graph 11. Changes of pH during dynamic digestion method (representative example)

This graph shows the experimental values of pH, where the theoretical values are:

- *Stomach:* the initial pH is 5.5.

Table 18. pH profile in the stomach

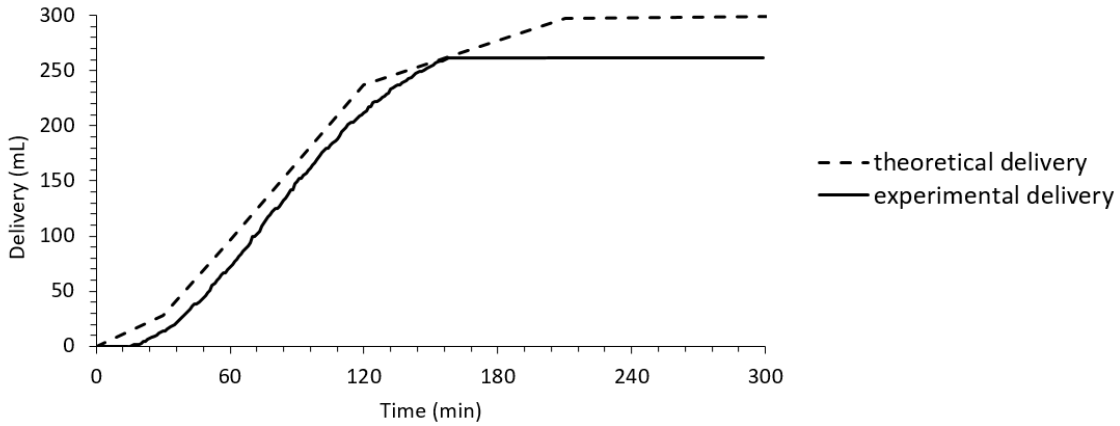
Time (min)	***pH***
0	5.5
30	4.5
60	3.0
120	2.0
210	1.7
300	1.7

- *Duodenum:* constant pH of 5.9.
- *Jejunum:* constant pH of 6.5.
- *Ileum:* constant pH of 7.4.

This is a difference between dynamic and static digestion method. During static digestion method the sample with secretions are mixed at constant pH of 3.0 for the gastric phase and at constant pH of 7.0 for the intestinal phase.

Gastric emptying

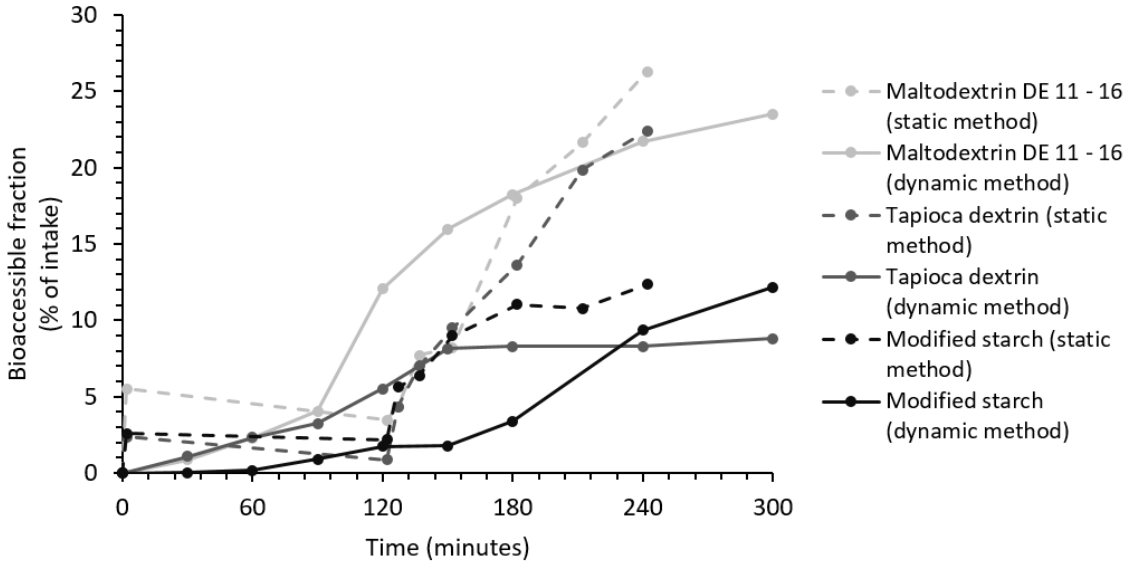
In Graph 12 gastric emptying for dynamic digestion method is depicted.



Graph 12. Stomach delivery (dynamic digestion method)

This graph shows another difference between 2 methods. In the static digestion method, the meal is incubated during a fixed time, fixed pH and the concentration of sample/secretions is kept constant. However, in the dynamic digestion method, the concentration of secretions and sample were changing during the digestion process following the protocol Water Fed HFM (High Fat Meal).

In Graph 13 the released glucose is depicted for static and dynamic digestion method and in dependence of the carbohydrate sources (Maltodextrin DE 11 – 16, Tapioca Dextrin, Modified starch).



Graph 13. Comparison between static and dynamic digestion method (glucose)

Graph 13 shows that the profiles between static and dynamic digestion method are different. During the first 90 min, the bio-accessible fraction of glucose is lower than 5% of intake and there are no major variations. After 90 min all the profiles are different.

For static digestion method, bio-accessible fractions increase drastically at 120 min for all because in this moment small intestinal phase started and alpha-amylase was added. Then, for Maltodextrin DE 11-16 and Tapioca Dextrin there is a large similar increase of glucose in the bio-accessible fraction. This increase is not so important for "Modified starch" and it looks like that after 4 h the digestion is not completed for "Modified starch". This result is confirmed by the value of recovery after 4 h.

Table 19. Recovery of different methods

	Recovery (% of intake)	
	Static digestion method	***Dynamic digestion method***
Maltodextrin DE 11-16	26.3	23.5
Tapioca Dextrin	22.4	8.79
"Modified starch"	12.3	12.2

Table 19 shows that the values of recovery for static digestion method are very similar to those obtained for dynamic digestion method, except for Tapioca Dextrin. Recovery obtained in the static method of different carbohydrate sources are more or less as expected because the largest polysaccharide chain has the lower recovery and vice versa. So the low value of recovery for Tapioca Dextrin can be a result of experimental errors, because a value of recovery for Tapioca Dextrin between Maltodextrin DE 11 – 16 and "Modified starch" was expected.

For dynamic digestion method, the bio-accessible fractions of glucose increase in a similar way during the first 90 min for Maltodextrin DE 11-16 and Tapioca Dextrin. Then, bio-accessible fraction of glucose for Maltodextrin DE 11-16 increases drastically while bio-accessible fraction of glucose for Tapioca Dextrin increases moderately and seems to reach a constant value at 150 min.

The increase of glucose in the bio-accessible fraction of "Modified starch" is much slower. As we already mentioned, the "Modified starch" is the polysaccharide with the longest chain, i.e. the one with slowest digestion. Consequently, it's not surprising that the released glucose from "Modified starch" is delayed. However, it's more surprising that it is not completed, even after 300 min, what is confirmed by the low value of recovery for "Modified starch" after 5 h.

5. CONCLUSIONS

A series of *in vitro* digestions were carried out to study digestibility and bio-accessibility of three different carbohydrates sources. The obtained results show the dependency of length chain of the carbohydrate source and glucose release from the carbohydrate source, except for Tapioca Dextrin when the dynamic digestion method was used. However, only one experiment for each carbohydrate source with dynamic digestion method was carried out, and these results could be caused by experimental errors.

The values of the recovery of glucose (Table 19) are lower than expected, and it seems that the digestion is not complete. Those results could be explained due to glucose being the last product of the starch digestion. Before reaching free glucose monomers the starch chains are broken into oligosaccharides that are consequently broken into tri- di- and monosaccharides.

One of the initial hypothesis was that the matrix of the clinical nutrition products could affect digestibility of polysaccharide, but the results show that there are no significant differences between the different used matrixes. Only it was confirmed that by pre-treatment at 121°C favors the solubilization of the polysaccharide and consequently the recovery is higher than without pre-treatment.

At the same time, two methods of *in vitro* digestion have been studied to know the advantages and drawbacks of each one, and to find out in possible future studies which method is more convenient (depending on the target of study). The results, as expected, show that the values of the recovery are similar between both methods, which is important for future experiments. It was also proved that the static digestion method is faster and easier because two experiments can be carried out at the same day. However, the results with dynamic method are more reliable, because the method mimics better human digestion than static digestion method.

Furthermore, effect of the volume was studied. The obtained results take us to the conclusion that static digestion method with small volumes can be carry out because it does not affect the final result and also it saves resources.

5.1. Following steps

Due to the complexity of the used methods (enzymes, static method, dynamic method, …) only a limited number of experiments were able to be performed under the scope of a master's thesis. Therefore, the repetition of these experiments could be the next step for more accurate results.

The obtained values of the recovery are lower than as expected, but we have to take into account that the monosaccharide glucose is the last product of starch digestion, and for this reason, the next step could be the measurement of the concentration of maltose (two molecules of glucose).

Looking the obtained results of the study of matrix effect, it could be interesting to try more complex matrixes, to study how a more complex matrix could affect the digestibility of these polysaccharides.

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