# Bioavailability of Nutrients and Micronutrients: Advances in Modeling and In Vitro Approaches

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5. Conclusion

Abstract

The bioavailability of food nutrients and micro-constituents is recognized as a determinant factor for optimal health status. However, human and animal studies are expensive and limited by the large amount of potential food bioactive compounds. The search for alternatives is very active and raises many questions. On one hand, in vitro digestion systems are good candidates, but to date mainly bioaccessibility is correctly assessed. To go further, to what degree should they reproduce natural processes? What techniques can be used to measure the changes in food properties and structures in situ in a non-invasive way? On the other hand, modeling approaches have a high potential, but their development is time-consuming. What compromises should be done between food and physiology realism and computational ease? This review of the literature addresses these questions by identifying the most promising dynamic in vitro systems, highly resolved analytical methods, and detailed computer models and simulations.

1. Introduction

Foods we eat are complex systems. They are made of various compounds: nutrients and micronutrients, but also micro-constituents and non-digestible nutrients (specific polysaccharides called dietary fibers, only fermentable in the colon). All of them may interact chemically, and are organized at several scales in structural units constituting the food matrix, which differs widely among diverse foods (Aguilera 2006).

Recently, food research focused on the role of these structures in the bioavailability of food compounds. This was shown to highly depend on the food matrix and on its processing (Parada & Aguilera 2007). The first step towards bioavailability is called bioaccessibility,

consisting in the gastrointestinal release of food compounds, and is especially influenced by food structures (Marze 2013; Marze 2015). Those indeed need to be broken down mechanically, and nutrients need to be hydrolyzed by enzymes before they can be released in an absorbable form. Compelling examples are given by vegetal foods composed of cells, such as almond seed, vegetables, or legumes. Their cell walls must be disrupted mechanically to allow the release of lipid, carotenoid, or starch, respectively (Ellis et al. 2004; Lemmens et al. 2014; Dhital et al. 2016).

McClements et al. (2015) proposed a bioavailability classification scheme taking many factors into account, the three major ones being bioaccessibility, absorption, and transformation of nutrients within the gastrointestinal tract (e.g. by chemical degradation or metabolism, as sub-factors of transformation). However, the study of these factors is complicated and restricted in human, such that animal, in vitro, and modeling alternatives are developed.

In this review, the focus is on the upper part of the digestive tract, where most of the digestive processes take place, before absorption in the small intestine. The large intestine (colon) is left out as it only plays a minor role in nutrient and micronutrient absorption. Advanced techniques are presented, principally for the investigation of bioaccessibility. Specific methods indeed exist to investigate the structural aspects of the food matrix within the digestive tract. First, in vitro digestion systems are introduced, with emphasis on dynamic systems. Then, innovative methods allowing dynamic non-invasive measurements are described. Finally, modeling approaches relevant to the study of both local mechanisms and global bioavailability are reported.

## 2. In vitro systems

The first requirement for in vitro studies is the use of realistic digestion systems, generating results at least qualitatively comparable to in vivo ones. This is an essential aspect benefiting from important research efforts. The main characteristics usually reproduced are temperature, pH, residence or transit time, and digestive fluid composition. A distinction is commonly made between static (maintaining constant quantities) and dynamic (allowing time-dependent quantities) systems. Although there were precedents, the systematic development of various digestion systems took off in the 1990s, mostly to study food digestion on one hand, and bioaccessibility of contaminants or soil minerals on the other hand (Marze 2015). In this section, the stress is on dynamic and advanced systems, as early ones were already reviewed thoroughly (Hur et al. 2011; Guerra et al. 2012; Marze 2013; Carbonell-Capella et al. 2014; Alminger et al. 2014).

### 2.1. Static

The basis of most static food digestion systems is continuously stirred and thermostated glass bottles. Classically, three bottles represent the main three steps, namely oral, gastric, and intestinal, an additional colonic step being used in some microbiology studies. Temperature is usually set to 37 °C. Stirring is set so that homogeneous mixing is achieved. In the first bottle, liquid food or homogenized solid food is mixed with artificial saliva fluid for a given duration (several seconds to several minutes), constituting the bolus. Then, this bolus is mixed with artificial gastric fluid in the second bottle for a given duration (several hours), constituting the chyme. Finally, this chyme is mixed with artificial intestinal fluid in the third bottle for a given duration (several hours), constituting the chyle. The adjustment of pH is done at the very beginning of each step. Artificial fluids are usually composed of physiological electrolytes, enzymes of animal origin (more rarely of human origin), and other specific compounds (e.g. mucin, bile).

On this basis, many static protocols were developed in the 2000s, yet using different parameters, even when similar studies were conducted. In order to harmonize research works, a European network was formed to identify the average values adapted to the human adult. An international consensus was reached, leading to a published set of recommendations (Minekus et al. 2014).

Another type of static system is cultured cells. The most common setup is a monolayer of Caco-2 cells, resembling small intestine ones with microvilli, tight junctions, and various enzymatic activities. This is used to investigate absorption (sometimes called uptake or transport in this context), or crossing from the apical side to the basolateral side (sometimes inadequately termed in vitro bioavailability). Cultured cells can be coupled with a digestion system to obtain a more realistic sequence of processes (Alminger et al. 2014).

### 2.2. Dynamic

Contrary to a static system, which is often used as a screening method, a dynamic one seeks to replace clinical studies. Thus, it is scarcer, as it requires much more work to be developed and quantitatively validated against in vivo data. Early efforts were mostly related to mastication, as the in vivo validation of the characteristics of food fragments and bolus is relatively simple. More recently, the attention extended to the rest of the digestive tract.

#### 2.2.1. Mastication

Early works focused on the particle size distribution in the bolus for various foods masticated by humans (Manly & Braley 1950; Lucas & Luke 1983a; Olthoff et al. 1984). But the investigation of the role of oral breakdown on subsequent digestion is much more recent. A typical example is the case of almond seed, presenting resistant cell walls which need to be broken down by processing or mastication in order to release the intracellular lipid. This was indeed shown to control the postprandial lipemia (a measurement of lipid bioavailability), as well as the fecal lipid content (Ellis et al. 2004; Berry et al. 2008).

To study the various aspects of oral processing, some in vitro mastication systems were developed, already reviewed by Morell et al. (2014) and Peyron & Woda (2015). In summary, the main goals are to reproduce the bolus characteristics, such as particle size distribution, saliva incorporation, rheological or mechanical properties, release behavior of flavor or taste compounds, or several aspects together. Most of the systems are dynamic, so these characteristics can be investigated during time. In some cases, they were correlated to texture, flavor, or taste perceptions in humans.

In the context of digestion, the use of such systems to reproduce the bolus formation of highly structured foods should be of high interest. Particle size distribution and saliva incorporation are expected to be the most relevant parameters influencing subsequent digestion processes.

#### 2.2.2. Digestion

Several types of in vitro dynamic systems were developed, mostly mechanical and physicochemical ones.

##### 2.2.2.1. Mechanical

As previously seen, mouth has an essential mechanical role. In the stomach, a grinding process completes the oral breakdown of the bolus. In the small intestine, the mechanical aspects become a matter of fluid mechanics. In general, as food progresses down the digestive tract, the mechanical stress decreases and the transit time increases, as reviewed quantitatively by Marze (2016).

Kong & Singh (2008; 2010) were the first reporting a dynamic artificial stomach with mechanical capabilities. Their first version was able to apply continuous or periodic anisotropic forces, but did not include dynamic secretions, pH, and gastric emptying (Kong & Singh 2008). Their second version (called the human gastric simulator HGS) was able to reproduce typical peristaltic forces, as well as dynamic secretions, pH, and gastric emptying (Kong & Singh 2010). Both were evaluated by studying the disintegration of solid foods due

to mechanical grinding and enzymatic hydrolysis. The HGS was then used to investigate structural evolutions or micro-constituent release for various foods: rice (Kong et al. 2011), fortified almond butter (Roman et al. 2012), and protein emulsion gel (Guo et al. 2014). No systematic comparison between HGS and in vivo digestion was conducted so far.

The other major dynamic gastric system including mechanical aspects is the dynamic gastric model (DGM), developed for both pharmacological and nutrition applications (Vardakou et al. 2011). It is able to apply shear forces using a piston/barrel device, and to control dynamic secretions, pH, and gastric emptying. It was used in combination with a static intestinal system to study the bioaccessibility of various micro-constituents (López de Lacey et al. 2012; Mandalari et al. 2013), and the digestion of human milk proteins (only gastric, Zhang et al. 2014), of cereal starch (Ballance et al. 2013), and of almond lipid (Mandalari et al. 2014). The DGM was validated against in vivo data in some pharmacological studies, and in one nutrition study, where the glycemic index could be predicted in vitro (Ballance et al. 2013). Recently, another dynamic system was developed (advanced gastric compartment TIMagc), reproducing the stomach shape in three parts, the differential pressures in these parts using flexible walls, and the dynamic secretions, pH, and gastric emptying (Bellmann et al. 2016). All these aspects were quantitatively validated against in vivo data.

Simpler gastric systems focusing on mechanical aspects were also developed, using oscillating probes to apply shear or compressive forces (Chen et al. 2011; Verrijssen et al. 2014; Do et al. 2016), or using rollers on deformable surfaces to apply peristaltic forces (Kozu et al. 2014; Wang et al. 2015). Only one of them accounted for the other dynamic parameters, namely secretions, pH, and gastric emptying (Do et al. 2016).

A few intestinal systems were developed with mechanical purposes, essentially capturing fluid mechanics. The first one reported, named the small intestinal model (SIM), reproduces peristaltic and segmentation motions, secretions, and allows nutrient mass transfer through a

semi-permeable cellulosic membrane with a cutoff of 8 *kDa* (Tharakan et al. 2010; Gouseti et al. 2014; Jaime-Fonseca et al. 2016). It is linked to a spectrophotometer for direct mass transfer analysis. Up to now, it was only used for carbohydrate digestion and absorption at room temperature, with no comparison to in vivo data. A comparable system including the same features was reported recently for the duodenum, with a more realistic shape, and the possibility to work at body temperature (Wright et al. 2016).

2.2.2.2. Physicochemical

This type of dynamic digestion system aims at reproducing the temporal change of pH, secretions, luminal flows, and absorption, usually for both the stomach and the small intestine. The mechanical aspects are usually basic (pressuring or stirring device). The pioneering systems are the TNO gastrointestinal model (TIM-1, Minekus et al. 1995) comprising four compartments (stomach, duodenum, jejunum, and ileum), and the simulator of the human intestinal microbial ecosystem (SHIME, Alander et al. 1999), comprising more compartments (three colonic ones). Although the SHIME was mainly dedicated to colonic microbiology, it was designed to reproduce most aforementioned parameters, except absorption. So, in principle, it could be used to study gastrointestinal food digestion.

On the other hand, the TIM-1 was extensively used to perform both pharmacological and nutrition studies. The latter focused on micronutrient or micro-constituent bioaccessibility, or on nutrient digestion for various food systems. The most representative examples are reported below. First, an evaluation of the bioavailability of iron and phosphorus was conducted in cereals (Larsson et al. 1997). Bioavailability was represented by the percentage of the mineral dialyzed through a cellulosic hollow-fiber membrane (cutoff 5-10 *kDa*) out of each absorptive intestinal compartment (jejunum and ileum). Such a measurement was later renamed bioaccessibility as the artificial membrane is not truly representative of the intestinal cell membrane. This analysis of dialysates was then used in many works: for fatty acids and

cholesterol from yogurt/oil/egg meals (Minekus et al. 2005), folate from orange juice (Öhrvik & Witthöf 2008), ferulic, *p*-coumaric, and sinapic acids from various breads (Anson et al. 2009), riboflavin from soy-zein protein microspheres in yogurt (Chen et al. 2010), simple sugars from ground wheats (Lafond et al. 2011), bivalent iron from whey protein gel particles (Martin & de Jong 2012), anthocyanins from various berries (Lila et al. 2012; Ribnicky et al. 2014), nitrogen from caseinates or cheeses (Havenaar et al. 2013), eicosapentaenoic acid from milk/yogurt meals (Domoto et al. 2013), β-carotene from two homogenized diets (Van Loo-Bouwman et al. 2014), carotenoids from cooked eggs (Nimalaratne et al. 2015), fatty acids from human milk or infant formulas (Fondaco et al. 2015), and fatty acids from fat/bread meals (Thilakarathna et al. 2016). Five other works used the TIM-1 without the artificial membranes, in order to investigate the fate of foods directly within the intestinal compartments. Blanquet-Diot et al. (2009) examined the stability of xanthophylls and carotenoids from various homogenized meals. Déat et al. (2009) studied the bioaccessibility of lycopene and α-tocopherol from a homogenized meal. Gervais et al. (2009) investigated the bioaccessibility of fatty acids from different milks. Villemejane et al. (2015; 2016) studied protein and starch hydrolysis in short-dough biscuits.

Recently, a new system mimicking the stomach and the small intestine (called the DIDGI) was developed, allowing the temporal control of secretions, pH, and gastric emptying (Ménard et al. 2014; Sánchez-Rivera et al. 2015). It was used to study milk protein digestion, and was validated for infants using in vivo data in piglets (Ménard et al. 2014).

Lately, a system resembling the TIM-1 was developed for both food and drug applications (the engineered stomach and small intestine ESIN). The main improvements were the inclusion of a mouth compartment, and the design of a gastric compartment allowing a differential emptying of liquids and of solids depending on particle size. It was validated for tablet drugs using an optional pharmaceutical basket retaining the tablet during disintegration

in the different compartments. The percentage of theophylline dialyzed through hollow fiber membranes was correlated to the percentage absorbed in vivo in healthy humans (Guerra et al. 2016).

In summary, the development of mechanical or physicochemical dynamic digestion system progressed quickly these last years, yielding more and more realistic devices, sometimes validated against in vivo data. A major limitation is still the difficulty to separate the enzyme substrates and their digestion products, done by absorption in vivo. It is indeed known for all nutrients that digestion products tend to inhibit further enzymatic hydrolysis. The use of semi-permeable membranes is valuable, but not adapted to all nutrients. The use of different cutoffs along the small intestine could provide an improvement. The systematic inclusion of a cultured cells compartment could be a good solution, but challenging in practice, due to variable survival conditions. Some other options will be discussed in the next section.

## 3. Innovative analytical methods

A major limitation of most in vitro digestion systems is the necessity to collect and store samples for further characterization. This questions the representativeness of the samples, which need to be incubated with enzyme inhibitors and/or snap frozen before storage. Frequently, they are also subjected to ultracentrifugation, solvent extraction, among other preparation steps. To avoid sampling, various methods were developed to characterize physicochemical quantities directly within a digestion system (in situ), in a non-invasive way. These techniques usually derive from well-established ones for food and nanoparticle characterization (Luykx et al. 2008). In the following, the focus is on methods allowing a continuous monitoring of digestion kinetics.

### 3.1. Scattering techniques

These techniques are all based on the scattering of radiation (wave and/or particle). They are used to study the geometrical features of ordered and disordered matter at the molecular and supramolecular scales. A very common one in the laboratory is dynamic light scattering (DLS), allowing the measurement of an equivalent particle diameter in a range about 1-1000 *nm*. However, to resolve all size, shape, and structure, neutron or X-ray scattering should be used, measuring in a range about 0.1-100 *nm*. Wide-angle X-ray instruments for crystalline matter are common in the laboratory, whereas small-angle ones for amorphous matter are scarcer. The main sources of neutron and X-ray beamlines are research nuclear reactors and synchrotrons, respectively.

The early kinetic studies using small-angle X-ray scattering (SAXS) were performed using laboratory instruments. Then, synchrotron sources were preferred due to their high intensity, allowing fast and highly resolved measurements.

The first kinetic study focused on the fasted intestinal digestion of drug nano-emulsions made of lipids, showing that a fast transition producing a lamellar phase occurs, followed by a slow transition producing a hexagonal phase (Fatouros et al. 2007). The subsequent investigations applied to both drug and food systems. For the fed state with food systems, the first study also showed fast transitions from emulsion droplets to various liquid crystalline or lamellar phases upon intestinal digestion, depending on pH and bile salt concentration. Vesicles and elongated micelles were found to be the dominant structures towards the end of the digestion (Salentinig et al. 2011). Then, the same author reported results on the intestinal digestion of milk fat. At a low bile salt concentration, inverse micelles formed inside the lipid droplets right from the digestion start (less than 1 *min*). Then, various liquid crystalline structures formed successively inside the lipid droplets. In contrast, at a high bile salt concentration, mainly vesicles formed in the aqueous phase, with slow kinetics (Salentinig et al. 2013; 2015a). At a normal adult bile salt concentration, Marze et al. (2015) studied the intestinal digestion of

emulsions and found no liquid crystalline structures, but the fast (2-3 *min*) appearance of coexisting micelles and vesicles (micelle being the dominant structure throughout digestion), growing by coalescence as digestion proceeded. This growth was reported to depend on the digested triglyceride, more significant for triolein than for tricaprylin. The characterization of these lipid structures is important as they are responsible for the transport of lipophilic micronutrients and micro-constituents. Mixed micelles composed of bile salt, phospholipid, fatty acid, and monoglyceride are classically evoked for this role, but these results show that liquid crystalline or lamellar structures dominate in certain conditions. This questions the mechanisms of transport and absorption in these situations.

In parallel, kinetic studies were also conducted for starch. The first results obtained by laboratory SAXS showed that 5 *nm* amylose crystals tend to form quickly upon digestion of a high-amylose starch (essentially amorphous), accompanied by an increase in double helical order and crystallinity. These crystals were hypothetically identified as the resistant starch fraction (Lopez-Rubio et al. 2008). Similar results were obtained for a semi-crystalline starch, the amorphous region being especially susceptible to enzymes (Zhang et al. 2010). In a SAXS and time-resolved small-angle neutron scattering (SANS) study, Blazek & Gilbert (2010) found that various semi-crystalline starch granules were all preferentially accessed by enzymes through the amorphous growth ring, unwinding double helices and hydrolyzing branching points, leading to the destabilization of the crystalline lamellae. The presence of granular pores and channels was found to enhance enzyme accessibility, and the length and rigidity of amylopectin spacers and branches were found to influence subsequent hydrolysis. In a multiscale investigation by the same research group, it was found that the main factor controlling maize starch digestibility was enzyme accessibility at the microscopic scale. Densely packed granules were slowly digested compared to granules presenting internal channels and pores. Mesoscopic and molecular features reported previously were found to

play a secondary role (Shrestha et al. 2012). To our opinion, more kinetic studies should be conducted to elucidate the structural determinants of the resistant starch fraction.

Synchrotron SAXS was recently used to monitor the structural changes of casein cluster during in vitro gastric digestion. It revealed surface fractals with dense primary clusters, changing to mass fractals with decreasing cluster density during digestion. The whole cluster as well as the primary clusters grew in size within the first 20 *min* of digestion (Jarunglumlert et al. 2015).

Another promising technique is coherent anti-Stokes Raman scattering (CARS), which was used as a label-free micro-spectroscopy tool to monitor the kinetics of lipid hydrolysis and lipophilic micronutrient release simultaneously during in vitro intestinal digestion of a tricaprylin emulsion (Day et al. 2010).

Finally, the use of diffusing wave spectroscopy (DWS), a technique similar to DLS but in the multiple scattering regime, was proposed as a non-invasive method to study in vitro intestinal digestion of emulsions. It was shown that the droplet size and diffusion coefficient distributions in turbid emulsions could be measured in situ, and the latter could be related to supramolecular structural changes. Vesicles were identified within 10 *min* of digestion, whereas micelles became progressively dominant after 2 to 4 hours of digestion, for tricaprylin and triolein, respectively (Marze et al. 2012).

Overall, these scattering techniques revealed the fast kinetics of digestion with regard to structural changes. In order to assess the unknown early processes, high intensity sources of radiation should be used, allowing fast measurements in a large spatial range.

### 3.2. Nuclear magnetic resonance

This technique was first applied to monitor the digestion of starch, this nutrient being structured at various scales. Some of the studies reported above used $^{13}C$ NMR spectroscopy to measure the evolution of the ordered and amorphous proportions of starch during digestion

(Lopez-Rubio et al. 2008; Zhang et al. 2010). The ordered proportion could be further resolved to single and double helix proportions (Shrestha et al. 2012). In parallel, time-resolved $^{1}$H NMR spectroscopy was used to construct the digestion kinetics of starch in terms of hydrolyzed α (1,4) link, and oligosaccharide then glucose production. Two regimes were observed, a fast one attributed to the initial hydrolysis of accessible starch chains, then a slow one attributed to the difficult access to inter-glucose linkages (Dona et al. 2011). The same technique was applied to a waxy rice starch in native or gelatinized states. The rate of hydrolysis by α-amylase and the number of α-glucan chains protruding from the starch granule could be monitored in situ. Both were higher in the gelatinized sate, interpreted as a preferential affinity of α-amylase for these protruding chains (Baldwin et al. 2015).

$^{1}$H NMR spectroscopy was also used to monitor the kinetics of in vitro intestinal digestion of emulsions in situ. The diffusion coefficient of several groups along the lipid alkyl chains was measured. This quantity initially represented the movement of the triglyceride chains in a triglyceride environment (in terms of viscosity), but quickly evolved to represent the movement of a fatty acid chain in a fatty acid environment. This showed that triglycerides were quickly hydrolyzed within 30-60 *min*, which was compared to the timescales for structural changes, either much shorter for the appearance of vesicles, or much longer for the predominance of micelles (Marze et al. 2012). A recent study extended the technique by using deuterated tricaprin examined by $^{2}$H NMR spectroscopy. It was also concluded that vesicles or micelles formed, but this was not quantified (Salentinig et al. 2015b). Note that other researchers also used $^{1}$H NMR spectroscopy to resolve the chemistry of lipid hydrolysis (quantification of substrates and products), but not its kinetics and not in situ, as extraction and solvation were needed before measurement (Nieva-Echevarría et al. 2014; 2015).

Investigations using both low-field NMR relaxometry and $^{1}$H NMR spectroscopy were reported for in vitro digestion of proteins from cheese or processed meat. Relaxometry was

done in situ, whereas spectroscopy needed dilution and centrifugation as preparation steps. The former technique allowed the kinetic characterization of the physical matrix/digestive fluid interactions. The latter technique allowed the kinetic quantification of proteins, peptides, and amino acids (Bordoni et al. 2011; 2014).

Finally, another research group used low-field NMR relaxometry to study the swelling behavior of chitosan/BSA microparticles during in vitro digestion. An increase of the transverse relaxation time in the gastric compartment was attributed to the microparticle swelling. This result was confirmed in vivo using magnetic resonance imaging (Pregent et al. 2012).

On the whole, the use of NMR techniques is valuable to investigate the molecular aspects of digestion, although it is not always suitable for non-invasive measurements. Developing methods that enable physical and chemical characterizations in situ would constitute an important advance.

### 3.3. Mass spectroscopy techniques

The analysis of protein digestion entered the proteomic era recently. Coincidentally, proteomics developed on the basis of trypsin digestion as a preparation step for protein characterization. An interesting review marks the advent of various mass spectroscopy techniques to study the so-called protein digestomics (Picariello et al. 2013). This essentially consists in the identification of peptide fragments and sequences upon gastrointestinal digestion, then searching for their potential bioactivities. In one study, human milk was submitted to a dynamic gastric digestion, and the abundance of 413 proteins was quantified as a function of time (Zhang et al. 2014). In two other studies, the gastric digestion kinetics of specific proteins from cow milk were investigated, namely purified lactoferrin (Grosvenor et al. 2014), or caseins from skimmed milk powder (Sánchez-Rivera et al. 2015). Other works also included the intestinal digestion step, again for caseins in dairy products (Sánchez-Rivera

et al. 2014), or for ovalbumin from egg white (Nyemb et al. 2014), or for proteins from pork meat (Wen et al. 2015). However, these latter studies did not include kinetics, only examining the peptides profile at the end of the digestion.

Scarcer developments were made in the way of lipid digestomics. One research group coupled high performance liquid chromatography (HPLC) and mass spectroscopy to determine the digestion and oxidation substrates and products of rapeseed oil submitted to in vitro gastrointestinal digestion. Altogether, they were able to quantify 155 compounds (Tarvainen et al. 2010; 2011; 2012). Another research group used mass spectroscopy to identify the stereospecific isomers produced during in vitro intestinal digestion of tricaprin deuterated at different sn-positions (Salentinig et al. 2015b). Up to date, only the end products of lipid digestion were analyzed, and no kinetics was reported.

All in all, mass spectroscopy techniques are promising as they allow the characterization of many compounds simultaneously. They have a high potential for the discovery of novel bioactivities (beneficial or detrimental). However, they usually need invasive preparation steps and are too complex in their set up and interpretation to consider systematic kinetic studies for the time being.

### 3.4. Interface techniques

These techniques are designed to isolate interfacial from bulk interactions, for instance by studying lipid in a single droplet, or protein only located at model interfaces. For example, Langmuir trough was used by enzymologists to investigate lipases and lipolysis at planar interfaces (Verger & de Haas 1976). Latter, similar studies were conducted using rising oil drop tensiometry (Nury et al. 1987; Labourdenne et al. 1994; 1997).

These approaches were recently reexamined for the systematic analysis of lipid and protein digestion at interfaces. The dynamic interfacial tension of various lipases and lipolysis products at the oil-water interface was studied using pendant aqueous drop tensiometry. The

digestion kinetics could be monitored for triglycerides, diglycerides, or monoglycerides, the lipolysis products inducing a decrease of the interfacial tension. However, fungal lipases were used, having different stereospecificities compared to gastrointestinal lipases, and no bile salt was included (Reis et al. 2008; 2009). To be more realistic, a purified pancreatic lipase and a bile salt extract were used to study the intestinal digestion of olive oil with a similar technique. By adding compounds sequentially, the interfacial effects due to the adsorption of either the bile salts or the digestion products could be separated (both decrease the interfacial tension). In the absence of the bile salt extract, lipase was completely inhibited by the presence of a poloxamer, and only partially by the presence of a phospholipid (Torcello-Gómez et al. 2011). This approach was further refined using a purified pancreatic lipase and a purified bile salt. As rising oil drop tensiometry was used, both the kinetics of lipolysis and of micellar solubilization could be monitored. Those were influenced by the type of triglyceride (tricaprylin or triolein), but not by the type of emulsifier initially present at the interface (β-lactoglobulin or sodium oleate). Interfacial tension showed that bile salt dominates at the interface, making it fluid as evidenced by the decrease of the interfacial dilatational viscoelasticity moduli (Marze & Choimet 2012). In a second work, the sequential digestion (mouth, gastric, and intestinal) of two fish oils in the presence or absence of β-lactoglobulin was examined. The digestion fluids were much more complex, notably including amylases and proteases, adsorbing at the interface. The supplemental effects of adsorption competition and interfacial proteolysis could be identified (Marze et al. 2013). Rising oil drop tensiometry was also applied to monitor solely the proteolysis of interfacial protein (β-lactoglobulin or β-casein), using pure gastrointestinal proteases (and no lipase). This revealed that the production of peptides increases the interfacial tension, except in the presence of bile salts where all other compounds are removed from the interface, proteolysis then likely taking place in the aqueous phase (Macierzanka et al. 2009). Interfacial proteolysis was further demonstrated at the air-

water interface using pendant aqueous drop tensiometry, and was imaged by AFM using Langmuir-Blodgett films transferred from a Langmuir trough. The protein network as imaged by AFM was significantly disrupted upon proteolysis only when a lipid emulsifier was present in the system (Maldonado-Valderrama et al. 2010). Recently, pendant aqueous drop tensiometry at the oil-water interface was applied to the sequential digestion of protein (β-lactoglobulin or β-casein) by pepsin, then of lipid by pancreatic lipase and bile salt. The interfacial tension was confirmed to increase upon proteolysis and to decrease upon lipolysis. The decrease of the interfacial dilatational viscoelasticity was confirmed to be mostly due to bile salt, although lipase also played a minor role (Maldonado-Valderrama et al. 2013). Similar results were found with an additional trypsinolysis step before the lipolysis step. A pulsed xenon light treatment of β-lactoglobulin at the interface had minor effects on the interfacial dilatational viscoelasticity, whereas the interfacial shear interfacial elasticity was significantly increased. The latter decreased mainly upon pepsin digestion as the protein network was disrupted, trypsin digestion and bile salt being required to fluidize the interface only in the most extreme treatment condition (del Castillo-Santaella et al. 2014).

Another approach allowing the isolation of the interfacial phenomena is the study of digestion at the level of the individual droplet. Such a scale was already investigated using light microscopy in the 1980s. However, the droplet size was polydisperse, and bulk interactions (droplet flocculation and coalescence) could occur during digestion (Patton & Carey 1979; Patton et al. 1985). In order to control these parameters, Marze et al. (2014) designed a microfluidic device allowing the generation of 100 $\mu m$ monodisperse oil droplets which can be subsequently retained inside a digestion chamber in individual traps, thus not in interaction. Sequential digestion (mouth, gastric, and intestinal) can be performed by continuously flowing digestive fluids through the chamber, what also removes the digestion products. The decrease in droplet size due to lipid digestion was monitored using an inverted

microscope recording images. The type of triglyceride was found to be the main parameter controlling the kinetics, in agreement with emulsion studies. However, the digestion rates were much higher than those reported for emulsions. This was explained by the highly diluted state of oil in this system, and also by the absence of flocculation and coalescence. Interfacial investigations could be performed by varying the digestive fluids composition and the initially adsorbed emulsifier (protein, lipid, polysaccharide…).

Overall, these techniques are very interesting to resolve interfacial mechanisms and interactions in a controlled way, but they should always be compared to bulk behaviors to evaluate the weight of their contribution among all the phenomena.

In summary, the application of innovative techniques to monitor in vitro digestion allows much more precise quantifications. From the physical point of view, scattering techniques are able to resolve structures at various scales with high temporal resolution, and interface techniques can probe the molecular interactions, providing indirect indications about the interfacial structures and mechanisms. From the chemical point of view, methods including a mass spectroscopy technique have the potential to identify all digestion products (including non-digestive reactions like oxidation), and new developments enable the interpretation of such complex systems for the first time. NMR is versatile and can be applied to quantify both structures and chemical species.

4. Modeling approaches

A very promising way to understand food digestion is to simulate one or several processes mathematically. This was specifically reviewed recently, including an historical perspective comparing the pharmacology and nutrition advances (Marze 2016). Briefly, two approaches are commonly used to design a model, either focusing on a single process (mechanical,

chemical, physical) with a high level of details, or considering the whole digestive tract (sometimes including the systemic circulation) with a limited amount of details. In this section, both approaches are presented, but the mathematical formalisms are not included, as they were already reported before (Marze 2016).

### 4.1. Mouth

The main aspect modeled in the oral cavity is the mechanical breakdown by mastication. This is a very important process for the selection of food particles before swallowing and further digestion (initiated by enzymes in the saliva, although to a minor extent). The first attempts to model mastication intended to predict the reduction in particle size. The evolution of the particle size distribution of carrot could be calculated as a function of the number of chews (Lucas and Luke 1983b). Then, with a similar model with 2D geometric considerations, it was also possible to simulate the cohesive force between food particles, interpreted as a determinant of bolus swallowing (Prinz & Lucas 1997).

Nowadays, computational techniques allow a full 3D representation of the oral cavity. This was already used about 10 years ago to simulate the muscle forces during a chewing cycle, solved by the finite element method (Röhrle & Pullan 2007). The discrete element method was also used with a 3D representation of teeth to evaluate the stresses acting on a model food depending on the rate and mode of chewing (Zhang & Hui 2015). The most advanced simulation to date is based on the smoothed particle hydrodynamics method. It was validated for elastobrittle agar gels with a 3D representation including jaws, teeth, tongue, palate, cheeks, and lips. The simulated particle size distributions after two chewing cycles compared successfully to the experimental ones. Moreover, the force, strain, and stress could be predicted as a function of chewing time (Harrison et al. 2014b). In a second study, the mechanical effects of the type of food (elastoplastic or brittle solids) and of the incorporation of saliva were investigated. The deformation or the fracture behaviors were predicted, as well

as the fluid velocity in the anterior oral cavity induced by tongue movement (Harrison & Cleary 2014). For real foods, many other phenomena may occur in the mouth, such as melting due to temperature. The mixing of food and saliva may also modify the cohesion, adhesion, and lubrication properties of the bolus, and induce a softening of food, which needs to be accounted for. The release of taste and aroma compounds could also be considered, based on all the mechanisms above plus some dissolution, diffusion, and advection effects. For aroma compounds, the gas flow in the oral and nasal cavities should also be simulated. Finally, the inclusion of chemical reactions and their relation to sensory perceptions and digestion would complete the model. Preliminary works concerning the thermomechanical coupling, the heterogeneous nature of the food (solid and liquid parts), and the diffusion or release of taste compounds were performed for various model foods, constituting an excellent start for further improvements (Harrison et al. 2014a).

### 4.2. Stomach

The gastric step of digestion is the last stage for particle breakdown and selection. The breakdown is obtained by peristalsis, and the selection by recirculation and differential emptying through the pyloric valve, only allowing particles smaller than about 1 *mm*. Although this step also plays an important role in enzymatic digestion, only the mechanical aspects for liquid foods were simulated so far. First, the pressure and velocity fields were calculated for a 2D stomach representation using a lattice-Boltzmann method. The mixing and gastric emptying behaviors were simulated, depending on the fundic and antral contractions. Retropulsive jet-like motion at the pylorus and recirculation between fundus and antrum due to vortices were evidenced (Pal et al. 2004). A role for the antral contraction waves in gastric emptying was demonstrated in a second study (Pal et al. 2007). Note that another 2D model with a non-realistic stomach geometry recovered the retropulsive and recirculation flows. This

is the only model where pepsin was included, although not considering hydrolysis, but only mixing (Kozu et al. 2010).

Most of the results above (except the gastric emptying which was not simulated) were later confirmed with a 3D stomach representation using a computational fluid dynamics solver (Ferrua & Singh 2010). The role of the liquid food viscosity was studied, demonstrating that a viscosity above 0.17 *Pa.s* inhibited the formation of the retropulsive jet-like motion and of the vortices, hence of the recirculation. The stretching behavior was also quantified, obeying the same viscosity threshold in the fundus part of the stomach. The authors concluded that low-viscosity motions should not play a major role in gastric mixing as the very low threshold (around the viscosity of tomato juice) could often be exceeded, except for water-like fluids (Ferrua et al. 2014).

Further works could examine the effects of a solid food phase, in terms of liquid-solid mixing or segregation, and of particle breakdown and selection, including differential gastric emptying. For specific foods, the role of gastric lipase, or pepsin, or the residual activity of salivary α-amylase should be accounted for.

### 4.3. Small intestine

This part of the digestive tract is the one where absorption takes place, but hydrolysis has to be completed before. Moreover, intestinal flows are not well-known, so it is generally assumed that absorption occurs by diffusion, which is actually true only at the intestinal cell level. To clarify this matter, a multiscale lattice Boltzmann method was used to study advection and diffusion in the lumen and in the mucus layer. Macroscale bulk flow, microscale villi motion (assumed to be pendular), and absorption were simulated. Flows were dominated by advection, generating vortices at both scales. In between, a layer was evidenced where transport occurred by diffusion. The motion of the villi was shown to increase the absorption rate, suggesting an important function related to fluid mechanics (Wang et al.

2010a; 2010b). A similar model confirmed these results at the villi scale, neglecting the lumen flow. The motion of the villi was assumed to follow lateral relaxation/contraction cycles due to mucosal folding. Absorption was found to be mainly dependent on the villi motion producing vortices, with a small effect of chyle rheology (Newtonian or shear-thinning). Nutrient diffusivity also influenced absorption, although to a minor extent (Lentle et al. 2013; Lim et al. 2015). Another lattice Boltzmann model was developed to explore the fluid mechanics in the intestinal lumen. Both longitudinal and circular contractions were simulated. The first type of contraction generated relatively high shear rates able to mix viscous fluids and accelerate the diffusive mass transfer. The second type of contraction had a smaller effect on fluid mixing, even in the presence of a pylorus outflow, but could probably be involved in the presence of solids (de Loubens et al. 2013). Using the same model, the concentration of a dye tracer mixed by longitudinal contractions ex-vivo was successfully simulated. However, the mixing at the periphery of the small intestine was underestimated, the authors concluding that the contribution of mucosal folding must be considered (de Loubens et al. 2014).

Other works did not include intestinal anatomy, but focused on chyle structural parameters instead. Most of them were based on mass transfer or transport, modeled using differential equations. For lipid, mathematical expressions were derived for the mass transfer from emulsion droplets to the aqueous phase, representing the release of free fatty acids due to hydrolysis and solubilization during intestinal digestion. The first expression obtained was based on a zeroth-order equation including the physicochemical parameters describing the droplet size and composition (Li & McClements 2010). Although the initial differential equation was correct, there was an error during the derivation of the solution, which was recently corrected (Gaucel et al. 2015). In the interval, an alternative calculation method was used, leading to the correct solution (Marze & Choimet 2012). Note that the initial differential equation was also directly used to analyze hydrolysis and solubilization data (Giang et al.

2015; 2016), thus avoiding the use of the erroneous solution. First-order differential equations were also obtained, allowing the inclusion of the Kelvin solubility equation depending on droplet size and composition, but also on interfacial tension. The latter could also be decomposed into interfacial viscoelastic properties. Various solutions were calculated and tested on droplet size and release of digestion products data. Better agreements were obtained for the first-order equations compared to the zeroth-order one, especially when the measured interfacial viscoelastic properties were included in the model. For starch, it was also reported that first-order kinetics best describes the whole course of hydrolysis, although Michaelis-Menten kinetics was appropriate for the early stage (Goñi et al. 1997; Mahasukhonthachat et al. 2010). In fact, it was recently shown that a fast regime and a slow regime may follow, both obeying first-order kinetics. This result was evidenced using a logarithm of the slope analysis. The type of starch and its state (native, gelatinized, or retrograded) were found to influence these regimes (Butterworth et al. 2012; Patel et al. 2014; Edwards et al. 2014).

Models were also developed assuming a generic behavior for all nutrient types. They usually included both intestinal hydrolysis and absorption. The first ones were based on ideal or nonideal chemical reactors, supposing Michaelis-Menten kinetics (Jumars 2000a; 2000b). Then, a similar approach was followed with an additional term for advection, using Michaelis-Menten or first-order kinetics for the nutrient consumption rate by hydrolysis and absorption (Logan et al. 2002; 2003). Recently, a more comprehensive model was proposed, adding the effect of the peristaltic contractions on advection, and accounting for all species in the small intestine, including enzymes, substrates, solubilized and non-solubilized products, endogenous and exogenous water. The processes in the stomach were indirectly accounted for. First-order kinetics was assumed for both hydrolysis and absorption (Taghipoor et al. 2012; 2014).

Another approach was proposed to model lipid digestion and bioaccessibility, based on multi-agents simulation. Briefly, it consists in constructing a system made of various agents (representing various species) with specific properties and interaction rules (Hunt et al. 2009). In the case of lipid digestion, each type of molecule of a given mass was represented by a type of particle with specific physicochemical properties and interaction rules. Triglyceride and lipophilic vitamin particles were organized in a droplet (oil phase) surrounded by an aqueous phase containing digestive particles (lipase and/or bile salt). All particles were set to diffuse in their respective phase. Interaction between particles was governed by contacts through interfacial particles set at the droplet interface. Triglyceride particles were set to hydrolyze after a given number of contacts, inversely proportional to experimental hydrolysis rates. Hydrolysis products and lipophilic vitamin particles were set to solubilize after a given number of contacts, inversely proportional to experimental solubilization ratios (mass of solubilizate per mass of bile salt). Various types of oil (triglycerides, non-digestible oil), lipophilic vitamins, and digestion conditions were simulated. The results were in qualitative agreement with experimental data, highlighting a strong relationship between lipophilic vitamin bioaccessibility and fatty acid bioaccessibility, especially when mixed micelles were considered (Marze 2014; 2015).

### 4.4. Gastrointestinal

Only a few models explicitly represented both stomach and small intestine compartments. Most of them followed a compartmental simulation approach and were applied to pig digestion (ruminants are not considered in this review as the rumen considerably differs from the human stomach). Contrary to the last models presented above for the small intestine alone, simulated bioavailability, characterized as the amount of a nutrient leaving the small intestine by absorption, was compared to experimental bioavailability. The first comprehensive model accounted for endogenous secretions, gastrointestinal transit, all nutrients including digestible

and non-digestible cell walls, and minerals. Hydrolysis and absorption were supposed to occur only in the small intestine. Although the temporal trends were correct, nutrients bioavailability was systematically overestimated. This was attributed to the fact the intestinal cell metabolism was neglected (Bastianelli et al. 1996). This model was later refined by including an inhibitory effect of dietary fiber on proteolytic enzymes. Hydrolysis rate was supposed to depend on enzyme concentration, and nutrients absorption was supposed to be active (transporter-mediated). Endogenous and exogenous proteins were considered separately, as they were shown to obey different hydrolysis and absorption behaviors. Similar results were obtained, again with an overestimation of the experimental bioavailability of the nutrients (Strathe et al. 2008). Recently, a compartmental model was specifically developed for the gastrointestinal digestion of milk protein gels, with a stomach comprised of two parts and a blood plasma compartment to account for the distribution of amino acid into the body. Contrary to the previous works, hydrolysis was not represented. In the first stomach compartment, milk clotting due to acidity and aggregation was simulated. In the second one, endogenous secretions (including water), and gastric emptying were simulated. Different gel structures were obtained experimentally and fed to mini-pigs to investigate the kinetics of amino acid bioavailability. The model was able to reproduce these results quantitatively, correctly discriminating the various types of protein gel (Le Feunteun et al. 2014). A compartmental model was also developed for the gastrointestinal digestion of starch, including gastric emptying, fluid mechanics, hydrolysis, and absorption (no blood plasma compartment). Hydrolysis was assumed to obey Michaelis-Menten kinetics. Both convective and diffusive absorption modes were accounted for by using an effective mass transfer coefficient. Absorption was found to increase with decreasing chyle viscosity, increasing gastric emptying rate, and increasing hydrolysis rate (Moxon et al. 2016).

An interesting appropriation of these works is the comprehensive compartmental model of George van Aken (2016). In addition to most parameters mentioned above, it also includes some hormonal and neural feedbacks. This allows the regulation of food intake, gastrointestinal transit, and secretions, which can then be related to hunger and fullness. Moreover, the gastric tone is estimated from the volume inside the stomach, and the pH is calculated throughout the gastrointestinal tract.

All in all, the modeling of digestion is still a relatively new field, with various approaches and degrees of complexity. In general, models focusing on one phenomenon or on one compartment can account for food features, so they have a potential for food formulation and structuration to obtain a specific digestion effect. In the contrary, very comprehensive digestive tract models lack the food details, but provide a better understanding of the physiological aspects, probably more related to some long-term health effects.

## 5. Conclusion

In the field of food digestion, many developments were achieved lately. From the in vitro methodology point of view, a harmonized static protocol was published, and various dynamic systems were designed. They are able to reproduce many features of the human digestive tract, and some were validated against in vivo data. However, only a few can deal with real foods within the whole digestive tract, and there are still some issues with the inclusion of artificial absorption systems, which require specific studies. Innovative analytical methods were proposed to monitor digestion kinetics in situ in a non-invasive way. Structural and chemical aspects were explored in simple systems, and most techniques show a potential for the investigation of complex systems. Inclusion of a measurement technique in a dynamic digestion system was also tested using spectrophotometry, and such approach should be

further explored. Nevertheless, some techniques are currently too demanding for routine laboratory use, so more developments are necessary. Finally, although mathematical modeling of digestion started long ago, significant contributions to understand the role of food structure and composition are recent, and so are those with high anatomical and physiological realism. In the future, detailed numerical simulations could benefit from the gathering of the different compartments from a physiological perspective.

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Acronyms and Definitions list:

Micronutrients: essential compounds needed in microgram (for some milligram) amount, including exclusively vitamins and minerals.

Micro-constituents: non-essential bioactive compounds present in microgram (for some milligram) amount in the diet, including carotenoids, polyphenols, and phytosterols.

Bioavailability: proportion of a specific food compound reaching the systemic circulation after absorption.

Bioaccessibility: proportion of a specific food compound released from the food matrix in the gastrointestinal tract in an absorbable form.

Intestinal lumen: interior of the small intestine, with a diameter around 40 *mm* excluding the wall.

Intestinal villi: 1 *mm* finger-like projections protruding from the wall, lined with intestinal cells and covered by a mucus layer.