Static and Dynamic In vitro Models for Studying Secondary Plant Metabolite Digestion and Bioaccessibility

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Abstract

There is an increased interest on secondary plant metabolites, such as polyphenols and carotenoids, due to their proposed health benefits. This attention includes their bioavailability, a prerequisite for assigning further physiological functions. As human studies are time-consuming, costly, and restricted by ethical concerns, *in vitro* models for investigating changes of these compounds during digestion have been developed and employed for predicting their release from the food matrix (bioaccessibility) and changes in their profiles prior to absorption.

Most typically, models simulate digestion in the oral cavity, the stomach, the small intestine, and, occasionally, the large intestine. A plethora of models have been reported, the choice mostly driven by the type of phytochemical studied, whether the purpose is screening or studying under close physiological conditions, and the availability of the model systems. Unfortunately, the diversity of model conditions has hampered the possibility to compare results across different studies. For example, there is substantial variability in the time of digestion, concentrations of salts, enzymes, and bile acids used, pH, the inclusion of various digestion stages; and whether chosen conditions are static; (with fixed concentrations of enzymes, bile salts, digesta, and so on) or dynamic (varying concentrations of these constituents). This review presents an overview of models that have been employed to study the digestion of both lipophilic...
and hydrophilic phytochemicals (to compare digestive conditions \textit{in vitro} and \textit{in vivo}) and, finally, recommends a set of parameters for both static and dynamic models that resemble physiological conditions.

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\textbf{Abbreviations}

AM2 – Artificial masticatory advanced machine
CVD – Cardiovascular diseases
DGM – Dynamic gastric model
DNS – Dinitrosalicylic acid color assay
EPI – Echo-Planar magnetic resonance Imaging
FDA – Food and Drug Administration
GI – Gastrointestinal
GIT – Gastrointestinal tract
HGS – Human gastric simulator
HPH – High-pressure homogenization
HPLC – High performance liquid chromatography
HPP – High-pressure processing
IFCC – International Federation of Clinical Chemistry
LPH – Lactase phlorizin hydrolase
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Static models
Phytochemicals are a large and structurally diverse group of secondary plant metabolites that are non essential for humans, that is, their non consumption does not cause any specific deficiency symptoms. For the plant, these are also non essential
compounds, but they aid, among others, in fending off herbivores (polyphenols), or stabilizing photosynthetic pigments (carotenoids). From a chemical point of view, phytochemicals include very diverse compounds, from the rather polar polyphenols, to the rather non polar carotenoids, phytosterols, and terpenes.

There has been increased interest in phytochemicals as their consumption and body tissue levels have been associated with several health benefits, especially in relation to the prevention of chronic diseases such as diabetes, cancer, cardiovascular diseases (CVD) and neurodegenerative diseases (Krzyzanowska and others 2010). This is especially true for their consumption of whole fruits and vegetables, even though there is controversy about the compounds and mechanisms responsible for the observed health benefits. Nevertheless, a number of prospective studies have related the consumption of phytochemicals, such as of polyphenols and carotenoids, to whole fruits or vegetables with the prevention of chronic diseases (He and others 2007; Carter and others 2010). For example, in various meta-analyses, the consumption of carotenoids and several types of polyphenols such as flavonoids were inversely related to the incidence of CVD (Arts and Hollman 2005; Hamer and Chida 2007).

The biological response of the human body to phytochemicals is greatly determined by the bioavailability of these bioactive molecules. The most abundant phytochemicals in our diet are not necessarily those able to result in the highest tissue concentrations or those revealing biological effects, owing to considerable differences in bioavailability (Manach and others 2005). Phytochemical bioavailability depends on a large number of factors and may differ according to the types of compounds studied, their differing associations with the plant matrix, variation in polarity, molecular mass, presence in crystalline or amorphous state, digestion by gastrointestinal enzymes, active vs. passive absorption into the enterocytes, and many more. Among the most important factors
determining bioavailability, and a prerequisite for intestinal absorption, is release from
the food matrix and solubilization during digestion, also termed bioaccessibility (Parada
and Aguilera 2007), which is therefore describing the fraction of a compound
potentially available for further uptake and absorption. The amount of any
phytochemical released and therefore potentially available for further absorption may
differ greatly from its total concentration in the native food matrix. For some
compounds that are poorly released and solubilized, such as carotenoids (Bohn 2008),
or that are degraded prior to reaching their site of absorption, such as anthocyanins, the
portion that is bioaccessible may be below 10% (Minekus 1995; Bouayed and others
2011). Thus, a thorough understanding of changes occurring during digestion (such as
mechanical action, enzymatic activities, and altered pH) is crucial for the understanding
of bioaccessibility and estimating bioavailability and bioactivity, as only bioavailable
phytochemicals will exert fully their potential beneficial effects. Because animal and
human studies are very lengthy and costly to conduct, and also have limitations due to
ethical considerations, in vitro systems have been developed that enable the prediction
of phytochemical changes during oral and gastro-intestinal digestion. This has allowed
the screening of comparatively large numbers of samples and/or conditions, studying
the separate and combined impacts of each stage of digestion on the release and
availability of phytochemicals, which would hardly be possible in vivo.

A major obstacle for the interpretation of phytochemical bioaccessibility based on in
vitro studies is the large number of models published and presented in the scientific
literature since the description of the first model developed for studying iron
bioaccessibility (Miller and others 1981). The diversity of models has hampered the
comparison of results across studies, and increased the chances of finding contradictory
results. The employed models mainly differ in the inclusion of various stages of
digestion (oral, gastric, small intestinal, large intestinal); digestion times (typically ranging from a few minutes per stage to up to 3 h); pH; the nature of digestive enzymes involved and concentrations of salts and bile acids. Finally, while most of the models are operated in static conditions, that is with pre-fixed concentrations and volumes of digested materials, enzymes, salts etc. (though during digestion phases are mixed and concentrations may change), there are also a limited number of continuous models that mimic the dynamic changes of the physicochemical conditions (and go along with a more constant change of digested material enzymes, salts etc. during various phases of digestion), and which aim to better simulate the passage of the bolus/digesta through the human digestive tract. However, these models are much more labor- and cost-intensive than the batch models.

The aim of this review is to summarize frequently employed models for studying phytochemical bioaccessibility, to compare conditions to the situation *in vivo*, and to suggest a set of variables and values that appear closest to conditions *in vivo*, in order to contribute to the standardization of *in vitro* models. One of the major differences between the reported models, apart from being static or dynamic, is their application to either hydrophilic or lipophilic compounds (Figure 1).

For practical reasons, this review focuses on 2 major groups of phytochemicals: polyphenols as the major water-soluble phytochemicals and carotenoids, as the major lipid-soluble phytochemicals, aiming to elucidate factors affecting the choices of the appropriate model for each application, in order to simulate *in vivo* conditions to the best of present knowledge. Thus, the review is structured, first, into a discussion of general digestion considerations, then to provide more thorough insights into the individual digestion phases themselves.
Parameters that drive the choice of model

There are a number of factors that drive the choice of a model system (Figure 1). The most important is the desired outcome of the study. In some studies, the prime objective is to understand the effect of simulated gastrointestinal digestion on a certain class of phytochemicals (hydrophilic or lipophilic). For a limited selection of samples, in-depth simulation of a dynamic system may be more appropriate as it allows simulation of the effects of multiple digestive parameters on a small number of samples. Larger-scale studies may require screening of the effect of *in vitro* digestion on multiple samples (such as different source materials or the effects of processing/cooking) and a relatively simple static model may be more appropriate (Figure 1).

In some cases, the function of *in vitro* digestion is to provide samples that are more physiologically-relevant for further studies on potential bioactivities, as with the preparation of “colon-available” samples for effects on colon cancer models (Brown and others 2012) or the preparation of dietary fiber fractions such as β-glucans (Beer and others 1997).

Of course, there is considerable flexibility in the approaches. Initial hypotheses could be tested in the static models and then extended in dynamic model experiments; and insights gained from dynamic models could be fed back into the design of morephysiologically appropriate screening methods (Figure 1).

Overview on parameters affecting the release and chemical changes of lipophilic and hydrophilic phytochemicals during digestion
Digestion of phytochemicals is a complex process, and the bioaccessibility of phytochemicals depends on both the characteristics of the food matrix and the physiological conditions encountered in the various compartments of the gastrointestinal tract (including enzyme concentration and pH). Additionally, the physicochemical properties of the phytochemicals themselves are important parameters. For example, the hydrophilicity/lipophilicity balance is crucial in driving the solubilization of hydrophilic phenolic compounds into the aqueous phase of the intestinal digesta and the restructuring of lipophilic carotenoids into mixed micelles.

Since plant foods are often divers in composition or eaten in conjugation with other foods, food bolus constituents are likely to modulate the bioaccessibility and stability of phytochemicals. This may contribute to the rather small fraction of dietary phytochemicals that is typically absorbed and utilized by humans (Schramm and others 2003). Therefore, defining the conditions that influence their absorption can provide significant insights into methods for maximizing the utilization of these sometimes health-promoting constituents. The main food components are proteins, carbohydrates, fiber, and fat, and their interactions with phytochemicals are often not considered. When considering in vitro bioaccessibility studies, chemical reactions (such as oxidation/reduction, complexation), biochemical reactions (enzyme/substrate interaction), or physical constraints (diffusion) occurring within food must be taken into account. For polyphenols, in particular, these types of interactions have rarely been taken into account when determining polyphenol digestion (Ortega and others 2009).

**Lipophilic phytochemicals**

Although carotenoids are lipophilic compounds and considered as relatively labile under acidic conditions, no significant chemical modification in the human stomach has
been described (Tyssandier and others 2003). Some isomerization was observed in the stomach of ferrets (Boileau and others 1999) and relatively high recoveries of dietary carotenoids (65-91%) have been observed after gastrointestinal in vitro digestion (Granado-Lorencio and others 2007; Failla and others 2009). The digestive stability of carotenoids in different food matrices has been investigated in a dynamic in vitro model simulating the stomach and small intestine (TIM 1) (Blanquet-Diot and others 2009; Déat and others 2009). Zeaxanthin and lutein (xanthophylls) were found to be stable during the whole digestion, whereas lycopene and β-carotene (carotenes) were stable in the gastric and duodenal compartments but partly degraded in the jejunal and ileal compartments of the small intestine, perhaps due to delayed release from the matrix and later micellarization at this stage of these carotenoids (Blanquet-Diot and others 2009). Although an enhanced release from the matrix can contribute to higher bioaccessibility, the released carotenoids may be more susceptible to degradation and isomerization (Failla and others 2008a). In the study by Blanquet-Diot and others (2009), a degradation of β-carotene and all-trans lycopene, which could not be directly linked with the formation of cis isomers, was observed in the lowest part of the small intestine. As suggested by the authors, the results might be due to breakdown to non-detected metabolites (such as oxidation products) or enzyme-catalyzed cleavage products during small intestinal digestion, but no precise data could support this hypothesis. The absorption of lipophilic phytochemicals mainly occurs after the disruption of the food matrix, enabling the release and emulsification into lipid droplets in the stomach, followed by incorporation into mixed micelles. Apart from the food matrix, carotenoid bioavailability may be influenced by the presence of other nutrients and non-nutrients within the food. For example, a competition between carotenoids and other fat-soluble nutrients such as vitamin E at the absorption stage has been reported (Faulks and others...
Differences in location and form will also affect carotenoid release and bioavailability. Carotenoids are usually associated with proteins, for example, lutein in green leafy vegetables is located in chloroplasts, whereas carotenes are found in chloroplasts in oil droplets, such as in fruits or semi-crystalline membrane-bound solids like in carrot, tomato, and papaya (Faulks and others 1998, Schweiggert and others 2011).

The effect of physicochemical properties on carotenoid bioaccessibility and transport to storage tissues was recently studied by Sy and others (2012a). The efficiency by which pure carotenoids were transferred from dietary lipids into synthetic mixed micelles was assessed using a modified method of the in vitro digestion model developed by Garrett and others (1999). Sy and others (2012a) found that lutein was more readily micellarized than the other carotenoids and especially compared with lycopene, which was the least micellarized carotenoid. The apparent poor solubility and bioaccessibility of lycopene may be due to its elongated shape that could cause the molecule to protrude from the micelles into the surrounding aqueous environment and similar effects could be expected for other lipophilic phytochemicals.

Hydrophilic phytochemicals

Phenolic phytochemicals can greatly vary in their chemical structure and properties, ranging from simple molecules (such as phenolic acids) to highly polymerized molecules (proanthocyanidins) (Manach and others 2004). This chemodiversity results in different bioaccessibility. Factors in the bioaccessibility of polyphenols include the release from the food matrix, particle size, the hydrophilic/lipophilic balance as related to their glycosylation, different pH-dependent transformations (degradation, epimerization, hydrolysis and oxidation during gastrointestinal digestion), and also
interactions between polyphenols and food components (Stahl and others 2002; Karakaya 2004). Phenolic compounds can have strong affinities with human salivary proline- and histidine-rich proteins and form both non covalent and covalent associations depending on the size of the phenolic compound (de Freitas and Mateus 2001; Wroblewski and others 2001). High-molecular-weight polyphenols (such as tannins) interact strongly with fibers and proteins, but their affinity is related to their size and their solubility in water.

More hydrophobic compounds have stronger binding to proteins (Le Bourvellec and Renard 2011). Laurent and others (2007) investigated the behavior of low molecular weight flavonoids from grape seed extract during in vitro digestion (with α-amylase from human saliva, porcine pepsin, pancreatin and bile extract), combined with a Caco-2 cell model to evaluate the impact of brush border proteins. Their results showed that flavan-3-ol monomers ((+)-catechin and (−)-epicatechin) and procyanidin dimers (B2 and B3) were stable during oral and gastric digestion but those interactions with proteins occurred during the intestinal step with pancreatic digestion, and in the presence of brush border cell proteins. Simulated digestion of anthocyanins from, for example, red berries, red wine, and red cabbage have shown that these compounds appear to be stable at the acidic conditions of the stomach but less stable at the small intestinal pH (Gil-Izquierdo and others 2002; McDougall and others 2005a, 2007). The total recovery of anthocyanins from red cabbage was low (around 25%), possibly due to degradation into new phenolic components by the combination of the elevated pH and the presence of oxygen during pancreatic digestion (McDougall and others 2007). As recently shown in the investigation by Oidtmann and others (2012), a possible mean to enhance the stability and protect anthocyanins from degradation in the small intestine...
might be to use encapsulation techniques, such as microcapsule systems composed of polysaccharide pectin amide with or without shellac coating or whey proteins.

In summary, the digestive stability of carotenoids depends on the molecular nature and the food matrix in which they are included, with xanthophylls being more stable than carotenes. The absorption of carotenoids depends on an efficient release from the food matrix and subsequent solubilization in mixed micelles. By contrast, no micellarization is required prior to cellular uptake for phenolic compounds, and, thus, there are possibly fewer possibilities for impacting bioaccessibility such as by varying enzyme concentrations; however, some constituents such as anthocyanins may be rapidly degraded due to increasing pH (McDougall and others 2007). The affinity of polyphenols for proteins (Dangles and Dufour 2005, 2008) may lead to a major modulation of both polyphenol absorption and reactivity in the stomach and in the upper intestine.

Before modeling: Considerations with respect to pre treatments, meal size, and choice of test meals

Food composition, how it is processed and the interaction of phytochemicals with other food components (be they lipophilic or hydrophilic), may modify the amount of phytochemicals released from the food matrix and, therefore, potentially increase or decrease their bioaccessibility.

Influence of the plant matrix and food bolus

Plant cell walls acts as a barrier to digestion (Ellis and others 2004; Mandalari and others 2010). When a plant cell is broken through mastication or crushing in industrial or domestic processing, phytochemicals may associate with dietary fibers leading to a
modulation of their relative bioaccessibilities. In a recent study, comparing the stability and bioaccessibility of carotenoids in pure forms (synthetic β-carotene or retinyl palmitate solution) or from food (carrot juice and raw or cooked spinach), Courraud and others (2013) demonstrated the protective effect of the food matrix on dietary carotenoids. Their results showed that vitamin A and carotenoid standards (synthetic β-carotene or retinyl palmitate solution) were unstable, whereas food carotenoids were generally better protected by the food matrix (30-100% recovery versus 7-30% for standards). Although the susceptibility of carotenoids to degradation and isomerization has been found to increase after their release from the food matrix (Failla and others 2008b), interactions with other compounds released from the food matrix (including soluble fibers) and viscosity may affect their bioaccessibility (McClements and others 2008; Schweiggert and others 2012). For example, the bioaccessibility of β-carotene is known to be influenced by strong binding to pectins (Ornelas-Paz and others 2008).

Dietary fibers are the main carriers for phenolic compounds and thus influence their bioaccessibility, as fiber-entrapped polyphenols are both poorly extractable and barely soluble in the GI fluids. High-molecular-weight proanthocyanidins and hydrolyzable tannins which represent more than 75% of all food polyphenols ingested (Arranz and others 2010) may bind tightly to dietary fibers and this restricts their accessibility. Soluble and insoluble polysaccharides can bind phenolic compounds and limit their diffusion, they increase the medium viscosity, and limit substrate-enzyme contacts during GI digestion (Eastwood and Morris 1992). During the in vitro digestion of cocoa powder, protease and glycosidase actions as well as gut microflora activity were shown to take part in the release of flavanols from matrix fibers and proteins (Fogliano and others 2011). Additionally, the extractability of phenolic acids, flavonoids, and proanthocyanidins appeared to be improved in the presence of fat, increasing by a 1.5-3
factor for cocoa liquor (50% fat content) compared to cocoa powder (15% fat content) (Ortega and others 2009).

The affinity of milk and egg proteins as well as gelatins for polyphenols depends on both the protein and phenolic structures (Bohin and others 2012). For example, chlorogenic acid associates with milk caseins rather than with β-lactoglobulin and this complexation was relatively stable in simulated gastric and intestinal steps (Dupas and others 2006). Despite these interactions, chlorogenic acid absorption by Caco-2 cells and rats was not reduced by milk addition to coffee. In tea, more than 60% of green tea flavanols (such as ECG, EGC, and EGCG), which are very prone to oxidation, disappeared in the intestinal phase during in vitro digestion (Haratifar and Corredig 2014). A protective effect was caused by the addition of pure ascorbic acid, by citrus juices as well as by bovine, rice, and soy milks. While ascorbic acid contribution reflects its superior antioxidant capacity compared to tea flavanols, the protection by proteins was partially reversed by increasing the content of digestive enzymes, suggesting non covalent interactions between bovine milk proteins and galloylated tea flavanols (Green and others 2007).

Soy isoflavones appear to be more bioaccessible from fruit juices and chocolate bars compared to cookies in in vitro conditions, perhaps due to their lower diffusion rate from the carbohydrate/protein matrix of the cookies (de Pascual-Teresa and others 2006). However, a complementary human intervention study did not point out any significant difference in the bioavailability parameters (AUC, t_max or c_max) of these isoflavones. Similarly, the in vitro bioaccessibility of catechin recoveries was significantly higher in beverages than in confections (Neilson and others 2009). Higher amounts of isoflavones were also released in vitro from custards thickened with starch rather than with carboxymethylcellulose (Sanz and Luyten 2006). This effect was attributed to the
hydrolysis of starch by $\alpha$-amylase which occurs from the mouth to the intestine. Finally, bile salts improved the \textit{in vitro} bioaccessibility of isoflavone aglycones from soy bread through micellarization of these poorly-soluble molecules concentrations appeared to be a critical factor in the bioaccessibility of isoflavones from soy bread (Walsh and others 2003).

Impact of processing

Previous studies (Garrett and others 1999) have indicated that food processing and dietary fat can enhance carotenoid bioaccessibility. However, it is notable that only a little proportion of carotenoids (5-25\%) is efficiently liberated from the food matrix. Cooking and heat treatment may enhance carotenoid bioaccessibility due to disruption of plant tissue and denaturation of carotenoid-protein complexes which enhance release from the food matrix (Veda and others 2006; Failla and others 2009; Aherne and others 2010). However, cooking enhanced the bioaccessibility and bioavailability of all-trans $\beta$-carotenes but also caused carotenoid isomerization (Aherne and others 2010).

There are many reports describing that thermal processing improves lycopene bioaccessibility due to the breakdown of the tomato matrix (Gartner and others 1997; Porrini and others 1998; Van Het Hof and others 2000). However, depending on the processing methods, differences in lycopene bioaccessibility have been reported. Yilmaz and Karakaya (2007) reported that lycopene bioaccessibility in raw tomato (29\%) and canned tomato were similar (22\%). On the other hand, bioaccessibility of lycopene from sun-dried tomatoes reached 58\% (Yilmaz and Karakaya (2007). High-pressure homogenization (HPH) and HPH combined with heat processing (90 °C for 30 min) caused a decrease in the \textit{in vitro} bioaccessibility of lycopene. In addition, an inverse relationship between the homogenization pressure and lycopene \textit{in vitro}
bioaccessibility was reported (Colle and others 2010). It was suggested that the fiber network formed by HPH entrapped lycopene, making it less accessible for digestive enzymes and bile salts. High-pressure processing (HPP), however, had no effect on α-carotene and β-carotene bioaccessibility in carrots. Lutein bioaccessibility in green beans was increased by pressure treatment at 600 MPa (p<0.05), whereas β-carotene bioaccessibility was reduced by HPP at both 400 or 600 MPa (McInerney and others 2007), which suggests effects due to the matrix and compound structure.

In wheat bran, ferulic acid and para-coumaric acid are mostly bound to arabinoxylans and lignin and are thus insoluble, whereas sinapic acid is mainly found in soluble conjugate forms esterified to sugars and other compounds. It was reported that the bioaccessibility of sinapic acid from bran-rich breads was much higher than that of ferulic acid and para-coumaric acid (Hemery and others 2010). Food processing, especially grinding of the bran fractions, increased the bioaccessibility of phenolic acids. (Hemery and others 2010). This increase in bioaccessibility was correlated to the presence of very small particles (diameter < 20 µm) for sinapic acid and ferulic acid and that of larger particles for para-coumaric acid (between 20 and 100 µm). Additionally to particle size reduction, exogenous ferulase and xylanase treatments contributed to the pool of free and exposed ferulic acid residues as demonstrated by the increased antioxidant capacity displayed by treated fractions in an in vitro model of digestion (Rosa and others 2013a, b).

**Impact of starting meal size**

Adjustment of the ratio of the amount of the test meal to water present to mimic dietary bolus during digestion phases has an impact on viscosity. Both this ratio and
meal particle size are important factors influencing phytochemical release during digestion.

During transit in the oral cavity, the stomach, and the small intestinal compartments, the dietary bolus will be diluted as a consequence of addition of saliva and other secretions. The amount and type of food influence the composition and secretion rates. Apart from the volume and composition of the secretions, mechanical forces will also have an impact on the disintegration and dissolution of a meal and on the rate of transfer through the GI tract. In general, dynamic models are able to process complex foods through mechanical and enzymatic digestions at volumes equivalent to “standard” meals.

**Digestion models for studying phytochemical bioaccessibility - static vs. dynamic models**

Depending on the type of research question, for example, if constituting a screening application or a confirmative study, the type and amount of sample present, static or dynamic *in vitro* models can be used to simulate different phases of digestion (Figure 1). Practically, static models provide a feasible and inexpensive means to assess multiple experimental conditions, allowing large numbers of substrates to be tested. Dynamic multistage continuous models facilitate long-term studies and probably come closest to *in vivo* conditions. These complex computer controlled systems, however, are expensive to set up, more labor-intense and time-consuming (maximum one experiment /day) and require higher operating costs in terms of working volumes and continuous addition of substances mimicking the gastrointestinal fluids.

**Static models**
The simulation of the digestive process can be divided into 2 major stages: simulating gastric and small intestinal digestions, with conditions generally based on the method described by Miller and others (1981). Adaptations to this model have been made to modify the conditions and the procedures for studies of digestibility and bioaccessibility of phytochemicals, but the “physiological conditions” chosen vary considerably across different static in vitro studies.

The comparative simplicity of static methods have allowed their adaptation to measuring the bioaccessibility of many phytochemicals from various fruits and vegetables, including phytosterols (Bohn 2008), glucosinolates (Iori and others 2004), carotenoids (Garret and others 1999; Failla and others 2008b) and many types of polyphenols (Gil-Izquierdo and others 2002). This simplicity allows the running of multiple samples in parallel. However, contrary to dynamic models, these static models typically fail to take into account dynamic physiological responses to the introduction of a food bolus, such as pH increase and following decrease in the stomach, and enzyme secretions in response to the food bolus introduced (Isenman and others 1999).

However, adaptations of the static model have been carried out for the investigation of various phytochemicals, such as ultracentrifugation and/or filtration, to study the micellar phase of lipophilic constituents. While this is normally not done for polyphenol bioaccessibility, additional steps such as dialysis have occasionally been introduced (Bouayed and others 2012).

**Dynamic models**

Compared to static models, dynamic models have the advantage that they can simulate the continuous changes of the physicochemical conditions including variation
of pH from the mouth to the stomach and the intestine, altering enzyme secretion concentrations, and peristaltic forces in the gastrointestinal tract.

Different dynamic gastric models have been developed and designed for detailed measurement of gastric biochemistry and mixing. Due to their closer resemblance to *in vivo* conditions, but much lower throughput, they are more suitable to further confirm results obtained in static models and to gain more detailed insights into changes occurring during digestion. The dynamic gastric model (DGM), developed at the Institute of Food Research (Norwich, UK), is composed of 2 successive compartments (Vardakou and others 2011). The model reproduces gastric emptying and secretion according to data derived from echo-planar magnetic resonance Imaging (EPI) and the rates of GI digestion obtained from human studies (Golding and Wooster 2010). The system was originally constructed to assess the impact of the first stages of digestion on the bioaccessibility and delivery profiles of nutrients to the duodenum. It simulates the physical mixing, transit, and breakdown forces (including flow, shear, and hydration), pH gradients, and gastric secretions.

The human gastric simulator (HGS), a model developed at the University of California-Davis is composed of a latex chamber surrounded by a mechanical driving system to effectively simulate the frequency and intensity of the peristaltic movements in the stomach (Kong and Singh 2010). HGS is designed to mimic the gastric shear forces and stomach grinding. This appears to be important for bioaccessibility studies as the rate of release of phytochemicals, from fibrous particles, into the surrounding intestinal fluid is inversely proportional to particle size, and is directly proportional to phytochemical gradient. It is furthermore affected by the physical state of the phytochemical, the physical structure, and the surface properties of the particle (Palafox-Carlos and others 2011). To allow a closer simulation of *in vivo* physiological
processes occurring within the lumen of the stomach and small intestine, some of the main parameters of digestion such as peristaltic mixing and transit, secretions, and pH changes, have been applied in some models. The TNO gastrointestinal model (TIM-1) developed by TNO in Zeist (The Netherlands), has been used for a broad range of studies (Minekus 1995). The system consists of 4 different compartments, representing the stomach, duodenal-jejunal and ileal parts of the gastrointestinal tract. Each compartment is composed of 2 glass jackets lined with flexible walls. The TIM-1 system enables simulation of gastric emptying rate, peristaltic movements, and transit time through the small intestine and gradual pH changes in the different compartments (Minekus 1995), and has given useful information on the parameters affecting the release and digestive stability of carotenoids from different food matrices through the gastrointestinal tract (Minekus 1995; Blanquet-Diot and others 2009). This model has also been extensively used to assess both folate and folic acid bioaccessibility from foods (Öhrvik 2008; Öhrvik and others 2010).

For polyphenols, there is not enough evidence as to which method is the most appropriate for measuring bioaccessibility, especially as it has become clear that the colon is greatly involved in the metabolism and absorption of these compounds (Bolca and others 2012; Czank C and others 2013; Ludwig IA and others 2013). Thus both static and dynamic models, those that do not take into account the simulation of the colon, will have limitations in predicting the bioavailability of polyphenols. However, with the development of additional models aiming to simulate colonic fermentation, such as the TIM-2 model, the non bioaccessible fraction following gastric and small intestinal digestion may be studied, such as was done for phenolic compounds in wheat bread (Mateo Anson and others 2009).
An adapted model of TIM-1, a computer-controlled gastrointestinal model called Tiny-TIM, has more recently been used to assess the bioaccessibility of phenolic acids in breads (Hemery and others 2010). The model is a simplified and downscaled TIM-1 for rapid screening. The main characteristics of the system are the same as for TIM-1, but instead of four compartments, the Tiny-TIM model consists of 2 compartments that represent the stomach and the small intestine. The results were found to be consistent both with the data from a previous study evaluating the bioaccessibility of phenolic acids in TIM-1 (Kern and others 2003) and a human study (Mateo Anson and others 2009). To our knowledge, except for the comparison between the results obtained in the TIM-1 and Tiny-Tim model, so far no comparisons between the different dynamic models have been made.

Setting up the model

Digestion simulation of the oral cavity

The oral cavity is the portal of entry of nutrients. Due to its unique constituents it may also be considered a “bioreactor” (Gorelik and others 2008; Mathes and others 2010; Ginsburg and others 2012). Whole saliva is a very dilute fluid composed of more than 99% water. It contains a variety of minerals, various proteins (the major being the mucin glycoproteins, albumin, and digestive enzymes), and nitrogenous compounds as urea and ammonia (Ginsburg and others 2012). An intensive mixing of simulated saliva and the introduced food bolus is usually desired, usually in a ratio of 1:1, keeping in mind practicality and the basal flow of saliva during ingestion estimated at 1-3 mL/min (Engelen and others 2003). An ingested food or beverage undergoes a number of chemical, biochemical, and mechanical processes in the mouth, although not so
significant for liquids due to short residence time. There may occur changes in pH, ionic strength, and temperature, action of various digestive enzymes (notably lingual lipase, amylase, protease); interactions with biopolymers in the saliva (mucin); interactions with sensory receptors of the tongue and mouth; and particle size reduction of bolus by chewing (mastication). These are all major factors to take into consideration when designing an in vitro digestion step that simulates the human mouth (McClements and Li 2010).

Particle size reduction

A few studies have paid attention to how mechanical breakdown during the oral phase affects phytochemical bioaccessibility. Mastication consists of grinding food into smaller pieces and impregnating these pieces with saliva to form a bolus that can be swallowed. Decreasing the particle size enlarges the surface area available for hydration and action by digestive enzymes, thus increasing the overall digestion efficiency and gastrointestinal absorption of phytochemicals (Kulp and others 2003). A partial and short mastication might affect the availability of major phytochemicals from vegetables, fruits. However the inter-individual variability in the particle size of food boluses at the end of chewing is considered to be insignificant for overall bioaccessibility (Woda and others 2010), and the use of one individual to chew the meal and expectorate it prior to swallowing was found to be acceptable (Ballance and others 2012). However, more studies are needed to confirm that one subject is sufficient for investigating the effect of mechanical breakdown on phytochemical bioaccessibility during the oral phase. When studying bioaccessibility of carotenoids, techniques such as grinding or homogenizing, with a stomacher laboratory blender for different intervals in the presence of artificial saliva, were compared with physically masticated foods by humans (Lemmens and...
The average particle size distribution after human chewing was investigated and this information was used to simulate average mastication \textit{in vitro} by a blending technique.

To produce food boluses with properties similar to those resulting after natural chewing, the Artificial Masticatory Advanced machine (AM2) has been developed and validated against human subjects chewing raw carrots (cylindrical samples height 1 cm, diameter 2 cm, 4 g) and peanuts (3.5 g) (Mishellany-Dutour and others 2011). It was concluded that AM2 produces a food bolus with similar granulometric characteristics to human chewing, although no bioaccessibility parameters for phytochemicals were evaluated.

\textbf{Chemical and biochemical processes}

Due to the usually very short interaction of oral enzymes with the food bolus prior to reaching the stomach, their influence is much less clear and rather limited to carbohydrate-rich foods such as cereal-based foods (Hur and others 2011). For example, it is estimated that nearly 5% of the consumed starch is already degraded in the mouth cavity by salivary amylase (Hall 1996). Usually, \textit{in vitro} methods are initiated using $\alpha$-amylase at pH around 7 (Table 1).

Ginsburg and others (2012) suggested that saliva has an important role in the solubilization of polyphenols present in fruits and plant beverages and thus substantially increases their availability. Moreover, saliva can increase the stickiness to oral surfaces of polyphenols and their prolonged retention in the oral cavity and thus it contributes to the enhancement of the redox status of the oral cavity. Salivary albumin, mucins, and proline-rich proteins may be of particular importance affecting the digestibility and absorption of specific polyphenols, for example, tannins may be precipitated and
retained by such proteins (Bennick 2002) through hydrogen bonding and hydrophobic
interactions.

In summary, an oral digestion phase may be recommended for carbohydrate-rich
foods. Alternatively, starting with particles of small size (50-1,000 µm) may be
appropriate, as this mimics the particle size following the chewing process for
vegetables and fruits (Hoebler and others 2000; Lemmens and others 2010,). If oral
digestion is left out, dry samples may be introduced at a ratio of approximately 1:4
(food:liquid), considering common meal sizes of approximately 200-300 g and a gastric
juice volume of about 1L (Sergent and others 2009). A fluid of physiological salt
concentration (saline) should be employed.

The gastric phase of digestion

The knowledge of disintegration of food inside the stomach is crucial for assessing
the bioaccessibility of phytochemicals for both static and dynamic methods. Food
disintegration in the stomach is a complex process including mechanical actions and
activity of gastric fluids.

Gastric juice contains hydrochloric acid (HCl), pepsinogens, lipase, mucus,
electrolytes and water. The rate of secretion varies from approximately 1-4 mL/min
under fasting conditions to between 1 and 10 mL/min after food intake (Wisen and
Johansson 1992; Brunner and others 1995). The presence of HCl contributes to the
denaturation of proteins and it activates pepsin.

Peristaltic waves originating from the stomach participate to the size reduction of
solid foods down to a diameter of 1 to 2 mm (Kong and Singh 2010). Stomach
emptying is a critical step in the digestion process. Several factors may influence the
gastric emptying of food and fluids including volume, viscosity, and pH. The speed of
the emptying of liquid meals is directly proportional to the volume present in the stomach. Solid foods are emptied more slowly, in a biphasic pattern with a lag phase during which little emptying occurs, followed by a linear emptying. The duration depends on the physical properties and approximately 3 to 4 h are needed for a complete emptying of the stomach (Schulze 2006).

A nutrient-driven feedback regulation from the small intestine, limiting the gastric emptying to a maximum of about 3 kcal/min has been suggested (Lin and others 2005, Kwiatek and others 2009) when other data point to a nutrient-dependent emptying pattern with emulsion fat emptying faster than glucose and protein (Goetze and others 2007). Furthermore, the presence of dietary fibers is known to slow down gastric emptying of complex meals (Marciani and others 2001).

\textbf{pH}

The gastric pH in the fasted state in healthy human subjects is in the range of 1.3 to 2.5. The intake of a meal generally increases the pH to above 4.5 depending on the buffering capacity of the food. For example, in nasogastrically intubated humans fed a western-type diet enriched in either tomato, or spinach or carrot purees, the stomach pH sharply increased to 5.4–6.2 after meal intake, then continuously decreased to reach 1.8–2.9 after 3 h of digestion (Tyssandier and others 2003). Similarly, after ingestion of a cocoa beverage, the gastric pH reached 5.4 within 3 min before returning to the baseline pH of 1.9 (Rios and others 2002). Most static in vitro studies have been conducted at a pH below 2.5, which are conditions related to the human fasting state rather than to real food digestion. Only a few authors have considered as relevant a pH of 4 associated with the mid-step of digestion (Reboul and others 2006; Dhuique-Mayer and others 2007). The decay of gastric pH is however taken into consideration in
dynamic models as shown for the digestion of tomato carotenoids in the TIM system (pH 6 to 1.6) (Blanquet-Diot and others 2009).

**Enzymes**

Pepsin, which is readily available as porcine pepsin, has been integrated in most *in vitro* models of gastric digestion, although in varying amounts (Table 2). Pepsin content should be assessed as enzymatic activity per weight of protein for the sake of comparison. Gastric lipase is usually omitted. However, existence of lipolysis in the human stomach by gastric lipase is known (Carriere and others 1993; Armand and others 1994). Most of the dietary lipids are present in the form of emulsified droplets, in the range of 20-40 μm, and it was suggested that gastric lipolysis can help to increase emulsification in the stomach (Armand and others 1994), which would thus enhance lipophilic phytochemical bioaccessibility. It was reported that human gastric lipase secretion ranged from 10 to 25 mg/3 h and that the percentage of intra-gastric lipolysis during gastric digestion was 5-40% (Carriere and others 1993: Armand 2007). Lipolysis catalyzed by gastric lipase has been found to primarily occur within the first hour of digestion (Armand and others 1994).

Because human gastric lipase is unavailable, fungal lipases from *Aspergillus niger* or *Aspergillus oryzae* have been used, as in the TIM model. However, *A. niger* lipase has a wide pH optimum of 2.5-5.5 compared to 4.5 to 6 for human gastric lipase (Carriere et al., 1991). The fungal lipase can hydrolyze both the sn-1 and sn-3 positions of the triacylglycerol molecule, with a slight preference for the sn-1 position, whereas gastric lipase is most active at the sn-3 position (Van Aken and others 2011). Alternatively, a mammalian lipase such as rabbit gastric lipase could be used as Capolino and others (2011) demonstrated that its specificity is close to that of human lipase. At the present
time, a combination of rabbit gastric lipase and porcine pancreatic extract is favored to simulate *in vitro* gastrointestinal lipolysis.

*Oxygen, dietary iron and antioxidant activity of phytochemicals and micronutrients*

The presence of other food components may alter polyphenol and carotenoid stability in the gastric tract. After food intake, dietary iron, dioxygen, and emulsified lipids come into close contact and lipid oxidation may take place. This was demonstrated for heme (metmyoglobin) and nonheme iron (Fe$^{II}$/Fe$^{III}$) forms in emulsion systems modeling the physical state of triacylglycerols (Lorrain and others 2012). Dietary polyphenols such as rutin, (+)-catechin, and chlorogenic acid proved to be better inhibitors of the metmyoglobin-initiated lipid oxidation than α-tocopherol and vitamin C (Lorrain and others 2010). The antioxidant activity of polyphenols depended on an emulsifying agent (proteins, phospholipids) and pH. In this process, polyphenols were however consumed, giving rise to oxidation products which themselves retain antioxidant properties (Lorrain and others 2010). In this *in vitro* model of gastric digestion, lycopene and β-carotene proved to be less efficient inhibitors of lipid oxidation compared to bacterial carotenoids (mainly glycosylated apolycopenoids) (Sy and others 2012b). Phenolic compounds and carotenoids had complementary mechanisms of action: the former inhibited the initiation step of lipid peroxidation by reducing the prooxidative Fe$^{III}$ species of myoglobin when the latter inhibited the propagation phase by direct scavenging of the lipid peroxyl radicals. Oxygen may thus impact phytochemical and micronutrient stability in the gastric tract. The level of dissolved O$_2$ increases during mastication of food (Gorelik and others 2005), whereas the presence of a marked oxygen partial pressure gradient from the proximal to the distal GI tract was
evidenced in living mice from 58 torr in the mid-stomach, 32 torr in the mid-duodenum, 11 torr in the mid-small intestine and mid-colon to 3 torr in the distal sigmoid colon-rectal junction (compared to 160 torr for O\textsubscript{2} in air) (He and others 1999). For this reason, some authors suggested flushing with nitrogen or argon for a few minutes to reduce the levels of dissolved O\textsubscript{2} (Bermudez-Soto and others 2007).

Static models

Static modeling of gastric digestion of phytochemicals is basically conducted by a pepsin hydrolysis of homogenized food under fixed pH and temperature for a period of time. The internal body temperature (37 °C) is classically used. Dynamic processes occurring during human digestion such as mechanical forces or continuous changes in pH and secretion flow rates are usually not reproduced (Guerra and others 2012). There are many studies on in vitro digestion of phytochemicals using static models, and they only differ slightly (Table 2). The major differences among the methods used for modeling gastric phase digestion are (i) addition or absence of phospholipid vesicles; (ii) addition or absence of lipase; (iii) incubation time between 0.5 h to 2 h; (iv) pH varying from 1.7 to 2.5; and (v) pepsin to substrate ratio.

For highly processed plant matrices, it appears that the large majority of polyphenols is already released in the gastric phase. Indeed, the polyphenol bioaccessibility from fruit juices, wines, green tea, or phenolic extracts, in the presence of simulated gastric juices (pH 1.7-2.5, pepsin, 1-4 h) is nearly 100% (Perez-Vicente and others 2002; McDougall and others 2005a; McDougall and others 2005b; Bermudez-Soto and others 2007; Greenand others 2007; McDougall and others 2007; Gumienna and others 2011) but can be only between 30-100% from solid matrices such as homogenized peaches,
apple, grape berries, cherries or carob flour (Fazzari and others 2008; Bouayed and others 2011; Ortega and others 2011; Tagliazucchi and others 2012).

Among phenolic compounds, apple flavanols (epicatechin and procyanidin B2), as well as chokeberry proanthocyanidin oligomers, were more degraded than caffeoylquinic derivatives, flavonols, or anthocyanins. Cocoa proanthocyanidins (trimers to hexamers) and apple procyanidin B2 were shown to undergo depolymerization in a simulated gastric juice (37 °C, pH 1.8-2.0) (Spencer and others 2000, Kahle and others 2011), whereas in vivo, this degradation was not validated, mainly because the stomach pH increased to 5.4 after the ingestion of the cocoa beverage and progressively decreased to the basal value as the stomach emptied (Rios and others 2002).

Certain epoxycarotenoids, such as violaxanthin and neoxanthin from spinach, were shown to undergo epoxide-furanoid transitions at pH 2 (Biehler and others 2011a). This transformation extent may clearly depend on the gastric acidity and time of exposure.

Dynamic models

Dynamic gastric models of digestion incorporate i) mixing of the non homogeneous gastric digesta which is best modeled by peristaltic movements as in the HGS model (Kong and Singh 2010), ii) acidification, iii) addition of gastric enzymes, and iv) delivery to the duodenum (Chen and others 2011). Usually, computer-controlled protocols are designed to deliver secretions and chime (digesta) in the normal physiologic range. Dynamic models are described in more details in the previous section “Digestion models for studying phytochemical bioaccessibility”. Up to now, few applications have been reported for phytochemicals compared to the numerous data in static models. For example, in the TIM-1 system, tomato (E)-beta-carotene and (E)-
lycopene proved to be stable, although the recovery yield was modulated by the tomato matrix (Blanquet-Diot and others 2009). The Tiny TIM-1 system was used to evaluate the bioaccessibility of phenolic acids in breads made from processed wheat bran fractions (Hemery and others 2010). The amount of bioaccessible phenolic acids was enhanced by using finer particles in bran-rich breads.

General considerations

The rapid release of the phenolic compounds in the stomach maximizes the potential for absorption in the small intestine. For lipophilic compounds, such as for carotenoids, such comparisons would not appear meaningful, as the formation and incorporation of the mixed micelles are mostly achieved during the small intestinal stage.

Several major aspects deserve consideration during the gastric digestion, including the limitation of oxygen, either by flushing with inert gasses or by reducing the headspace volume to a minimum, the inclusion of gastric lipase, especially for lipid-soluble compounds, and a sufficient protein degradation capacity to allow release of phytochemicals. An initial low pH (<3) is not physiological and should be avoided due to non optimal functioning of enzymes, especially of lipase.

Digestion in the small intestine

After food disintegration in the mouth and stomach, the main enzymatic digestion and absorption of nutrients take place in the small intestine. After stomach digestion, the acidic chyme is delivered to the small intestine and neutralized with sodium bicarbonate to give an appropriate pH for enzyme activities.
The *in vitro* small intestinal digestion of phytochemicals is generally applied by mimicking pH, temperature, time, and pancreatic juice including electrolytes, bile salts, and enzymes.

**pH, enzymes and bile salts**

In the fed state, pH can vary from 5.4-7.5 in the duodenum (Tyssandier and others 2003; Kalantzi and others 2006; Clarysse and others 2009), to 5.3-8.1 in the jejunum (Lindahl and others 1997; Perez de la Cruz Moreno and others 2006), and up to 7.0-7.5 in the ileum (Daugherty and Mrsný 1999) (Table 3).

Pancreatic enzymes including proteases, amylases, and lipases, as well as other digestive enzymes (brush border enzymes, like maltase, lactase, α-dextrinase, peptidases) produced by the brush border, a microvillus membrane at the luminal surface of the small intestine (Holmes and Lobley, 1989), all act together on the breakdown of food constituents.

*In vivo* bile salt concentrations were found to be higher in the fed state (3-12 mM range) than in the fasted state and variable between duodenum and jejunum (Table 3).

The major differences among the methods are the forms of enzymes (pancreatin or individual enzymes) and biliary acids used (bile salt mixtures, real fresh bile, or individual bile salts) (Table 3). Very few models use individually prepared bile salts and enzymes (including porcine pancreatic lipase, porcine colipase, porcine trypsin, bovine chymotrypsin, and porcine amylase), although this may give better control over enzymatic activity (Mandalari and others 2010). Several studies have reported that the presence of bile salts and pancreatic enzymes is essential for the efficient micellarization of lipophilic compounds (Garrett and others 1999; Hedrén and others, 2002, Wright and others 2008; Biehler and others, 2011a). In the study by Biehler and
others (2011a), carotenoid micellarization from spinach was strongly reduced in the absence of pancreatin and bile salts, while it was not significantly impacted by the omission of pepsin during gastric digestion (Biehler and others 2011a). Minimal bile salt concentration of 2.4 mg/mL (about 5 mM), within the in vivo concentration range, was required for optimal transfer of lutein and beta-carotene from lipid droplets into mixed micelles (Garrett and others 1999, Wang and others 2012). It was also shown that the maximum beta-carotene transfer was obtained at pH 6, in relation to the activity of pancreatic lipase, which is most efficient at this pH, and with a pancreatic lipase concentration of 0.4 mg/mL (Wang and others 2012). At higher bile salt concentration, beta-carotene micellarization could depend on the activity of pancreatic colipase-dependent lipase (Wright and others 2008). As to polyphenols, the hydrophilic forms such as glycosylated flavonols or quinic acid derivatives of hydroxycinnamic acids may readily solubilize in the aqueous phase when less soluble flavonoid aglycones or procyanidins will bind to dietary fibers and proteins for transport. A bile salt-dependent micellarization has however been suggested for isoflavone aglycones (Walsh, Zhang, Vodovotz, Schwartz and Failla 2003). In the intestinal conditions, the bioaccessibility and stability of polyphenols depends mainly on pH. In near neutral conditions and in the presence of oxygen as it occurs in most in vitro models, some phenolic compounds may be degraded through non enzymatic oxidation (Bergmann and others 2009). Examination of the recovery of specific classes revealed that flavan-3-ols were poorly recovered following the digestion of a grape-orange-apricot juice (Cilla and others 2009) but not in chokeberry juice (Bermudez-Soto and others 2007). Pure (+)-catechin was recovered at only 42% after incubation with pancreatin (Bermudez-Soto and others 2007), while (-)-epicatechin and procyanidin B2 from homogenized apple were not recovered after the intestinal step (Bouayed and others 2012). The high affinity of
monic and oligomeric flavanols for proteins and dietary fibers may also lead to their loss during the solid removal step by centrifugation (Le Bourvellec and Renard 2011). For green tea flavanols, the stability order was epicatechin > epicatechin gallate > epigallocatechin = epigallocatechin gallate, in agreement with the higher oxidizability of the 1,2,3-trihydroxyphenyl moiety compared to the 1,2-dihydroxyphenyl one (Green and others 2007). The recovery of caffeoylquinic acids appears to be more affected by the intestinal step than by the gastric step as observed for apple, a grape-orange-apricot beverage, and red wine (Cilla and others 2009; Gumienna and others 2011; Bouayed and others 2012, ). Chlorogenic acid (5-caffeoylquinic acid) may autooxidize, although regio-isomerization is a major pathway as described for $p$-coumaroyl- and caffeoylquinic acids by Kahle and others 2011. Anthocyanins appear to be the most sensitive class and may largely disappear in the intestinal step (McDougall and others 2005a, b, 2007; Bermudez-Soto and others 2007; Tagliazucchi and others 2010, 2012). The quantification of anthocyanins is complicated by a pH-dependent equilibrium of the red flavylium cation to several related structures at pH above 2. The hydration of the flavylium cation produces a colorless hemiketal which is in equilibrium with colorless (E)- and (Z)-chalcone forms. In the near-neutral conditions of intestinal digestion, a first deprotonation of the flavylium cation provides neutral quinonoidal bases ($p$Ka $\approx$ 4) which can further be deprotonated to ionic quinonoidal bases ($p$Ka $\approx$ 6), both bases displaying blue and violet hues (Brouillard and others 1991; Clifford 2000). Thus, the detection of anthocyanins in simulated gastrointestinal conditions can be challenging as it is influenced by pH and copigment molecules. For example, Perez-Vicente and others (2012) evaluated the recovery of pomegranate anthocyanins to be 18% when measured at the pH of the intestinal digesta and 70% following acidification of the digesta at pH 2. Analysis of anthocyanins at pH lower than 2 should be favored as it is more
convenient to evaluate the flavylium cation form by high-performance liquid chromatography (HPLC) or colorimetric tests.

When exposed to acids or bases, ester bonds in ellagitannins **and caffeoylquinic acids** are hydrolyzed and the hexahydroxydiphenic acid is spontaneously rearranged into the water-insoluble ellagic acid (Clifford and Scalbert 2000). Daniel and others (1991) showed that ellagic acid could be released from raspberry ellagitannins at pH 7 and optimally at pH 8. Furthermore, Gil-Izquierdo and others (2002) observed a 5-to 10-fold increase in ellagic acid from strawberry ellagitannins during incubation with pancreatic enzymes in mild alkaline conditions (Gil-Izquierdo and others). This may be the mechanism behind the relative increases in smaller ellagitannin molecules noted during **in vitro** digestion of raspberry and strawberry extracts (McDougall and others 2007; Brown and others 2012,). In the mildly alkaline conditions of **in vitro** digestion, orange flavanones form less soluble chalcone forms which precipitate (Gil-Izquierdo and others 2003). However, more than 90% of orange flavanones and 80% of soy isoflavone glycosides were recovered after the intestinal step outlining their high stability toward autoxidation (Walsh, Zhang, Vodovotz, Schwartz and Failla 2003, Gil-Izquierdo, Gil, Tomas-Barberan and Ferreres 2003). The sensitivity to autoxidation is probably overestimated in **in vitro** digestion models as oxygen is known to largely disappear in the gastric tract. Last, it should be noted that proteolytic enzymes could play a role in polyphenol bioaccessibility by releasing phenolic compounds bound to dietary proteins as observed in the gastric tract for pepsin. However, more data support a role for phenolic compounds as inhibitors of intestinal enzymes such as trypsin and lipase (Gonçalves and others, 2007; He and others, 2006).
**Static models**

Conditions used in *in vitro* static models simulate quite well the physiology of intestinal digestion with the use of porcine pancreatin, biliary extract or bile salts, and a pH ranging between 6.0 and 7.5 (Table 3). However, the time allowed for this step is highly variable (0.5-2.5 h). A too short digestion time may lead to trapping of carotenoids in triglycerides, and thus underestimation of carotenoid bioaccessibility (Sy and others 2012a). Different carotenoids show differing micellarization. Xanthophylls (lutein and beta-cryptoxanthin) showed higher micellarization compared to alpha- and beta-carotenes, while lycopene was only slightly micellarized (Garrett and others 2000; Reboul and others 2006; Thakkar and Failla 2008). There have also been differences noted between (E)-carotenoids and their (Z)-isomers (Chitchumroonchokchai and others 2004; Bengtsson and others 2010; Biehler and others 2011b), with the latter commonly found in processed foods, also tending to be better micellarized (Bohn 2008). It could also be speculated that a prolonged time of small intestinal digestion will favor the formation of more Z-isomers. However, the *in vivo* data showed no significant isomerization either in the stomach or in the duodenum for beta-carotene and lycopene (Tyssandier and others 2003).

In most *in vitro* studies, the stability of phenolic compounds has been assessed by determining total phenolic content such as the Folin-Ciocalteu method (Singleton L and Rossi 1965), which does not yield information on the reactivity of specific phenolic classes or molecules. The intestinal step, when compared to the gastric step, did not influence the recovery of total phenolic compounds for homogenized prunes (81% of the initial conc. in fruit) (Tagliazucchi and others 2012), grape berries (62%) (Tagliazucchi and others 2010), cherries (127%) (Fazzari and others 2008), pomegranate juice (100%) (Perez-Vicente and others 2002) and red cabbage extract...
Howeve\text{r}, a loss in total phenolics during the intestinal step was observed for plums (44%), peaches (37%), tomato (31%) (Tagliazucchi and others 2012), chokeberry juice (73%) (Bermudez-Soto and others 2007), raspberry extract (86%) (McDougall and others 2005b), and red wine (47% and 58%) (Gumienna and others 2005b), many of which contain labile anthocyanins. In conclusion, the analysis of specific phenolic compounds should be addressed in order to avoid conflicting results. Additionally, findings on the recovery of different classes in one fruit/vegetable cannot be readily extended to other sources as stability \textit{in vitro} is influenced by interactions with the other phenolic compounds in the mixture and vitamin C (for example sacrificial oxidation).

\textit{Dynamic models}

To simulate the \textit{in vivo} conditions of the small intestine, dynamic models can be used to reproduce pH changes and secretion of pancreatic juice and bile. In the TIM model, the intestinal transit time and pH conditions in the human digestive tract are simulated through pre-programmed pH and delivery curves (Minekus 1995). Porcine pancreatin, bile salts, electrolytes, and NaHCO$_3$ are secreted by computer-controlled pumps. The model does not mimic brush border secretions. pH usually increases between the duodenal, jejunal, and ileal compartments, for example, from 6.4 to 7.2 for the digestion of a tomato-containing Western diet (Blanquet-Diot and others 2009). The gastrointestinal transit time may greatly influence the bioaccessibility of phytochemicals by affecting the release from the food matrix. Additionally, the solubility and stability of different compounds may be affected by the time they are exposed to the conditions in the intestinal tract. Apart from the integration of key parameters of digestion as peristaltic mixing, transit time, and transport, the ability to remove digested material by
passive absorption of water and digested molecules through a dialysis system is also an important feature of *in vitro* models. In particular, removal of digested molecules should prevent product inhibition of the pancreatic enzymes (Minekus 1995).

The TIM-1 and Tiny-TIM systems have shown their usefulness in studying the digestive stability of carotenoids from tomato, and phenolic acids present in bread, respectively (Blanquet-Diot and others 2009, Hemery and others 2010). The TIM-1 system can be equipped with semi-permeable hollow fiber membrane filters (with a molecular weight cut-off ranging between 3-5 kDa to 5-8 kDa, depending on filter type) connected to the jejunal and ileal compartments in order to remove degraded compounds and to simulate absorption of water soluble nutrients. For the estimation of the bioaccessibility of lipophilic carotenoids, the incorporation into micelles is crucial and for this purpose the TIM system needs to be equipped with a specific membrane that separates the micellar phase from the fat phase (Minekus 1995). The formation of micelles which are less than 10 nm in size is dependent, among other factors, on the presence of fat and bile salts, and the digestion protocol should be adequately designed to ensure triglyceride hydrolysis and micellarization by bile salts.

**General considerations**

The contribution of the intestinal step to the bioaccessibility of phenolic compounds is clearly influenced by several parameters. First, the action of intestinal enzymes on the residual matrix could increase the phenolic content. Next, phenolic compounds are chemically reactive in near-neutral conditions and their degradation or isomerization may be catalyzed by the presence of oxygen and/or transition-metal ions. Additionally, specific absorption by the small intestine can occur by passive diffusion or active transport, as demonstrated for aglycones and their glucosylated forms. The latter forms
can be actively transported by the sodium-glucose-linked transporter 1 (SGLT1) found in the enterocytes. Extracellular hydrolysis can be promoted by lactase phlorizin hydrolase (LPH) in the brush border and be followed by diffusion of the resulting aglycone into the enterocyte (Day and others 2000). A transcellular transport involving multidrug resistance protein and P-glycoprotein transporters appears to be favored for hydroxycinnamic acid and flavonol aglycones (Poquet and Clifford 2008, Barrington and others 2009). These 2 phenomena cannot be readily modeled in vitro. Therefore, in vitro digestion methods may over estimate the levels of these phenolic components. In summary, a further limitation in oxygen, an inclusion of brush border enzymes or analogs with α-glucosidase activity, a sufficient bile salt concentration, and the presence of lipolytic, amylolytic and proteolytic enzymes for specific nutrient digestion are all of importance for an optimal release of phytochemicals. While remaining triglycerides may trap lipid-soluble phytochemicals, incompletely digested proteins and polysaccharides will bind to water-soluble phytochemicals, making them unavailable in the small intestine.

Large intestinal bioconversions

The colon contains a highly complex microbial ecosystem, which is capable of fermenting food components not digested in the upper GI tract. Some undigested food ingredients, including certain polyphenols, can act as substrate for the indigenous bacterial community (Possemiers and others 2011). In addition, microbial bioconversion products can influence the overall intestinal ecosystem and the bioavailability of the parent compounds. Carotenoids are typically not studied in colonic models, as they are primarily absorbed in the small intestine, and colonic metabolites have not been reported so far. Colonic bioconversion of polyphenols is most well-described for
flavonoids (Table 4) and phytoestrogens, lignans and isoflavonoids. The complexity of
in vitro colonic models used to study the metabolism of phenolic compounds is diverse,
ranging from batch fecal incubations using a strictly anaerobic and dense fecal
microbiota suitable for metabolic studies (Barry 1995; Gross and others 2010; Aura and
others 2012) to more complex continuous models involving one or multiple connected,
pH-controlled vessels representing different parts of the human colon (Fogliano and
others 2011) or in vitro dynamic gastrointestinal-colonic system models (Gao and others
2006; Van Dorsten and others 2012), which are applicable also to study effects of food
components on the microbial population.

Characterization of phenolic metabolites using in vitro colonic models is
complementary to the metabolic bioconversion by the small intestine or the liver
(methylation, sulfation, and glucuronidation) of the native forms in which they are
present in foods (Scalbert and others 2002) and shows the diversity of structural
transformations occurring in the colon prior to absorption (Aura 2008; Selma and others
2009). Colonic metabolism of phenolic compounds starts with the transient appearance
of aglycones and the subsequent formation of hydroxylated aromatic compounds and
phenolic acids (Rechner and others 2004; Aura 2008). Flavones, flavanones, flavanols,
proanthocyanidins, and phenolic acids share hydroxyphenylpropionic acid metabolites
(Rechner and others 2004; Aura 2008), whereas flavonols (quercetin, myricetin) and
ferulic acid dimers share hydroxylated phenylacetic acid metabolites (Aura and others
2002, Braune and others 2009). Moreover, flavanols also yield hydroxyphenylvaleric
acids and corresponding valerolactone derivatives (Aura and others 2008; Sanchez-
Patan and others 2012). Anthocyanins yield benzoic acids, hydroxylated benzaldehydes,
and acetaldehydes (Aura and others 2005; Fleschhut and others 2006; Czank and others
2013). Complex microbial metabolites, such as lactones formed from plant lignans or
ellagitannins (Heinonen and others 2001; Cerda and others 2004), are re-absorbed from the colon and are subject again to liver metabolism and the conjugate derivatives are excreted via urine (Adlercreutz and others 1995). Thus plasma and urine excretions reflect both the hepatic and colonic metabolism of polyphenols (Table 4).

Limitations of in vitro colonic models include that they may not fully represent the microbiota present in the colonic lumen and mucosa and that the combined rates of catabolism and absorption that occur in vivo are not reproduced. However, the use of colonic models provides information on the types of microbial metabolites formed (Table 4) and helps to elucidate the pathways involved. Batch models are of particular interest for a first assessment of colonic metabolism of phenolic compounds, which is characterized by a high inter-individual variability (Gross and others 2010), or for comparison of different sources or doses of compounds (Bolca and others 2009). The anaerobic batch colonic model developed by Barry and others (1995), which uses pooled human feces from several healthy donors, has been particularly suitable as coupled with a metabolomics platform to investigate the effects of structure and dose of fruit proanthocyanidin fractions on the efficiency of microbial metabolism and structure of flavanol monomers (Aura and others 2012; Aura and others 2008).

Dynamic, multi-compartment colonic models are useful for long-term experiments needed to evaluate the spatial and temporal adaptation of the colonic microbiota to dietary phenolic compounds and the microbial metabolism of these phytochemicals. These models are designed to and should harbor a reproducible microbial community that should be stable upon inoculation, colon region-specific, and relevant to in vivo conditions (Macfarlane and others 1998; Van den Abbeele and others 2010). Dynamic colonic models have shown that microbial metabolism of black tea and red wine (Van Dorsten and others 2012) and cocoa (Fogliano and others 2011) is dependent on colon
location. In addition, dynamic models may be used to enrich the colonic microbiota with polyphenol-converting species such as *Eubacterium limosum* to increase the production of 8-prenylnaringenin from hop extracts (Possemiers and others 2008). Dynamic colonic simulators have integrated new tools to improve modeling the physiological colonic conditions, such as the incorporation of a mucosal environment (Macfarlane and others 2005; Van den Abbeele and others 2012) and a mucus layer combined with epithelial cells (Marzorati and others 2011). The models can differentiate between the luminal microbiota with a large metabolic degradation capacity and the mucosa-associated microbiota able to closely interact with the host.

An important element to be considered for designing colonic model experiments is the use of one or multiple fecal donors in terms of diversity of the microbiota population, as high-and low-polyphenol metabolizing phenotypes can skew the extent of metabolism of certain compounds (Selma and others 2009; Bolca and others 2012). Meanwhile, comparison of human gut metagenomes has suggested the classification of individuals into three distinct enterotypes (Arumugan and others 2011). The maintenance of anaerobic conditions during stool processing and inoculation to the models is crucial for microbial and enzymatic activities. Another important matter to be considered is the pH adjustment needed to avoid suppression of particularly minor conversion activities, for example slow enterolactone formation (Aura 2008). In summary, *in vitro* colonic models are the preferred choice to study mechanisms of polyphenol microbial metabolism as well as the polyphenol-induced modulation of gut microbiota. However, the ability of colonic models to simulate the *in vivo* conditions is limited by the lack of studies involving the formation of microbial biofilms adhering to the colonic epithelium. The simulation of intestinal absorption to remove end products
of microbial metabolism is also relevant to prevent inhibition of the colonic microbiota during *in vitro* studies.

**Determination of bioaccessible fraction and further coupling techniques following digestion and/or colonic fermentation**

During the past few years *in vitro* digestion model systems have been used to analyze the structural and chemical changes that occur in different foods under simulated gastrointestinal conditions. These methods either simulate either disintegration, food matrix and digestion processes only (for bioaccessibility) or both digestion and absorption processes (for bioavailability estimates). According to the desired endpoints of the studies, there are considerable differences in the type of experimental parameters measured after digestion. These include chemical changes (such as hydrolysis of macronutrients), gastric solubilization of drugs, nutrient availability, release of encapsulated components, studying competitive processes, and structural changes (such as break-down of specific structures), aggregation, droplet coalescence, or droplet disruption (Chen and others 2011). Thus, samples obtained by *in vitro* digestion, either following small intestinal digestion or following further colonic fermentation *in vitro*, have been used in a variety of ways. In addition, the obtained fractions have been coupled to further investigation procedures, allowing for example the estimation of uptake into or transport through the intestinal epithelium.

**Estimation of bioaccessibility**

The estimation of the bioaccessibility of non-polar food constituents such as carotenoids has been made both by measuring the transfer of carotenoids from the food
matrix to the aqueous layer obtained after *in vitro* digestion and centrifugation (Hedrén and others 2002; Bengtsson and others 2009) or by filtering the aqueous fraction through a 0.22 µm membrane to obtain micelles (Reboul and others 2006; Huo and others 2007), or both. Since the micellarized carotenoids are considered to be the form in which these compounds will ultimately be absorbed by the intestinal cells, it has been suggested that assessment of carotenoid bioaccessibility must include the isolation, extraction and measurement of carotenoids in micelles (Etcheverry and others 2012). Reboul and others (2006) showed a high correlation (r = 0.90) of the *in vitro* bioaccessibility of α- and γ-tocopherol, β-carotene, and lycopene with the *in vivo* values measured in the micellar phase from human duodenum during digestion of a carotenoid-rich meal. Their findings suggest that estimation of carotenoid micellarization *in vitro* can be indicative of the amount available for uptake in the gastrointestinal tract *in vivo*.

For polyphenols, Bouayed and others (2011, 2012) studied bioaccessibility following simulated gastric and intestinal *in vitro* digestion of fresh apple. They used a cellulose semi-permeable membrane, chosen as a simplified mechanical model for the epithelial barrier to identify dialyzable polyphenols after intestinal digestion. They suggested that dialyzable polyphenols in the intestinal phase could potentially be taken up by the enterocytes and suggested it may be a practical step prior to coupling to cellular methods due to increased purity of the dialysate, preventing negative impacts on cell viability. Similar studies were performed by other researchers (Liang and others 2012, Rodriguez-Roque and others 2013). At the same time, it is difficult to study the *in vivo* changes and digestive stability of different food constituents during their passage through the digestive tract, albeit some approaches, such as studying ileostomists, have allowed some comparisons to *in vitro* small intestinal digestion (Walsh and others 2007; Erk and others 2012).
Bioaccessibility following colonic fermentation

In vitro digestion procedures have also been employed to produce berry samples that are characteristic of components that survive digestion, and therefore more physiologically relevant, for studies on bioactivities relevant to colon cancer models (Brown and others 2012).

Due to the limited sampling possibilities (and intra- and inter-individual variations), the function and the composition of ileal microbiota is hard to study in vivo. The effect of small intestinal microflora on the enzymatic hydrolysis of phenol glycosides was studied in an ex vivo ileostomy model (Knaup and others 2007). Ileostomy effluents from 3 healthy subjects were used for incubation with synthetic quercetin and p-nitrophenol glycosides. The conclusion was that the hydrolysis of phenol glycosides is influenced both by the structural components of the phenols and the microflora in the small intestine. Schantz, Erk and Richling (2010) have also reported evidence of degradation of polyphenols in the small intestine, using an ex vivo ileostomy model to study the microbial metabolism and chemical stability of green tea catechins and gallic acid. According to studies in ileostomy patients, the ileal microbiota is restored 6 months after surgery (Hove and Mortensen, 1996) which may resemble the reflux situation occurring in subjects with a healthy colon, or even take the role of colon fermentation to some extent in ileostomy patients.

Phenolic microbial metabolites are relevant in terms of human health because they appear in plasma and are excreted in urine (Aura 2008). Pharmacokinetic studies show that microbial metabolite concentrations are elevated for up to 24-48 h in the bloodstream after a single dose of their precursors before returning to baseline values (Sawai and others 1987; Gross and others 1996; Kuijsten and others 2005).
Enterolactone, enterodiol, and urolithins are excreted via urine as hepatic conjugates (Heinonen and others 2001; Cerda and others 2004), whereas microbial phenolic acid metabolites appear in urine mainly in a free form in contrast to beverage-derived phenolic acids which are excreted mainly as sulphates and glucuronides (Sawai and others 1987; Stalmach and others 2009). In a recent work, Ludwig and others (2013) show that after ingestion of coffee, the main colon-derived metabolites found in plasma and/or in urine were dihydrocaffeic acid, dihydroferulic acid, and their sulfated and glucuronidated metabolites. As the metabolites described above and their hepatic conjugates are found in plasma and urine, therefore they circulate through the body and may exhibit both local and systemic effects. Phenolic metabolite levels in plasma range from low to high nano molar concentrations (Sawai and others Ando 1987; Kilkkinen and others 2001; Kern and others 2003; Johnsen and others 2004; Kuijsten and others 2006), whereas urinary levels are at the micro molar range. In peripheral tissues, the concentrations can be anticipated to be even lower.

A good example of studies including in vitro digestion models and colon conversion and pharmacokinetic studies in human volunteers was performed by Mateo Anson and others (2009, 2011). The group showed that bioprocessing of wheat bran with enzymes (xylanase, cellulose, β-glucanase, and feruloyl esterase) and yeast enhanced the bioaccessibility of ferulic acid, para-coumaric acid, and sinapic acid from white wheat bread matrix in the in vitro gastrointestinal models TIM-1 and TIM-2 by 5-fold. Since the release of para-coumaric acid and sinapic acid occurred mainly in the TIM-1 model simulating the upper intestine, the microbial conversion products (3-(3'-hydroxyphenyl) propionic acid and 3-phenylpropionic acid) from the TIM-2 colon model were shown to be related to matrix bound ferulic acid content (Mateo Anson and others 2009). In a subsequent pharmacokinetic in vivo study volunteers consumed 300 g white wheat
bread samples fortified with either native or bioprocessed wheat bran, and then phenolic acids and their metabolites were followed for 24 hours. The release and conversion of microbial metabolites were enhanced by bioprocessing of bran by 2- to 3-fold and their time course profiles in plasma were altered by bioprocessing of bran (Mateo Anson and others 2011).

Coupling digesta to uptake and transport models of the intestinal epithelium

More recently, human enterocyte cell culture models (such as Caco-2 cells) was coupled with a simulated gastric model. Small intestinal digestive processes or following further colonic fermentation have been widely used as a predictive tool for the absorption of bioactive components from foods (Chitchumroonchokchai and Failla 2006). Caco-2 is a cell line originating from human colonic carcinoma that exhibits some morphological and functional characteristics similar to those of differentiated epithelial cells that line the intestinal mucosa (Sambruy and others 2001). The in vitro digestion/Caco-2 cell culture model developed by Glahn and others (1998) offers a rapid, low-cost method for screening foods and food combinations for iron uptake before more definitive human trials (Hur and others 2011). Most Caco-2 cell model studies were carried out to model iron uptake and many researchers reported that the estimation of iron bioavailability, but also that of other phytochemicals such as carotenoids from the in vitro digestion/Caco-2 cell culture model has been well correlated, qualitatively and quantitatively, with human data (Garrett and others 1999; Mahler and others 2009). Caco-2 cells have also been applied to a number of uptake and transport studies for both hydrophilic constituents (such as polyphenols) and lipophilic compounds (such as carotenoids). Garret and others (1999, 2000) developed a coupled digestion/Caco-2 human intestinal cell system to examine cellular acquisition of
micellarized carotenoids and other lipophilic components from digested foods, supplements, and meals. While the majority of studies have focused on simple uptake employing a biphasic model with the apical membrane and the cell layer, uptake models including also an additional basolateral compartment are also available to allow the study of fluxes and, therefore, kinetic parameters through the cell layer (Reboul and others 2006; Manzano and Williamson 2010; Biehler and others 2011a). However, the latter requires transwell inserts, which are more costly, and the concentrations to be determined are usually lower and may require more sophisticated analytical instruments for detection, such as mass spectrometry, and may not be feasible for easily studying minor food constituents. More detailed discussion on characteristics and limitations of standard in vitro digestion methods coupled with a Caco-2 cell model can be found in review articles by Failla and others (2008a) and by Biehler and others (2011a). More recently, the Caco-2 cell model has been extended by adding a layer of mucus-producing cells (such as HT-29 MTX cells) on top of the Caco-2 cells. However, only preliminary data are available on how this system performs compared to Caco-2 cells alone, although this may represent a more realistic approach, which may further hamper uptake of more lipophilic constituents due to the additional mechanical barrier (Nollevaux and others 2006). Also, Ussing chambers are used, in order to obtain a better understanding of the transepithelial transport processes on a molecular basis. This is a model that simulates the mucosa and its luminal/apical side (Bergmann H and others 2009; Clarke LL 2009). For example, Deusser H and others (2013) have used the Ussing chamber to evaluate apple polyphenol transport and their effect on mucosal integrity.

Conclusions and Summary
Many considerations have to be taken into account when determining bioaccessibility of phytochemicals by means of in vitro digestion models. Two important criteria are whether the focus of research is on biochemical transformation of food components and metabolomics, favoring the metabolic batch models, or if the close simulation of dynamic physiological conditions and changes in microbial population are the primary aims, the use of continuous models such as the TIM models can be recommended. An additional criterion is the lipophilicity of the phytochemicals of interest. While for hydrophilic compounds such as for polyphenols, often associated with fiber or complex carbohydrates, amylase digestion and perhaps particle size appear to play predominant roles. Whereas, for lipophilic compounds, (such as carotenoids) emulsifying agents, (presence of dietary fats, bile salts, and sufficient lipolytic activity), appear crucial, thus their use during digestion should be well considered and standardized. This includes adjusting pH values and sufficient digestion times to allow for optimal enzyme functioning comparable to the in vivo situation. The suggested conditions for static digestion models are outlined in Table 5. In addition, lipophilic phytochemicals require separation of the micellar fraction prior to further investigation, such as via ultracentrifugation (static model), filtration, or employing a membrane (dynamic model). Coupling the cell-based uptake model with large intestinal digestion model is a comparatively novel but important completion of modeling digestion. This may especially be suitable for compounds such as polyphenols, which are metabolized and taken up from the colon.

Until now, the lacking of consensus values for the different digestion parameters has hampered the possibility to compare results across different studies. The suggested conditions are based in relevant in vivo data, yet further studies should be done to
validate its use and limitations in phytochemicals digestion. While still having their limitations, much insightful information has been gained from applying *in vitro* digestion models to phytochemical research. The recent improvements in our understanding and the advances in the technology warrant continuous research in the important area of bioavailability.

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**Conflicts of Interest**

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.
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reproducible, colon region specific, and selective for Bacteroidetes and Clostridium cluster


Table 1: Concentrations of enzymes and concentrations employed during the oral phase of *in vitro* (A) and human studies *in vivo* (B) studies.

### A - *in vitro* studies

<table>
<thead>
<tr>
<th>Type of study</th>
<th>α-Amylase activity (U/mL)</th>
<th>pH of digestion</th>
<th>Time of digestion (min)</th>
<th>Temperature (°C)</th>
<th>Study/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digestion of grape seed flavonoids (human saliva)</td>
<td>Not specified</td>
<td>6.9</td>
<td>10</td>
<td>37</td>
<td>(Laurent and others 2007)</td>
</tr>
<tr>
<td>Digestibility of soya bean, cowpea and maize</td>
<td>ca. 1</td>
<td>7</td>
<td>30</td>
<td>37</td>
<td>(Kiers and others 2000)</td>
</tr>
<tr>
<td>Bioactivity of wheat bread; changes in the antioxidant activities of vegetables</td>
<td>200</td>
<td>6.75</td>
<td>10</td>
<td>37</td>
<td>(Gawlik-Dziki, 2009, 2012)</td>
</tr>
<tr>
<td>Developing digestion procedure with mammalian enzymes</td>
<td>25-125</td>
<td>7</td>
<td>15</td>
<td>37</td>
<td>(Lebet and others 1998)</td>
</tr>
<tr>
<td>β-Carotene micellarization</td>
<td>900</td>
<td>6.5 ±0.2</td>
<td>10</td>
<td>37</td>
<td>(Thakkar and others 2007)</td>
</tr>
<tr>
<td>β-Carotenoid bioaccessibility</td>
<td>300</td>
<td>6.7-6.8</td>
<td>10-15</td>
<td>37</td>
<td>(Bengtsson and others 2009, 2010)</td>
</tr>
<tr>
<td>β-Carotene bioaccessibility (human saliva from n=9)</td>
<td>12.5°F</td>
<td>6.7-6.9</td>
<td>10</td>
<td>37</td>
<td>(Schweiggert and others 2012)</td>
</tr>
<tr>
<td>β-Carotene bioaccessibility from sweet potato</td>
<td>35</td>
<td>7.0</td>
<td>10</td>
<td>37</td>
<td>(Poulaert and others 2012)</td>
</tr>
<tr>
<td>Polyphenol release during digestion</td>
<td>150</td>
<td>6.9</td>
<td>10</td>
<td>37</td>
<td>(Tagliazucchi and others 2012)</td>
</tr>
</tbody>
</table>
### B - Human studies (in vivo)

<table>
<thead>
<tr>
<th>Type of study</th>
<th>α-Amylase activity (U/L)**</th>
<th>Time of digestion (min)</th>
<th>pH</th>
<th>Study/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Studying impact of saliva process on lipophilic polyphenol availability</td>
<td>Not specified</td>
<td>0.5</td>
<td>nd</td>
<td>(Ginsburg and others 2012)</td>
</tr>
<tr>
<td>Physiology of human saliva including mucin and electrolytes</td>
<td>Not specified</td>
<td>nd</td>
<td>7.0</td>
<td>(Aps and Martens, 2005)</td>
</tr>
<tr>
<td>Human salivary α-amylase activity</td>
<td>4-1653, mean 284f</td>
<td>nd</td>
<td>nd</td>
<td>(Suska and others 2012)</td>
</tr>
<tr>
<td>List of reference values</td>
<td>60-282, mean 170f</td>
<td>nd</td>
<td>nd</td>
<td>(Jakob, 2008; Kopf-Bolanz and others 2012)</td>
</tr>
<tr>
<td>Stress and alpha-amylase</td>
<td>220-500 between 8am and 20pm.f</td>
<td>nd</td>
<td>nd</td>
<td>(Nater and others 2010)</td>
</tr>
<tr>
<td>Oral digestion of cereals by humans</td>
<td>52-77(basal )</td>
<td>5</td>
<td>7.1±0.1</td>
<td>(Hoebler and others 1998)</td>
</tr>
<tr>
<td>Saliva activity measurements</td>
<td>190f</td>
<td>nd</td>
<td>nd</td>
<td>(Rohleder and Nater, 2009)</td>
</tr>
</tbody>
</table>

**“Sigma units”, unless stated otherwise: 1 unit will liberate 1 mg maltose from starch in 3 min at 20 °C at pH 6.9. Often done via the dinitrosalicylic acid (DNS) color assay (540 nm).

Conversion to IFCC and Phabedas: when expressed as same mass unit (mmol not mg), 1 DNS unit = ca. 2.5 IFCC units (Bassinello and others 2002). For results given in mg, conversion factor from DNS to IFCC is x 2.5/342 = x 0.0073.

** Units are expressed here in final volume of salivary fluid. 1 unit will cleave 1μmol glucosidic linkage from starch per min, however substrate may differ. Both methods presented here (IFCC EPS and Phabedas) yield comparable results.

f: Phabedas (Magle AB, Lund, Sweden) test: blue color from starch breakdown measured at 620 nm. Conversion from µkat to U according to http://www.phadebas.com/data/phadebas/files/document/Instructions_Phabedas_Amylase_Test.pdf, 60U=1µkat

f releases 1 µmol/min p-nitrophenol from 4,6-ethylidene-G7-p-nitrophenol-D-maltoheptasoid (ethylidene-G7PNP, measured at 405 nm. 60 U=1 µkat (IFCC EPS method, Ethylen protected substrate)

---

Table 2: Typical concentrations of gastric enzymes in human studies and in vitro experiments.
### A - *In vitro* studies

<table>
<thead>
<tr>
<th>Type of study</th>
<th>Pepsin* (mg/mL)</th>
<th>Pepsin activity* (U/mL)</th>
<th>pH of digestion</th>
<th>Time of digestion (min)</th>
<th>Study/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bioavailability of iron</td>
<td>ca. 5</td>
<td>4,000-12,500</td>
<td>2.0</td>
<td>120</td>
<td>(Miller and others 1981)</td>
</tr>
<tr>
<td>Bioaccessibility of carotenoids</td>
<td>2.3</td>
<td>1,800-5,600</td>
<td>2.0</td>
<td>60</td>
<td>(Biehler and others 2011a, b)</td>
</tr>
<tr>
<td>Bioaccessibility of carotenoids</td>
<td>2.2</td>
<td>1,700-5,400</td>
<td>2.0</td>
<td>60</td>
<td>(Garrett and others 1999)</td>
</tr>
<tr>
<td>Bioaccessibility of carotenoids</td>
<td>1.7</td>
<td>1,400-4,300</td>
<td>2.0</td>
<td>60</td>
<td>(Hedrén and others 2002)</td>
</tr>
<tr>
<td>Bioaccessibility of carotenoids</td>
<td>1.2</td>
<td>900-3,000</td>
<td>4.0</td>
<td>30</td>
<td>(Dhuique-Mayer and others 2007)</td>
</tr>
<tr>
<td>Bioaccessibility of carotenoids</td>
<td>1.0</td>
<td>800-2,500</td>
<td>2.0</td>
<td>60</td>
<td>(Liu and others 2004)</td>
</tr>
<tr>
<td>Bioaccessibility of carotenoids</td>
<td>3.0</td>
<td>2,400-8,300</td>
<td>2.0</td>
<td>60</td>
<td>(Yonekura and Nagao 2009)</td>
</tr>
<tr>
<td>Bioaccessibility of polyphenols</td>
<td>nd</td>
<td>315-350</td>
<td>2.0</td>
<td>120</td>
<td>(Gil-Izquierdo and others 2002)</td>
</tr>
<tr>
<td>Bioaccessibility of polyphenols</td>
<td>2.2</td>
<td>1,800-5,600</td>
<td>2.0-2.5</td>
<td>60</td>
<td>(Bouayed and others 2011)</td>
</tr>
<tr>
<td>Bioavailability of polyphenols</td>
<td>16</td>
<td>15,600</td>
<td>2.0</td>
<td>120</td>
<td>(Cilla and others 2011)</td>
</tr>
<tr>
<td>Bioavailability of polyphenols</td>
<td>nd</td>
<td>158</td>
<td>2.0</td>
<td>120</td>
<td>(Tagliazucchi and others 2012)</td>
</tr>
<tr>
<td>Bioavailability of polyphenols</td>
<td>ca. 0.1</td>
<td>315</td>
<td>1.7</td>
<td>120</td>
<td>(McDougall and others 2005a, b)</td>
</tr>
</tbody>
</table>
**Table 2 cont.**

### B- Human studies (*in vivo*)

<table>
<thead>
<tr>
<th>Fluid investigated and type of study</th>
<th>Pepsin (U/mL)</th>
<th>Gastric residence time (h)</th>
<th>pH</th>
<th>Study/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digestion of adults</td>
<td>942(^5) (1207)(^7) (basal)</td>
<td>nd</td>
<td>nd</td>
<td>(Armand and others 1995)</td>
</tr>
<tr>
<td>Digestion of infants</td>
<td>ca. 85(^5) (109)(^7) (pp) 190(^5) (243)(^7) (basal)</td>
<td>nd</td>
<td>nd</td>
<td>(Armand and others 1996a)</td>
</tr>
<tr>
<td>Helicobacter pylori impact on stomach</td>
<td>47(^b) (174)(^7)</td>
<td>nd</td>
<td>1.41 (basal)</td>
<td>(Feldman and others 1998)</td>
</tr>
<tr>
<td>18 individuals, fasting juice</td>
<td>37±21 [7-70](^a) (3700)(^7)</td>
<td>nd</td>
<td>1-4, median 2 (basal)</td>
<td>(Ulleberg and others 2011)</td>
</tr>
<tr>
<td>Pepsin inhibitors in humans</td>
<td>20-260 µg/mL(^k)</td>
<td>nd</td>
<td>nd</td>
<td>(Pearson and Roberts, 2001)</td>
</tr>
<tr>
<td>Measurement gastric secretion</td>
<td>nd</td>
<td>nd</td>
<td>1.1 (basal); 3.5 (60 min. pp) 2.0(120 min. pp)</td>
<td>(Gardner and others 2002)</td>
</tr>
<tr>
<td>Characterization of digestive fluids</td>
<td>110-220 µg/mL (basal)(^k) 260-580 µg/mL (pp)</td>
<td>nd</td>
<td>2 (basal) 6 (60 min. pp) 5 (120 min. pp)</td>
<td>(Kalantzi and others 2006)</td>
</tr>
<tr>
<td>Helicobacter pylori impact on pepsin</td>
<td>114 to 1030 µg/mL(^k)</td>
<td>nd</td>
<td>2.4 (basal)</td>
<td>(Newton and others 2004)</td>
</tr>
<tr>
<td>Gastric residence time, solid meal</td>
<td>nd</td>
<td>3.5± 0.7</td>
<td>nd</td>
<td>(Mojaverian and others 1988)</td>
</tr>
<tr>
<td>Gastric residence time of capsule</td>
<td>nd</td>
<td>1.2± 0.45</td>
<td>1.5±0.04 (basal)</td>
<td>(Mojaverian, 1996)</td>
</tr>
<tr>
<td>Gastric passage time of capsule</td>
<td>nd</td>
<td>1.0</td>
<td>nd</td>
<td>(Worsoe and others 2011)</td>
</tr>
<tr>
<td>Digestability of rice pudding</td>
<td>nd</td>
<td>65% com-plete (1.5h)</td>
<td>nd</td>
<td>(Darwiche and others 1999)</td>
</tr>
</tbody>
</table>

---

\(^*\)“Sigma units”; pepsin typically used: 800 – 2500 units/mg such as by Sigma-Aldrich. 1 unit: One unit will produce a change in absorbance of 0.001 at 280 nm at 37 °C, in 1 min, at pH 2.0, with hemoglobin as substrate.

\(^5\) *IN VITRO* One pepsin unit has been defined as the amount of enzyme required to produce 0.1 µmole of tyrosine-containing peptides at 37 °C in 10 min at pH 1.8 from a 2% hemoglobin solution. 1 unit equivalent to approx. 1.28 “Sigma units” (http://www.worthington-biochem.com/pm/assay.html);

\(^6\) One unit of enzyme activity was defined as the amount (in mL) of gastric or duodenal juice giving a difference in absorbance of 1.0 at 280 nm at 37 °C and pH 3.0, in 10 min, with hemoglobin as substrate. 1 unit equivalent to approx. 100 “Sigma units”.

\(^7\) measured as international units, with 1 IU=3.7 Anson units.

\(^a\) nd= no data; \(^pp\)=post-prandial; \(^k\) µg enzyme/mL

Table 3: Concentrations of intestinal enzymes and bile salts in humans studies and *in vitro* experiments.
### A - *In vitro* studies

<table>
<thead>
<tr>
<th>Type of study</th>
<th>Bile salts (^+) (mmol/L)</th>
<th>Pancreatin(^+) concentration, ca. (mg/L)</th>
<th>Minimum pancreatin activity (U/mL)(^+)</th>
<th>pH</th>
<th>Digestion time (min)</th>
<th>Study/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bioaccessibility of iron</td>
<td>ca. 4 (2 g/L)</td>
<td>300</td>
<td>2.4</td>
<td>7.5</td>
<td>150</td>
<td>(Miller and others 1981)</td>
</tr>
<tr>
<td>Bioaccessibility of carotenoids</td>
<td>ca. 8.6 (4.3 g/L)</td>
<td>720</td>
<td>5.8</td>
<td>7-7.5</td>
<td>120</td>
<td>(Biehler and others 2011b)</td>
</tr>
<tr>
<td>Bioaccessibility of carotenoids</td>
<td>12 (6 g/L)</td>
<td>2500</td>
<td>20</td>
<td>7.5</td>
<td>120</td>
<td>(Tonekura and others 2009)</td>
</tr>
<tr>
<td>Bioaccessibility of carotenoids</td>
<td>4.4 (2.1 g/L)</td>
<td>390</td>
<td>3.1</td>
<td>7.5</td>
<td>120</td>
<td>(Garrett and others 1999)</td>
</tr>
<tr>
<td>Bioaccessibility of carotenoids</td>
<td>7.5 (3.75 g/L)</td>
<td>600</td>
<td>4.8</td>
<td>7.5</td>
<td>30</td>
<td>(Hedrén and others 2002)</td>
</tr>
<tr>
<td>Bioaccessibility of carotenoids</td>
<td>2.8 (1.44 g/L)</td>
<td>240</td>
<td>2.0</td>
<td>6.0</td>
<td>30</td>
<td>(Dhuique-Mayer and others 2007)</td>
</tr>
<tr>
<td>Bioaccessibility of carotenoids</td>
<td>3.0 (1.5 g/L)</td>
<td>250</td>
<td>2.0</td>
<td>ca. 7</td>
<td>120</td>
<td>(Liu and others 2004)</td>
</tr>
<tr>
<td>Bioaccessibility of carotenoids</td>
<td>4.3 (1.5 g/L)</td>
<td>250</td>
<td>2.0</td>
<td>5 to 7.5</td>
<td>120</td>
<td>(Gil-Izquierdo and others 2002)</td>
</tr>
<tr>
<td>Bioaccessibility of polyphenols</td>
<td>4.3 (2.2 g/L)</td>
<td>360</td>
<td>2.9</td>
<td>6.5-7.0-7.5</td>
<td>165</td>
<td>(Bouayed and others 2011)</td>
</tr>
<tr>
<td>Bioavailability of polyphenols</td>
<td>44 (22 g/L)</td>
<td>3,600</td>
<td>29</td>
<td>6.5</td>
<td>120</td>
<td>(Cilla and others 2011)</td>
</tr>
<tr>
<td>Bioavailability of polyphenols</td>
<td>10 (5 g/L)</td>
<td>800</td>
<td>6.4</td>
<td>7.5</td>
<td>120</td>
<td>(Tagliazucchi and others 2012)</td>
</tr>
<tr>
<td>Bioavailability of polyphenols</td>
<td>10 (5 g/L)</td>
<td>800</td>
<td>6.4</td>
<td>nd</td>
<td>120</td>
<td>(McDougall et al., 2005a, b)</td>
</tr>
</tbody>
</table>

Table 3 cont.
## B - Human studies (in vivo)

<table>
<thead>
<tr>
<th>Fluid investigated and type of study</th>
<th>pH</th>
<th>Bile salts (mmol/L)</th>
<th>Lipase activity (U/mL)</th>
<th>Study/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duodenal fluids; jejunal fluids;</td>
<td>7.0±0.4</td>
<td>0.6 - 5.5 (fasted)</td>
<td>n.d.</td>
<td>(Perez de la Cruz Moreno and others 2006)</td>
</tr>
<tr>
<td>Duodenal fluids</td>
<td>6.8±0.4</td>
<td>3.8–11.8 (fed)</td>
<td>6.8±0.4</td>
<td>(Van Deest and others 1968)</td>
</tr>
<tr>
<td>Duodenal fluids (standard meal)</td>
<td>nd</td>
<td>5 – 10 (fed)</td>
<td>n.d.</td>
<td>(Tabaqchali and others 1968)</td>
</tr>
<tr>
<td>Duodenal fluids (standard meal)</td>
<td>5.5-8.0, mean</td>
<td>20 (fed)</td>
<td>15-120 (fed) (mean 50)</td>
<td>(Borgstrom and others 1957)</td>
</tr>
<tr>
<td>Review article</td>
<td>nd</td>
<td>4 – 20 (fasted)</td>
<td>n.d.</td>
<td>(Garidel and others 2007)</td>
</tr>
<tr>
<td>Orlistat and enzyme activity</td>
<td>6-6.5</td>
<td>n.d.</td>
<td>1000 (fed)</td>
<td>(Sternby and others 2002)</td>
</tr>
<tr>
<td>Pancreatic enzyme examinations.</td>
<td>nd</td>
<td>n.d.</td>
<td>70-1000, mean 300</td>
<td>(Braganza and others1978)</td>
</tr>
<tr>
<td>Duodenal fluids (after regular diet)</td>
<td>n.d.</td>
<td>10 (fasted), 130 (fed)</td>
<td>n.d.</td>
<td>(Dukehart and others 1989)</td>
</tr>
<tr>
<td>Review lipolysis</td>
<td>n.d.</td>
<td>3-7 (fasted)</td>
<td>150-300</td>
<td>(Patton and Carey 1981; Zangenberg and others 2001a, b)</td>
</tr>
<tr>
<td>Fasting</td>
<td>5-9, mean</td>
<td>2.7±1.3</td>
<td>units not comparable to other tests</td>
<td>(Ulleberg and others, 2011)</td>
</tr>
<tr>
<td>18 individuals</td>
<td>6.2 (fasted)</td>
<td>2.6 (fasted)</td>
<td>n.d.</td>
<td>(Kalantzi and others2006)</td>
</tr>
<tr>
<td>6-14 individuals (median)</td>
<td>5.2-6.6 (fed)</td>
<td>11.2 (fed 30 min)</td>
<td>n.d.</td>
<td>(Armand and others1996b)</td>
</tr>
<tr>
<td>Duodenal fluids</td>
<td>6.0-7.0</td>
<td>5.9±1.8 (fasted)</td>
<td>600 (fasted)</td>
<td>(Armand, 2007)</td>
</tr>
<tr>
<td>Duodenal juices</td>
<td>n.d.</td>
<td>n.d.</td>
<td>80-7000</td>
<td>(Armand, 2007)</td>
</tr>
<tr>
<td>Small intestinal transit time (min)</td>
<td>90</td>
<td></td>
<td></td>
<td>(Kim, 1968)</td>
</tr>
<tr>
<td>GI passage times (min)</td>
<td>197</td>
<td></td>
<td></td>
<td>(Degen and Phillips, 1996)</td>
</tr>
<tr>
<td>GI passage times (min)</td>
<td>199</td>
<td></td>
<td></td>
<td>(Yu and others1996)</td>
</tr>
</tbody>
</table>

*Pancreatin typically used: 4 x US Pharmacopoeia specifications (2 USP units (U) lipase), 8 units; and both 25 USP, 100 USP units protease and amylase. Definition lipase: 1 unit liberates at least 1 μmole acid from olive oil/triolein per minute at 37 °C and pH 9 (http://www.pharmacopeia.cn/v29240/usp29nf24-0_m60320.html). Definition protease: hydrolyses casein at an initial rate such that there is liberated per min an amount of peptides not precipitated by trichloroacetic acid that gives the same absorption at
280 nm as 15 nmol of tyrosine (http://www.pharmacopeia.cn/v29240/usp29nf24s0_m60320.html). Definition amylase: decomposes starch at an initial rate such that 0.16 umol of glycosic linkage is hydrolyzed per min at pH 6.8 (and conditions further described for the amylase assay, http://www.pharmacopeia.cn/v29240/usp29nf24s0_m60320.htm).

* same as USP units.

Tributyrin units: 1 TBU (lipase unit) is the amount of enzyme which releases 1 mmol titratable butyric acid per min at 40 °C, pH 7.5. Yields comparable results to triolein units when expressed at same unit of molarity (McCoy and others 2002).

* values calculated from weight assuming a molecular weight of 500 g/mol and 100% purity.

*pentpostprandial
Table 4: Microbial phenolic metabolites identified from *in vivo* human studies and *in vitro* colonic models.

<table>
<thead>
<tr>
<th>Food</th>
<th>Metabolites <em>in vivo</em></th>
<th>Reference</th>
<th>Metabolites <em>in vitro</em></th>
<th>Colonic model</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tea</td>
<td>1.3-Dihydroxyphenyl-2-O-sulfate</td>
<td>(Daykin and others 2005)</td>
<td>3-Phenylpropionic acid</td>
<td>Batch</td>
<td>(Gross and others 2010)</td>
</tr>
<tr>
<td></td>
<td>5-(3',4'-Dihydroxyphenyl)-γ-valerolactone</td>
<td></td>
<td>3-(3',4'-Dihydroxyphenyl) propionic acid</td>
<td>Time: &lt;72 h</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5-(3',4',5'-Trihydroxyphenyl)-γ-valerolactone</td>
<td></td>
<td>3-(4-Hydroxyphenyl)propionic acid</td>
<td>pH: 6.92 ±0.26 (end)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hippuric acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1,3-Dihydroxyphenyl-2-O-sulphate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red wine, grapes</td>
<td>3-(3-Hydroxyphenyl)-propionic acid</td>
<td>(Jacobs and others 2012)</td>
<td>3-(3-Hydroxyphenyl)-propionic acid</td>
<td>Batch</td>
<td>(Sanchez-Patan and others 2012)</td>
</tr>
<tr>
<td></td>
<td>3-Hydroxyphenylacetic acid</td>
<td></td>
<td>3- and 4-Hydroxyphenylacetic acid</td>
<td>pH: 6.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3,5-Dimethoxy-4-hydroxybenzoic acid</td>
<td></td>
<td>5-(3',4'-Dihydroxyphenyl)-γ-valerolactone</td>
<td>Time: 48 h</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3-Hydroxyhippuric acid</td>
<td></td>
<td>γ-Valerolactone</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hippuric acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4-Hydroxyphenylacetic acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chocolate, cocoa</td>
<td>3-(3-Hydroxyphenyl)-propionic acid</td>
<td>(Llorach and others 2009)</td>
<td>3-(3-Hydroxyphenyl)-propionic acid</td>
<td>Batch</td>
<td>(Fogliano and others 2011)</td>
</tr>
<tr>
<td></td>
<td>5-(3,4-Dihydroxyphenyl) valerolactone and conjugates</td>
<td></td>
<td>3-Hydroxyphenylacetic acid</td>
<td>pH: monitored at each time point</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5-(3,4-Dihydroxyphenyl) valerate conjugates</td>
<td></td>
<td>3,4-Dihydroxybenzoic acid</td>
<td>Time: 0, 2, 4, 6, 8, and 24h</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4-Hydroxy-5-(3,4-dihydroxyphenyl)valeric acid Phenylvalerolactone derivatives</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soy</td>
<td>O-Demethylangolensin, Equol</td>
<td>(Joannou and others 1995)</td>
<td>O-Demethylangolensin, Equol</td>
<td>Batch</td>
<td>(Possemiers 2007)</td>
</tr>
<tr>
<td></td>
<td>Dihydrogenistein</td>
<td></td>
<td></td>
<td>Time: 72 h</td>
<td></td>
</tr>
<tr>
<td>Berries, nuts</td>
<td>4′-Hydroxymandelic acid, 3′,4′-Dihydroxyphenylacetic acid, 4′-Hydroxyhippuric acid, Hippuric acid, Urolithins</td>
<td>TIM2-2 colonic dynamic model pH: 5.8, 6.4 and 7.0 time: &lt;28 h (Gao and others 2006)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4-Hydroxybenzoic acid, 3,4-Dihydroxybenzoic acid, 3′-[3′,4′-Dihydroxyphenyl]propionic acid, 3′-[4′-Hydroxyphenyl]lactic acid, Urolithins</td>
<td>Batch Time: &lt;72 h pH: 7.2 (start), 6.2 (end) (González-Barrio and others 2011)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citrus fruits</td>
<td>4-Hydroxy-phenylpropionic glucuronide, 4-Hydroxy-benzoic acid glucuronide, 3-Methoxy-4-hydroxy-phenylacetic glucuronide, 3- and 4-Hydroxyphenylacetic glucuronide, Hippuric acid glucuronide</td>
<td>TIM-2 colonic dynamic model pH 5.8, 6.4 and 7.0 Time: &lt;28 h (Gao and others 2006)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1SHIME, Simulator of the Human Intestinal Microbial Ecosystem (Molly and others 1993).
2TIM, TNO Intestinal Model (Minekus 1995).
Table 5: Summarized conditions for simulated digestion under static conditions, based on common *in vitro* conditions applied, feasibility, and their similarity to *in vivo* conditions

<table>
<thead>
<tr>
<th>Phase of digestion</th>
<th>Common <em>in vitro</em> values</th>
<th>Common <em>in vivo</em> values</th>
<th>Tentatively suggested**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral phase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-α-amylase (U/mL)*</td>
<td>110</td>
<td>26</td>
<td>25-200</td>
</tr>
<tr>
<td>-time (min)</td>
<td>10</td>
<td>0.5-5</td>
<td>1-5</td>
</tr>
<tr>
<td>-pH</td>
<td>6.9</td>
<td>7.1±0.1</td>
<td>7.0±0.2</td>
</tr>
<tr>
<td>Gastric phase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-pepsin (U/mL) b,d</td>
<td>1,400-4,300</td>
<td>170-1200; 0.1-0.2 g/L</td>
<td>5,000-10,000</td>
</tr>
<tr>
<td>-time (min)</td>
<td>60</td>
<td>60-72; 140-210°</td>
<td>60; 120</td>
</tr>
<tr>
<td>-pH</td>
<td>2.0</td>
<td>2 (fasted); 3.5 (120 min (fed))</td>
<td>3.5±0.5</td>
</tr>
<tr>
<td>Small intestine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-lipase*</td>
<td>4.0</td>
<td>70-1000 (fed);</td>
<td>20-200</td>
</tr>
<tr>
<td></td>
<td>(0.5g/L)</td>
<td>10 (fasted)</td>
<td></td>
</tr>
<tr>
<td>-bile salts (mmol/L)</td>
<td>7.5</td>
<td>5 (fasted)</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>(3.8 g/L)</td>
<td>10 (fed)</td>
<td></td>
</tr>
<tr>
<td>-time (min)</td>
<td>120</td>
<td>200</td>
<td>120-200</td>
</tr>
<tr>
<td>-pH</td>
<td>7-7.5</td>
<td>6.8±0.4</td>
<td>7±0.2</td>
</tr>
<tr>
<td>Large intestine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-time (min)</td>
<td>42 (24-72)</td>
<td>35±2.1</td>
<td>35-45</td>
</tr>
<tr>
<td>-pH</td>
<td>6.6 (5.5-7.2, start)</td>
<td>6.2 (5.7-6.7)</td>
<td>6.2-6.6</td>
</tr>
<tr>
<td></td>
<td>6.6 (end)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* median value taken from Tables 1, 2, 3, and 4
** taking into account human trials (Tables 1, 2, 3 and 4) and herein reported physiological values

* Sigma units* (see Table 2). For conversion into IFCC units x 0.0073 (i.e. 140 units = 1.02 IFCC units).

*Sigma units*: Pepsin typically used: 800 – 2500 units/mg such as by Sigma-Aldrich. 1 unit: One unit will produce a change in absorbance of 0.001 at 280 nm at 37 °C, in 1 min, at pH 2.0, with hemoglobin as substrate. See footnotes table 2 for conversion factors.

Gastric lipase not required for water soluble compounds, however for lipophilic compounds such as carotenoids a concentration of 40-80 U/mL is recommended (Armand 1999, 2007). Gastric lipase (tributyrin units): 10-65 (mean 40, Armand 1999); 60-80 (Armand 2007). 1 TBU (lipase unit) is the amount of enzyme (g) which releases 1 μmol titratable butyric acid per minute under the given standard conditions.

† post-prandial

§ 1 unit liberates at least 1 μmole of acid from olive oil/triolein per minute at 37 °C and pH 9. Comparable to tributyrin units when expressed at same molarity.
Figure 1: Decision tree for choosing an in vitro digestion models: Some a-priori considerations. Major aspects concern lipophilicity of the phytochemical, as well as amount sample material and number of samples.

What type of phytochemicals?
- Hydrophilic (e.g. polyphenols)
- Lipophilic or both hydro+lipophilic (e.g. carotenoid or carotenoid+polyphenols)

What is the research?
- Screening/comparing, hypothesis building
- Confirmatory and sophisticated/physiological testing

Number of samples?
- Considerable number
- Few

Static
- +Lipid to test meal;
- Ultracentrifugation/filtration

Dynamic
- +Lipid to test meal;
- Membrane (e.g. TIM LIPID) for studying micellar fraction

Degradation products?
- No

Bioaccessibility?
- Consider dynamic model or additional modifications

Number of samples?
- Considerable number
- Few

Outputs adequate to the research question?
- No

Figure 1: Decision tree for choosing an in vitro digestion models: Some a-priori considerations. Major aspects concern lipophilicity of the phytochemical, as well as amount sample material and number of samples.