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

3

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27
28 Running Title: *In vitro* models for bioaccessibility and digestion studies
29
30 **Abstract**
31 There is an increased interest on secondary plant metabolites, such as polyphenols and
32 carotenoids, due to their proposed health benefits. This attention includes their
33 bioavailability, a prerequisite for assigning further physiological functions. As human
34 studies are time-consuming, costly, and restricted by ethical concerns, *in vitro* models
35 for investigating changes of these compounds during digestion have been developed and
36 employed for predicting their release from the food matrix (bioaccessibility) and
37 changes in their profiles prior to absorption.
38 Most typically, models simulate digestion in the oral cavity, the stomach, the small
39 intestine, and, occasionally, the large intestine. A plethora of models have been
40 reported, the choice mostly driven by the type of phytochemical studied, whether the
41 purpose is screening or studying under close physiological conditions, and the
42 availability of the model systems. Unfortunately, the diversity of model conditions has
43 hampered the possibility to compare results across different studies. For example, there
44 is substantial variability in the time of digestion, concentrations of salts, enzymes, and
45 bile acids used, pH, the inclusion of various digestion stages; and whether chosen
46 conditions are static; (with fixed concentrations of enzymes, bile salts, digesta, and so
47 on) or dynamic (varying concentrations of these constituents). This review presents an
48 overview of models that have been employed to study the digestion of both lipophilic

49 and hydrophilic phytochemicals (to compare digestive conditions *in vitro* and *in vivo*)
50 and, finally, recommends a set of parameters for both static and dynamic models that
51 resemble physiological conditions.
52
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57
58 **Abbreviations**
59 AM2 – Artificial masticatory advanced machine
60 CVD – Cardiovascular diseases
61 DGM – Dynamic gastric model
62 DNS – Dinitrosalicylic acid color assay
63 EPI – Echo-Planar magnetic resonance Imaging
64 FDA – Food and Drug Administration
65 GI – Gastrointestinal
66 GIT – Gastrointestinal tract
67 HGS – Human gastric simulator
68 HPH – High-pressure homogenization
69 HPLC – High performance liquid chromatography
70 HPP – High-pressure processing
71 IFCC – International Federation of Clinical Chemistry
72 LPH – Lactase phlorizin hydrolase

73 SGLT1 – Sodium-glucose linked transporter

74 SHIME - Simulator of Human Intestinal Microbial Ecosystem

75 T2D – Type 2 diabetes

76 TIM – TNO Gastro-Intestinal Model

77

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130 **Introduction**
131
132 Phytochemicals are a large and structurally diverse group of secondary plant
133 metabolites that are non essential for humans, that is, their non consumption does not
134 cause any specific deficiency symptoms. For the plant, these are also non essential

135 compounds, but they aid, among others, in fending off herbivores (polyphenols), or
136 stabilizing photosynthetic pigments (carotenoids). From a chemical point of view,
137 phytochemicals include very diverse compounds, from the rather polar polyphenols, to
138 the rather non polar carotenoids, phytosterols, and terpenes.

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

151 The biological response of the human body to phytochemicals is greatly determined
152 by the bioavailability of these bioactive molecules. The most abundant phytochemicals
153 in our diet are not necessarily those able to result in the highest tissue concentrations or
154 those revealing biological effects, owing to considerable differences in bioavailability
155 (Manach and others 2005). Phytochemical bioavailability depends on a large number of
156 factors and may differ according to the types of compounds studied, their differing
157 associations with the plant matrix, variation in polarity, molecular mass, presence in
158 crystalline or amorphous state, digestion by gastrointestinal enzymes, active vs. passive
159 absorption into the enterocytes, and many more. Among the most important factors

160 determining bioavailability, and a prerequisite for intestinal absorption, is release from
161 the food matrix and solubilization during digestion, also termed bioaccessibility (Parada
162 and Aguilera 2007), which is therefore describing the fraction of a compound
163 potentially available for further uptake and absorption. The amount of any
164 phytochemical released and therefore potentially available for further absorption may
165 differ greatly from its total concentration in the native food matrix. For some
166 compounds that are poorly released and solubilized, such as carotenoids (Bohn 2008),
167 or that are degraded prior to reaching their site of absorption, such as anthocyanins, the
168 portion that is bioaccessible may be below 10% (Minekus 1995; Bouayed and others
169 2011). Thus, a thorough understanding of changes occurring during digestion (such as
170 mechanical action, enzymatic activities, and altered pH) is crucial for the understanding
171 of bioaccessibility and estimating bioavailability and bioactivity, as only bioavailable
172 phytochemicals will exert fully their potential beneficial effects. Because animal and
173 human studies are very lenhtly and costly to conduct, and also have limitations due to
174 ethical considerations, *in vitro* systems have been developed that enable the prediction
175 of phytochemical changes during oral and gastro-intestinal digestion. This has allowed
176 the screening of comparatively large numbers of samples and/or conditions, studying
177 the separate and combined impacts of each stage of digestion on the release and
178 availability of phytochemicals, which would hardly be possible *in vivo*.

179 A major obstacle for the interpretation of phytochemical bioaccessibility based on *in*
180 *vitro* studies is the large number of models published and presented in the scientific
181 literature since the description of the first model developed for studying iron
182 bioaccessibility (Miller and others 1981). The diversity of models has hampered the
183 comparison of results across studies, and increased the chances of finding contradictory
184 results. The employed models mainly differ in the inclusion of various stages of

185 digestion (oral, gastric, small intestinal, large intestinal); digestion times (typically
186 ranging from a few minutes per stage to up to 3 h); pH; the nature of digestive enzymes
187 involved and concentrations of salts and bile acids. Finally, while most of the models
188 are operated in static conditions, that is with pre-fixed concentrations and volumes of
189 digested materials, enzymes, salts etc. (though during digestion phases are mixed and
190 concentrations may change), there are also a limited number of continuous models that
191 mimic the dynamic changes of the physicochemical conditions (and go along with a
192 more constant change of digested material enzymes, salts etc. during various phases of
193 digestion), and which aim to better simulate the passage of the bolus/digesta through the
194 human digestive tract. However, these models are much more labor- and cost-intensive
195 than the batch models.

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

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

209

210 **Parameters that drive the choice of model**

211

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

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

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

230

231 **Overview on parameters affecting the release and chemical changes of lipophilic**
232 **and hydrophilic phytochemicals during digestion**

233

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

242 Since plant foods are often divers in composition or eaten in conjugation with other
243 foods, food bolus constituents are likely to modulate the bioaccessibility and stability of
244 phytochemicals. This may contribute to the rather small fraction of dietary
245 phytochemicals that is typically absorbed and utilized by humans (Schramm and others
246 2003). Therefore, defining the conditions that influence their absorption can provide
247 significant insights into methods for maximizing the utilization of these sometimes
248 health-promoting constituents. The main food components are proteins, carbohydrates,
249 fiber, and fat, and their interactions with phytochemicals are often not considered. When
250 considering *in vitro* bioaccessibility studies, chemical reactions (such as
251 oxidation/reduction, complexation), biochemical reactions (enzyme/substrate
252 interaction), or physical constraints (diffusion) occuring within food must be taken into
253 account. For polyphenols, in particular, these types of interactions have rarely been
254 taken into account when determining polyphenol digestion (Ortega and others 2009).

255

256 Lipophilic phytochemicals

257 Although carotenoids are lipophilic compounds and considered as relatively labile
258 under acidic conditions, no significant chemical modification in the human stomach has

259 been described (Tyssandier and others 2003). Some isomerization was observed in the
260 stomach of ferrets (Boileau and others 1999) and relatively high recoveries of dietary
261 carotenoids (65-91%) have been observed after gastrointestinal *in vitro* digestion
262 (Granado-Lorencio and others 2007; Failla and others 2009). The digestive stability of
263 carotenoids in different food matrices has been investigated in a dynamic *in vitro* model
264 simulating the stomach and small intestine (TIM 1) (Blanquet-Diot and others 2009;
265 Déat and others 2009). Zeaxanthin and lutein (xanthophylls) were found to be stable
266 during the whole digestion, whereas lycopene and β-carotene (carotenes) were stable in
267 the gastric and duodenal compartments but partly degraded in the jejunal and ileal
268 compartments of the small intestine, perhaps due to delayed release from the matrix and
269 later micellarization at this stage of these carotenes (Blanquet-Diot and others 2009).
270 Although an enhanced release from the matrix can contribute to higher bioaccessibility,
271 the released carotenoids may be more susceptible to degradation and isomerization
272 (Failla and others 2008a). In the study by Blanquet-Diot and others (2009), a
273 degradation of β-carotene and all-*trans* lycopene, which could not be directly linked
274 with the formation of *cis* isomers, was observed in the lowest part of the small intestine.
275 As suggested by the authors, the results might to be due to breakdown to non-detected
276 metabolites (such as oxidation products) or enzyme–catalyzed cleavage products during
277 small intestinal digestion, but no precise data could support this hypothesis. The
278 absorption of lipophilic phytochemicals mainly occurs after the disruption of the food
279 matrix, enabling the release and emulsification into lipid droplets in the stomach,
280 followed by incorporation into mixed micelles. Apart from the food matrix, carotenoid
281 bioavailability may be influenced by the presence of other nutrients and non nutrients
282 within the food. For example, a competition between carotenoids and other fat-soluble
283 nutrients such as vitamin E at the absorption stage has been reported (Faulks and others

1998). 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

309 interactions between polyphenols and food components (Stahl and others 2002;
310 Karakaya 2004). Phenolic compounds can have strong affinities with human salivary
311 proline- and histidine-rich proteins and form both non covalent and covalent
312 associations depending on the size of the phenolic compound (de Freitas and Mateus
313 2001; Wroblewski and others 2001). High-molecular-weight polyphenols (such as
314 tannins) interact strongly with fibers and proteins, but their affinity is related to their
315 size and their solubility in water.

316 More hydrophobic compounds have stronger binding to proteins (Le Bourvellec and
317 Renard 2011). Laurent and others (2007) investigated the behavior of low molecular
318 weight flavonoids from grape seed extract during *in vitro* digestion (with α-amylase
319 from human saliva, porcine pepsin, pancreatin and bile extract), combined with a Caco-
320 2 cell model to evaluate the impact of brush border proteins. Their results showed that
321 flavan-3-ol monomers ((+)-catechin and (−)-epicatechin) and procyanidin dimers (B2
322 and B3) were stable during oral and gastric digestion but those interactions with
323 proteins occurred during the intestinal step with pancreatic digestion, and in the
324 presence of brush border cell proteins. Simulated digestion of anthocyanins from, for
325 example, red berries, red wine, and red cabbage have shown that these compounds
326 appear to be stable at the acidic conditions of the stomach but less stable at the small
327 intestinal pH (Gil-Izquierdo and others 2002; McDougall and others 2005a, 2007). The
328 total recovery of anthocyanins from red cabbage was low (around 25%), possibly due to
329 degradation into new phenolic components by the combination of the elevated pH and
330 the presence of oxygen during pancreatic digestion (McDougall and others 2007). As
331 recently shown in the investigation by Oidtmann and others (2012), a possible mean to
332 enhance the stability and protect anthocyanins from degradation in the small intestine

333 might be to use encapsulation techniques, such as microcapsule systems composed of
334 polysaccharide pectin amide with or without shellac coating or whey proteins.
335 In summary, the digestive stability of carotenoids depends on the molecular nature
336 and the food matrix in which they are included, with xanthophylls being more stable
337 than carotenes. The absorption of carotenoids depends on an efficient release from the
338 food matrix and subsequent solubilization in mixed micelles. By contrast, no
339 micellarization is required prior to cellular uptake for phenolic compounds, and, thus,
340 there are possibly fewer possibilities for impacting bioaccessibility such as by varying
341 enzyme concentrations; however, some constituents such as anthocyanins may be
342 rapidly degraded due to increasing pH (McDougall and others 2007). The affinity of
343 polyphenols for proteins (Dangles and Dufour 2005, 2008) may lead to a major
344 modulation of both polyphenol absorption and reactivity in the stomach and in the upper
345 intestine.
346
347 **Before modeling: Considerations with respect to pre treatments, meal size, and**
348 **choice of test meals**
349
350 Food composition, how it is processed and the interaction of phytochemicals with
351 other food components (be they lipophilic or hydrophilic), may modify the amount of
352 phytochemicals released from the food matrix and, therefore, potentially increase or
353 decrease their bioaccessibility.
354 Influence of the plant matrix and food bolus
355 Plant cell walls acts as a barrier to digestion (Ellis and others 2004; Mandalari and
356 others 2010). When a plant cell is broken through mastication or crushing in industrial
357 or domestic processing, phytochemicals may associate with dietary fibers leading to a

358 modulation of their relative bioaccessibilities. In a recent study, comparing the stability
359 and bioaccessibility of carotenoids in pure forms (synthetic β-carotene or retinyl
360 palmitate solution) or from food (carrot juice and raw or cooked spinach), Courraud and
361 others (2013) demonstrated the protective effect of the food matrix on dietary
362 carotenoids. Their results showed that vitamin A and carotenoid standards (synthetic β-
363 carotene or retinyl palmitate solution) were unstable, whereas food carotenoids were
364 generally better protected by the food matrix (30-100% recovery versus 7-30% for
365 standards). Although the susceptibility of carotenoids to degradation and isomerization
366 has been found to increase after their release from the food matrix (Failla and others
367 2008b), interactions with other compounds released from the food matrix (including
368 soluble fibers) and viscosity may affect their bioaccessibility (McClements and others
369 2008; Schweiggert and others 2012). For example, the bioaccessibility of β-carotene is
370 known to be influenced by strong binding to pectins (Ornelas-Paz and others 2008).

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

383 factor for cocoa liquor (50% fat content) compared to cocoa powder (15% fat content)
384 (Ortega and others 2009).

385 The affinity of milk and egg proteins as well as gelatins for polyphenols depends on
386 both the protein and phenolic structures (Bohin and others 2012). For example,
387 chlorogenic acid associates with milk caseins rather than with β-lactoglobulin and this
388 complexation was relatively stable in simulated gastric and intestinal steps (Dupas and
389 others 2006). Despite these interactions, chlorogenic acid absorption by Caco-2 cells
390 and rats was not reduced by milk addition to coffee. In tea, more than 60% of green tea
391 flavanols (such as ECG, EGC, and EGCG), which are very prone to oxidation,
392 disappeared in the intestinal phase during *in vitro* digestion (Haratifar and Corredig
393 2014). A protective effect was caused by the addition of pure ascorbic acid, by citrus
394 juices as well as by bovine, rice, and soy milks. While ascorbic acid contribution
395 reflects its superior antioxidant capacity compared to tea flavanols, the protection by
396 proteins was partially reversed by increasing the content of digestive enzymes,
397 suggesting non covalent interactions between bovine milk proteins and galloylated tea
398 flavanols (Green and others 2007).

399 Soy isoflavones appear to be more bioaccessible from fruit juices and chocolate bars
400 compared to cookies in *in vitro* conditions, perhaps due to their lower diffusion rate
401 from the carbohydrate/protein matrix of the cookies (de Pascual-Teresa and others
402 2006). However, a complementary human intervention study did not point out any
403 significant difference in the bioavailability parameters (AUC, t_{max} or c_{max}) of these
404 isoflavones. Similarly, the *in vitro* biacessibility of catechin recoveries was significantly
405 higher in beverages than in confections (Neilson and others 2009). Higher amounts of
406 isoflavones were also released *in vitro* from custards thickened with starch rather than
407 with carboxymethylcellulose (Sanz and Luyten 2006). This effect was attributed to the

408 hydrolysis of starch by α-amylase which occurs from the mouth to the intestine. Finally,
409 bile salts improved the *in vitro* bioaccessibility of isoflavone aglycones from soy bread
410 through micellarization of these poorly-soluble molecules concentrations appeared to be
411 a critical factor in the bioaccessibility of isoflavones from soy bread (Walsh and others
412 2003).

413

414 Impact of processing

415 Previous studies (Garrett and others 1999) have indicated that food processing and
416 dietary fat can enhance carotenoid bioaccessibility. However, it is notablethat only a
417 little proportion of carotenoids (5-25%) is efficiently liberated from the food matrix.
418 Cooking and heat treatment may enhance carotenoid bioaccessibility due to disruption
419 of plant tissue and denaturation of carotenoid-protein complexes which enhance release
420 from the food matrix (Veda and others 2006; Failla and others 2009; Aherne and others
421 2010). However, cooking enhanced the bioaccessibility and bioavailability of all-trans
422 β-carotenes but also caused carotenoid isomerization (Aherne and others 2010).

423 There are many reports describing that thermal processing improves lycopene
424 bioaccessibility due to the breakdown of the tomato matrix (Gartner and others 1997;
425 Porrini and others 1998; Van Het Hof and others 2000). However, depending on the
426 processing methods, differences in lycopene bioaccessibility have been reported.
427 Yilmaz and Karakaya (2007) reported that lycopene bioaccessibility in raw tomato
428 (29%) and canned tomato were similar (22%). On the other hand, bioaccessibility of
429 lycopene from sun-dried tomatoes reached 58% (Yilmaz and Karakaya (2007). High-
430 pressure homogenization (HPH) and HPH combined with heat processing (90 °C for 30
431 min) caused a decrease in the *in vitro* bioaccessibility of lycopene. In addition, an
432 inverse relationship between the homogenization pressure and lycopene *in vitro*

433 bioaccessibility was reported (Colle and others 2010). It was suggested that the fiber
434 network formed by HPH entrapped lycopene, making it less accessible for digestive
435 enzymes and bile salts. High-pressure processing (HPP), however, had no effect on α-
436 carotene and β-carotene bioaccessibility in carrots. Lutein bioaccessibility in green
437 beans was increased by pressure treatment at 600 MPa ($p<0.05$), whereas β-carotene
438 bioaccessibility was reduced by HPP at both 400 or 600 MPa (McInerney and others
439 2007), which suggests effects due to the matrix and compound structure.

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

453

454 Impact of starting meal size

455 Adjustment of the ratio of the amount of the test meal to water present to mimic
456 dietary bolus during digestion phases has an impact on viscosity. Both this ratio and

457 meal particle size are important factors influencing phytochemical release during
458 digestion.
459 During transit in the oral cavity, the stomach, and the small intestinal compartments, the
460 dietary bolus will be diluted as a consequence of addition of saliva and other secretions.
461 The amount and type of food influence the composition and secretion rates. Apart from
462 the volume and composition of the secretions, mechanical forces will also have an
463 impact on the disintegration and dissolution of a meal and on the rate of transfer through
464 the GI tract. In general, dynamic models are able to process complex foods through
465 mechanical and enzymatic digestions at volumes equivalent to “standard” meals.
466

467 **Digestion models for studying phytochemical bioaccessibility - static vs. dynamic**
468 **models**
469

470 Depending on the type of research question, for example, if constituting a screening
471 application or a confirmative study, the type and amount of sample present, static or
472 dynamic *in vitro* models can be used to simulate different phases of digestion (Figure
473 1). Practically, static models provide a feasible and inexpensive means to assess
474 multiple experimental conditions, allowing large numbers of substrates to be tested.
475 Dynamic multistage continuous models facilitate long-term studies and probably come
476 closest to *in vivo* conditions. These complex computer controlled systems, however, are
477 expensive to set up, more labor-intense and time-consuming (maximum one experiment
478 /day) and require higher operating costs in terms of working volumes and continuous
479 addition of substances mimicking the gastrointestinal fluids.
480

481 Static models

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

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

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

502

503 Dynamic models

504 Compared to static models, dynamic models have the advantage that they can
505 simulate the continuous changes of the physicochemical conditions including variation

506 of pH from the mouth to the stomach and the intestine, altering enzyme secretion
507 concentrations, and peristaltic forces in the gastrointestinal tract.

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

521 The human gastric simulator (HGS), a model developed at the University of
522 California-Davis is composed of a latex chamber surrounded by a mechanical driving
523 system to effectively simulate the frequency and intensity of the peristaltic movements
524 in the stomach (Kong and Singh 2010). HGS is designed to mimic the gastric shear
525 forces and stomach grinding. This appears to be important for bioaccessibility studies as
526 the rate of release of phytochemicals, from fibrous particles, into the surrounding
527 intestinal fluid is inversely proportional to particle size, and is directly proportional to
528 phytochemical gradient. It is furthermore affected by the physical state of the
529 phytochemical, the physical structure, and the surface properties of the particle
530 (Palafox-Carlos and others 2011). To allow a closer simulation of *in vivo* physiological

531 processes occurring within the lumen of the stomach and small intestine, some of the
532 main parameters of digestion such as peristaltic mixing and transit, secretions, and pH
533 changes, have been applied in some models. The TNO gastrointestinal model (TIM-1)
534 developed by TNO in Zeist (The Netherlands), has been used for a broad range of
535 studies (Minekus 1995). The system consists of 4 different compartments, representing
536 the stomach, duodenal- jejunal and ileal parts of the gastrointestinal tract. Each
537 compartment is composed of 2 glass jackets lined with flexible walls. The TIM-1
538 system enables simulation of gastric emptying rate, peristaltic movements, and transit
539 time through the small intestine and gradual pH changes in the different compartments
540 (Minekus 1995), and has given useful information on the parameters affecting the
541 release and digestive stability of carotenoids from different food matrices through the
542 gastrointestinal tract (Minekus 1995; Blanquet-Diot and others 2009). This model has
543 also been extensively used to assess both folate and folic acid bioaccessibility from
544 foods (Öhrvik 2008; Öhrvik and others 2010).

545 For polyphenols, there is not enough evidence as to which method is the most
546 appropriate for measuring bioaccessibility, especially as it has become clear that the
547 colon is greatly involved in the metabolism and absorption of these compounds (Bolca
548 and others 2012; Czank C and others 2013; Ludwig IA and others 2013). Thus both
549 static and dynamic models, those that do not take into account the simulation of the
550 colon, will have limitations in predicting the bioavailability of polyphenols. However,
551 with the development of additional models aiming to simulate colonic fermentation,
552 such as the TIM-2 model, the non bioaccessible fraction following gastric and small
553 intestinal digestion may be studied, such as was done for phenolic compounds in wheat
554 bread (Mateo Anson and others 2009).

555 An adapted model of TIM-1, a computer-controlled gastrointestinal model called
556 Tiny-TIM, has more recently been used to assess the bioaccessibility of phenolic acids
557 in breads (Hemery and others 2010). The model is a simplified and downscaled TIM-1
558 for rapid screening. The main characteristics of the system are the same as for TIM-1,
559 but instead of four compartments, the Tiny-TIM model consists of 2 compartments that
560 represent the stomach and the small intestine. The results were found to be consistent
561 both with the data from a previous study evaluating the bioaccessibility of phenolic
562 acids in TIM-1 (Kern and others 2003) and a human study (Mateo Anson and others
563 2009). To our knowledge, except for the comparison between the results obtained in the
564 TIM-1 and Tiny-Tim model, so far no comparisons between the different dynamic
565 models have been made.
566
567 **Setting up the model**
568
569 Digestion simulation of the oral cavity
570 The oral cavity is the portal of entry of nutrients. Due to its unique constituents it
571 may also be considered a "bioreactor" (Gorelik and others 2008; Mathes and others
572 2010; Ginsburg and others 2012). Whole saliva is a very dilute fluid composed of more
573 than 99% water. It contains a variety of minerals, various proteins (the major being the
574 mucin glycoproteins, albumin, and digestive enzymes), and nitrogenous compounds as
575 urea and ammonia (Ginsburg and others2012). An intensive mixing of simulated saliva
576 and the introduced food bolus is usually desired, usually in a ratio of 1:1, keeping in
577 mind practicality and the basal flow of saliva during ingestion estimated at 1-3 mL/min
578 (Engelen and others 2003). An ingested food or beverage undergoes a number of
579 chemical, biochemical, and mechanical processes in the mouth, although not so

580 significant for liquids due to short residence time. There may occur changes in pH, ionic
581 strength, and temperature, action of various digestive enzymes (notably lingual lipase,
582 amylase, protease); interactions with biopolymers in the saliva (mucin); interactions
583 with sensory receptors of the tongue and mouth; and particle size reduction of bolus by
584 chewing (mastication). These are all major factors to take into consideration when
585 designing an *in vitro* digestion step that simulates the human mouth (McClements and
586 Li 2010).

587

588 *Particle size reduction*

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

605 others 2010). The average particle size distribution after human chewing was
606 investigated and this information was used to simulate average mastication *in vitro* by a
607 blending technique.

608 To produce food boluses with properties similar to those resulting after natural
609 chewing, the Artificial Masticatory Advanced machine (AM2) has been developed and
610 validated against human subjects chewing raw carrots (cylindrical samples heigh 1 cm,
611 diameter 2 cm, 4 g) and peanuts (3.5 g) (Mishellany-Dutour and others 2011). It was
612 concluded that AM2 produces a food bolus with similar granulometric characteristics to
613 human chewing, although no bioaccessibility parameters for phytochemicals were
614 evaluated.
615

616 *Chemical and biochemical processes*

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

623 Ginsburg and others (2012) suggested that saliva has an important role in the
624 solubilization of polyphenols present in fruits and plant beverages and thus substantially
625 increases their availability. Moreover, saliva can increase the stickiness to oral surfaces
626 of polyphenols and their prolonged retention in the oral cavity and thus it contributes to
627 the enhancement of the redox status of the oral cavity. Salivary albumin, mucins, and
628 proline-rich proteins may be of particular importance affecting the digestibility and
629 absorption of specific polyphenols, for example, tannins may be precipitated and

630 retained by such proteins (Bennick 2002) through hydrogen bonding and hydrophobic
631 interactions.

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

640

641 The gastric phase of digestion

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

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

651 Peristaltic waves originating from the stomach participate to the size reduction of
652 solid foods down to a diameter of 1 to 2 mm (Kong and Singh 2010). Stomach
653 emptying is a critical step in the digestion process. Several factors may influence the
654 gastric emptying of food and fluids including volume, viscosity, and pH. The speed of

655 the emptying of liquid meals is directly proportional to the volume present in the
656 stomach. Solid foods are emptied more slowly, in a biphasic pattern with a lag phase
657 during which little emptying occurs, followed by a linear emptying. The duration
658 depends on the physical properties and approximately 3 to 4 h are needed for a complete
659 emptying of the stomach (Schulze 2006).

660 A nutrient-driven feedback regulation from the small intestine, limiting the gastric
661 emptying to a maximum of about 3 kcal/min has been suggested (Lin and others 2005,
662 Kwiatek and others 2009) when other data point to a nutrient-dependent emptying
663 pattern with emulsion fat emptying faster than glucose and protein (Goetze and others
664 2007). Furthermore, the presence of dietary fibers is known to slow down gastric
665 emptying of complex meals (Marciani and others 2001).

666

667 *pH*

668 The gastric pH in the fasted state in healthy human subjects is in the range of 1.3 to
669 2.5. The intake of a meal generally increases the pH to above 4.5 depending on the
670 buffering capacity of the food. For example, in nasogastrically intubated humans fed a
671 western-type diet enriched in either tomato, or spinach or carrot purees, the stomach pH
672 sharply increased to 5.4–6.2 after meal intake, then continuously decreased to reach
673 1.8–2.9 after 3 h of digestion (Tyssandier and others 2003). Similarly, after ingestion of
674 a cocoa beverage, the gastric pH reached 5.4 within 3 min before returning to the
675 baseline pH of 1.9 (Rios and others 2002). Most static *in vitro* studies have been
676 conducted at a pH below 2.5, which are conditions related to the human fasting state
677 rather than to real food digestion. Only a few authors have considered as relevant a pH
678 of 4 associated with the mid-step of digestion (Reboul and others 2006; Dhuique-Mayer
679 and others 2007). The decay of gastric pH is however taken into consideration in

680 dynamic models as shown for the digestion of tomato carotenoids in the TIM system
681 (pH 6 to 1.6) (Blanquet-Diot and others 2009).
682
683 *Enzymes*
684 Pepsin, which is readily available as porcine pepsin, has been integrated in most *in*
685 *vitro* models of gastric digestion, although in varying amounts (Table 2). Pepsin content
686 should be assessed as enzymatic activity per weight of protein for the sake of
687 comparison. Gastric lipase is usually omitted. However, existence of lipolysis in the
688 human stomach by gastric lipase is known (Carriere and others 1993; Armand and
689 others 1994). Most of the dietary lipids are present in the form of emulsified droplets, in
690 the range of 20-40 µm, and it was suggested that gastric lipolysis can help to increase
691 emulsification in the stomach (Armand and others 1994), which would thus enhance
692 lipophilic phytochemical bioaccessibility. It was reported that human gastric lipase
693 secretion ranged from 10 to 25 mg/3 h and that the percentage of intra-gastric lipolysis
694 during gastric digestion was 5-40% (Carriere and others 1993: Armand 2007). Lipolysis
695 catalyzed by gastric lipase has been found to primarily occur within the first hour of
696 digestion (Armand and others 1994).
697 Because human gastric lipase is unavailable, fungal lipases from *Aspergillus niger* or
698 *Aspergillus oryzae* have been used, as in the TIM model. However, *A. niger* lipase has a
699 wide pH optimum of 2.5-5.5 compared to 4.5 to 6 for human gastric lipase (Carriere et
700 al., 1991). The fungal lipase can hydrolyze both the sn-1 and sn-3 positions of the
701 triacylglycerol molecule, with a slight preference for the sn-1 position, whereas gastric
702 lipase is most active at the sn-3 position (Van Aken and others 2011). Alternatively, a
703 mammalian lipase such as rabbit gastric lipase could be used as Capolino and others
704 (2011) demonstrated that its specificity is close to that of human lipase. At the present

705 time, a combination of rabbit gastric lipase and porcine pancreatic extract is favored to

706 simulate *in vitro* gastrointestinal lipolysis.

707

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

709 *micronutrients*

710 The presence of other food components may alter polyphenol and carotenoid stability

711 in the gastric tract. After food intake, dietary iron, dioxygen, and emulsified lipids come

712 into close contact and lipid oxidation may take place. This was demonstrated for heme

713 (metmyoglobin) and nonheme iron (Fe^{II}/Fe^{III}) forms in emulsion systems modeling the

714 physical state of triacylglycerols (Lorrain and others 2012). Dietary polyphenols such as

715 rutin, (+)-catechin, and chlorogenic acid proved to be better inhibitors of the

716 metmyoglobin-initiated lipid oxidation than α-tocopherol and vitamin C (Lorrain and

717 others 2010). The antioxidant activity of polyphenols depended on an emulsifying

718 agent (proteins, phospholipids) and pH. In this process, polyphenols were however

719 consumed, giving rise to oxidation products which themselves retain antioxidant

720 properties (Lorrain and others 2010). In this *in vitro* model of gastric digestion,

721 lycopene and ß-carotene proved to be less efficient inhibitors of lipid oxidation

722 compared to bacterial carotenoids (mainly glycosylated apolycopenoids) (Sy and others

723 2012b). Phenolic compounds and carotenoids had complementary mechanisms of

724 action: the former inhibited the initiation step of lipid peroxidation by reducing the

725 prooxidative Fe^{III} species of myoglobin when the latter inhibited the propagation phase

726 by direct scavenging of the lipid peroxyl radicals. Oxygen may thus impact

727 phytochemical and micronutrient stability in the gastric tract. The level of dissolved O_2

728 increases during mastication of food (Gorelik and others 2005), whereas the presence of

729 a marked oxygen partial pressure gradient from the proximal to the distal GI tract was

730 evidenced in living mice from 58 torr in the mid-stomach, 32 torr in the mid-duodenum,
731 11 torr in the mid-small intestine and mid-colon to 3 torr in the distal sigmoid colon-
732 rectal junction (compared to 160 torr for O_2 in air) (He and others 1999). For this
733 reason, some authors suggested flushing with nitrogen or argon for a few minutes to
734 reduce the levels of dissolved O_2 (Bermudez-Soto and others 2007).

735

736 *Static models*

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

747 For highly processed plant matrices, it appears that the large majority of polyphenols
748 is already released in the gastric phase. Indeed, the polyphenol bioaccessibility from
749 fruit juices, wines, green tea, or phenolic extracts, in the presence of simulated gastric
750 juices (pH 1.7-2.5, pepsin, 1-4 h) is nearly 100% (Perez-Vicente and others 2002;
751 McDougall and others 2005a; McDougall and others 2005b; Bermudez-Soto and others
752 2007; Greenand others 2007; McDougall and others 2007; Gumienna and others 2011)
753 but can be only between 30-100% from solid matrices such as homogenized peaches,

754 apple, grape berries, cherries or carob flour (Fazzari and others 2008; Bouayed and
755 others 2011; Ortega and others 2011; Tagliazucchi and others 2012).
756 Among phenolic compounds, apple flavanols (epicatechin and procyanidin B2), as
757 well as chokeberry proanthocyanidin oligomers, were more degraded than
758 caffeoylquinic derivatives, flavonols, or anthocyanins. Cocoa proanthocyanidins
759 (trimers to hexamers) and apple procyanidin B2 were shown to undergo
760 depolymerization in a simulated gastric juice (37 °C, pH 1.8-2.0) (Spencer and others
761 2000, Kahle and others 2011), whereas *in vivo*, this degradation was not validated,
762 mainly because the stomach pH increased to 5.4 after the ingestion of the cocoa
763 beverage and progressively decreased to the basal value as the stomach emptied (Rios
764 and others 2002).
765 Certain epoxycarotenoids, such as violaxanthin and neoxanthin from spinach, were
766 shown to undergo epoxide-furanoid transitions at pH 2 (Biehler and others 2011a). This
767 transformation extent may clearly depend on the gastric acidity and time of exposure.
768
769 *Dynamic models*
770 Dynamic gastric models of digestion incorporate i) mixing of the non homogeneous
771 gastric digesta which is best modeled by peristaltic movements as in the HGS model
772 (Kong and Singh 2010), ii) acidification, iii) addition of gastric enzymes, and iv)
773 delivery to the duodenum (Chen and others 2011). Usually, computer-controlled
774 protocols are designed to deliver secretions and chime (digesta) in the normal
775 physiologic range. Dynamic models are described in more details in the previous
776 section "Digestion models for studying phytochemical bioaccessibillity". Up to now,
777 few applications have been reported for phytochemicals compared to the numerous data
778 in static models. For example, in the TIM-1 system, tomato (*E*)-beta-carotene and (*E*)-

779 lycopene proved to be stable, although the recovery yield was modulated by the tomato
780 matrix (Blanquet-Diot and others 2009). The Tiny TIM-1 system was used to evaluate
781 the bioaccessibility of phenolic acids in breads made from processed wheat bran
782 fractions (Hemery and others 2010). The amount of bioaccessible phenolic acids was
783 enhanced by using finer particles in bran-rich breads.

784

785 *General considerations*

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

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

796

797 Digestion in the small intestine

798 After food disintegration in the mouth and stomach, the main enzymatic digestion
799 and absorption of nutrients take place in the small intestine. After stomach digestion, the
800 acidic chyme is delivered to the small intestine and neutralized with sodium bicarbonate
801 to give an appropriate pH for enzyme activities.

802 The *in vitro* small intestinal digestion of phytochemicals is generally applied by
803 mimicking pH, temperature, time, and pancreatic juice including electrolytes, bile salts,
804 and enzymes.
805
806 *pH, enzymes and bile salts*
807 In the fed state, pH can vary from 5.4-7.5 in the duodenum (Tyssandier and others
808 2003; Kalantzi and others 2006; Clarysse and others 2009), to 5.3-8.1 in the jejunum
809 (Lindahl and others 1997; Perez de la Cruz Moreno and others 2006), and up to 7.0-7.5
810 in the ileum (Daugherty and Mrsny 1999) (Table 3).
811 Pancreatic enzymes including proteases, amylases, and lipases, as well as other
812 digestive enzymes (brush border enzymes, like maltase, lactase, α-dextrinase,
813 peptidases) produced by the brush border, a microvillus membrane at the luminal
814 surface of the small intestine (Holmes and Lobley, 1989), all act together on the
815 breakdown of food constituents.
816 *In vivo* bile salt concentrations were found to be higher in the fed state (3-12 mM
817 range) than in the fasted state and variable between duodenum and jejunum (Table 3).
818 The major differences among the methods are the forms of enzymes (pancreatin or
819 individual enzymes) and biliary acids used (bile salt mixtures, real fresh bile, or
820 individual bile salts) (Table 3). Very few models use individually prepared bile salts and
821 enzymes (including porcine pancreatic lipase, porcine colipase, porcine trypsin, bovine
822 chymotrypsin, and porcine amylase), although this may give better control over
823 enzymatic activity (Mandalari and others 2010). Several studies have reported that the
824 presence of bile salts and pancreatic enzymes is essential for the efficient
825 micellarization of lipophilic compounds (Garrett and others 1999; Hedrén and others,
826 2002, Wright and others 2008; Biehler and others, 2011a). In the study by Biehler and

827 others (2011a), carotenoid micellarization from spinach was strongly reduced in the
828 absence of pancreatin and bile salts, while it was not significantly impacted by the
829 omission of pepsin during gastric digestion (Biehler and others2011a). Minimal bile salt
830 concentration of 2.4 mg/mL (about 5 mM), within the *in vivo* concentration range, was
831 required for optimal transfer of lutein and beta-carotene from lipid droplets into mixed
832 micelles (Garrett and others 1999, Wang and others 2012). It was also shown that the
833 maximum beta-carotene transfer was obtained at pH 6, in relation to the activity of
834 pancreatic lipase, which is most efficient at this pH, and with a pancreatic lipase
835 concentration of 0.4 mg/mL (Wang and others 2012). At higher bile salt concentration,
836 beta-carotene micellarization could depend on the activity of pancreatic colipase-
837 dependent lipase (Wright and others 2008). As to polyphenols, the hydrophilic forms
838 such as glycosylated flavonols or quinic acid derivatives of hydroxycinnamic acids may
839 readily solubilize in the aqueous phase when less soluble flavonoid aglycones or
840 procyanidins will bind to dietary fibers and proteins for transport. A bile salt-dependent
841 micellarization has however been suggested for isoflavone aglycones (Walsh, Zhang,
842 Vodovotz, Schwartz and Failla 2003). In the intestinal conditions, the bioaccessibility
843 and stability of polyphenols depends mainly on pH. In near neutral conditions and in the
844 presence of oxygen as it occurs in most *in vitro* models, some phenolic compounds may
845 be degraded through non enzymatic oxidation (Bergmann and others 2009).
846 Examination of the recovery of specific classes revealed that flavan-3-ols were poorly
847 recovered following the digestion of a grape-orange-apricot juice (Cilla and others
848 2009) but not in chokeberry juice (Bermudez-Soto and others 2007). Pure (+)-catechin
849 was recovered at only 42% after incubation with pancreatin (Bermudez-Soto and others
850 2007), while (-)-epicatechin and procyanidin B2 from homogenized apple were not
851 recovered after the intestinal step (Bouayed and others 2012). The high affinity of

852 monomeric and oligomeric flavanols for proteins and dietary fibers may also lead to
853 their loss during the solid removal step by centrifugation (Le Bourvellec and Renard
854 2011). For green tea flavanols, the stability order was epicatechin > epicatechin gallate
855 > epigallocatechin = epigallocatechin gallate, in agreement with the higher oxidizability
856 of the 1,2,3-trihydroxyphenyl moiety compared to the 1,2-dihydroxyphenyl one (Green
857 and others 2007). The recovery of caffeoylquinic acids appears to be more affected by
858 the intestinal step than by the gastric step as observed for apple, a grape-orange-apricot
859 beverage, and red wine (Cilla and others 2009; Gumienna and others 2011; Bouayed
860 and others 2012,). Chlorogenic acid (5-caffeoylquinic acid) may autooxidize, although
861 regio-isomerization is a major pathway as described for *p*-coumaroyl- and
862 caffeoylquinic acids by Kahle and others 2011. Anthocyanins appear to be the most
863 sensitive class and may largely disappear in the intestinal step (McDougall and others
864 2005a, b, 2007; Bermudez-Soto and others 2007; Tagliazucchi and others 2010, 2012).
865 The quantification of anthocyanins is complicated by a pH-dependent equilibrium of the
866 red flavylium cation to several related structures at pH above 2. The hydration of the
867 flavylium cation produces a colorless hemiketal which is in equilibrium with colorless
868 (E)- and (Z)-chalcone forms. In the near-neutral conditions of intestinal digestion, a first
869 deprotonation of the flavylium cation provides neutral quinonoidal bases (pKa $\approx$ 4)
870 which can further be deprotonated to ionic quinonoidal bases (pKa $\approx$ 6), both bases
871 displaying blue and violet hues (Brouillard and others 1991; Clifford 2000). Thus, the
872 detection of anthocyanins in simulated gastrointestinal conditions can be challenging as
873 it is influenced by pH and copigment molecules. For example, Perez-Vicente and others
874 (2012) evaluated the recovery of pomegranate anthocyanins to be 18% when measured
875 at the pH of the intestinal digesta and 70% following acidification of the digesta at pH
876 2. Analysis of anthocyanins at pH lower than 2 should be favored as it is more

877 convenient to evaluate the flavylium cation form by high-performance liquid
878 chromatography (HPLC) or colorimetric tests.

879 When exposed to acids or bases, ester bonds in ellagitannins and caffeoylquinic acids
880 are hydrolyzed and the hexahydroxydiphenic acid is spontaneously rearranged into the
881 water-insoluble ellagic acid (Clifford and Scalbert 2000). Daniel and others (1991)
882 showed that ellagic acid could be released from raspberry ellagitannins at pH 7 and
883 optimally at pH 8. Furthermore, Gil-Izquierdo and others (2002) observed a 5-to 10-fold
884 increase in ellagic acid from strawberry ellagitannins during incubation with pancreatic
885 enzymes in mild alkaline conditions (Gil-Izquierdo and others). This may be the
886 mechanism behind the relative increases in smaller ellagitannin molecules noted during
887 *in vitro* digestion of raspberry and strawberry extacts (McDougall and others 2007;
888 Brown and others 2012,). In the mildly alkaline conditions of *in vitro* digestion, orange
889 flavanones form less soluble chalcone forms which precipitate (Gil-Izquierdo and others
890 2003). However, more than 90% of orange flavanones and 80% of soy isoflavone
891 glycosides were recovered after the intestinal step outlining their high stability toward
892 autoxidation (Walsh, Zhang, Vodovotz, Schwartz and Failla 2003, Gil-Izquierdo, Gil,
893 Tomas-Barberan and Ferreres 2003).The sensitivity to autoxidation is probably
894 overestimated in *in vitro* digestion models as oxygen is known to largely disappear in
895 the gastric tract. Last, it should be noted that proteolytic enzymes could play a role in
896 polyphenol bioaccessibility by releasing phenolic compounds bound to dietary proteins
897 as observed in the gastric tract for pepsin. However, more data support a role for
898 phenolic compounds as inhibitors of intestinal enzymes such as trypsin and lipase
899 (Gonçalves and others, 2007; He and others, 2006).

900

901

902 *Static models*

903 Conditions used in *in vitro* static models simulate quite well the physiology of
904 intestinal digestion with the use of porcine pancreatin, biliary extract or bile salts, and a
905 pH ranging between 6.0 and 7.5 (Table 3). However, the time allowed for this step is
906 highly variable (0.5-2.5 h). A too short digestion time may lead to trapping of
907 carotenoids in triglycerides, and thus underestimation of carotenoid bioaccessibility (Sy
908 and others 2012a). Different carotenoids show differing micellarization. Xanthophylls
909 (lutein and beta-cryptoxanthin) showed higher micellarization compared to alpha- and
910 beta-carotenes, while lycopene was only slightly micellarized (Garrett and others 2000;
911 Reboul and others 2006; Thakkar and Failla 2008). There have also been differences
912 noted between (E)-carotenoids and their (Z)-isomers (Chitchumroonchokchai and others
913 2004; Bengtsson and others 2010; Biehler and others 2011b), with the latter commonly
914 found in processed foods, also tending to be better micellarized (Bohn 2008). It could
915 also be speculated that a prolonged time of small intestinal digestion will favor the
916 formation of more Z-isomers. However, the *in vivo* data showed no significant
917 isomerization either in the stomach or in the duodenum for beta-carotene and lycopene
918 (Tyssandier and others 2003).

919 In most *in vitro* studies, the stability of phenolic compounds has been assessed by
920 determining total phenolic content such as the Folin-Ciocalteu method (Singleton L and
921 Rossi 1965), which does not yield information on the reactivity of specific phenolic
922 classes or molecules. The intestinal step, when compared to the gastric step, did not
923 influence the recovery of total phenolic compounds for homogenized prunes (81% of
924 the initial conc. in fruit) (Tagliazucchi and others 2012), grape berries (62%)
925 (Tagliazucchi and others 2010), cherries (127%) (Fazzari and others 2008),
926 pomegranate juice (100%) (Perez-Vicente and others 2002) and red cabbage extract

927 (100%) (McDougall and others 2007). However, a loss in total phenolics during the
928 intestinal step was observed for plums (44%), peaches (37%), tomato (31%)
929 (Tagliazucchi and others 2012), chokeberry juice (73%) (Bermudez-Soto and others
930 2007), raspberry extract (86%) (McDougall and others 2005b), and red wine (47% and
931 58%) (Gumienna and others 2005b), many of which contain labile anthocyanins. In
932 conclusion, the analysis of specific phenolic compounds should be addressed in order to
933 avoid conflicting results. Additionally, findings on the recovery of different classes in
934 one fruit/vegetable cannot be readily extended to other sources as stability *in vitro* is
935 influenced by interactions with the other phenolic compounds in the mixture and
936 vitamin C (for example sacrificial oxidation).
937
938 *Dynamic models*
939 To simulate the *in vivo* conditions of the small intestine, dynamic models can be used
940 to reproduce pH changes and secretion of pancreatic juice and bile. In the TIM model,
941 the intestinal transit time and pH conditions in the human digestive tract are simulated
942 through pre-programmed pH and delivery curves (Minekus 1995). Porcine pancreatin,
943 bile salts, electrolytes, and $NaHCO_3$ are secreted by computer-controlled pumps. The
944 model does not mimic brush border secretions. pH usually increases between the
945 duodenal, jejunal, and ileal compartments, for example, from 6.4 to 7.2 for the digestion
946 of a tomato-containing Western diet (Blanquet-Diot and others 2009). The
947 gastrointestinal transit time may greatly influence the bioaccessibility of phytochemicals
948 by affecting the release from the food matrix. Additionally, the solubility and stability
949 of different compounds may be affected by the time they are exposed to the conditions
950 in the intestinal tract. Apart from the integration of key parameters of digestion as
951 peristaltic mixing, transit time, and transport, the ability to remove digested material by

952 passive absorption of water and digested molecules through a dialysis system is also an
953 important feature of *in vitro* models. In particular, removal of digested molecules should
954 prevent product inhibition of the pancreatic enzymes (Minekus 1995).

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

968

969 *General considerations*

970 The contribution of the intestinal step to the bioaccessibility of phenolic compounds
971 is clearly influenced by several parameters. First, the action of intestinal enzymes on the
972 residual matrix could increase the phenolic content. Next, phenolic compounds are
973 chemically reactive in near-neutral conditions and their degradation or isomerization
974 may be catalyzed by the presence of oxygen and/or transition-metal ions. Additionally,
975 specific absorption by the small intestine can occur by passive diffusion or active
976 transport, as demonstrated for aglycones and their glucosylated forms. The latter forms

977 can be actively transported by the sodium-glucose-linked transporter 1 (SGLT1) found
978 in the enterocytes. Extracellular hydrolysis can be promoted by lactase phlorizin
979 hydrolase (LPH) in the brush border and be followed by diffusion of the resulting
980 aglycone into the enterocyte (Day and others 2000). A transcellular transport involving
981 multidrug resistance protein and P-glycoprotein transporters appears to be favored for
982 hydroxycinnamic acid and flavonol aglycones (Poquet and Clifford 2008, Barrington
983 and others 2009). These 2 phenomena cannot be readily modeled *in vitro*. Therefore, *in*
984 *vitro* digestion methods may over estimate the levels of these phenolic components. In
985 summary, a further limitation in oxygen, an inclusion of brush border enzymes or
986 analogs with α-glucosidase activity, a sufficient bile salt concentration, and the presence
987 of lipolytic, amylolytic and proteolytic enzymes for specific nutrient digestion are all of
988 importance for an optimal release of phytochemicals. While remaining triglycerides
989 may trap lipid-soluble phytochemicals, incompletely digested proteins and
990 polysaccharides will bind to water-soluble phytochemicals, making them unavailable in
991 the small intestine.
992
993 Large intestinal bioconversions
994 The colon contains a highly complex microbial ecosystem, which is capable of
995 fermenting food components not digested in the upper GI tract. Some undigested food
996 ingredients, including certain polyphenols, can act as substrate for the indigenous
997 bacterial community (Possemiers and others 2011). In addition, microbial bioconversion
998 products can influence the overall intestinal ecosystem and the bioavailability of the
999 parent compounds. Carotenoids are typically not studied in colonic models, as they are
1000 primarily absorbed in the small intestine, and colonic metabolites have not been
1001 reported so far. Colonic bioconversion of polyphenols is most well-described for

1002 flavonoids (Table 4) and phytoestrogens, lignans and isoflavonoids. The complexity of
1003 *in vitro* colonic models used to study the metabolism of phenolic compounds is diverse,
1004 ranging from batch fecal incubations using a strictly anaerobic and dense fecal
1005 microbiota suitable for metabolic studies (Barry 1995; Gross and others 2010; Aura and
1006 others 2012) to more complex continuous models involving one or multiple connected,
1007 pH-controlled vessels representing different parts of the human colon (Fogliano and
1008 others 2011) or *in vitro* dynamic gastrointestinal-colonic system models (Gao and others
1009 2006; Van Dorsten and others 2012) , which are applicable also to study effects of food
1010 components on the microbial population.

1011 Characterization of phenolic metabolites using *in vitro* colonic models is
1012 complementary to the metabolic bioconversion by the small intestine or the liver
1013 (methylation, sulfation, and glucuronidation) of the native forms in which they are
1014 present in foods (Scalbert and others 2002) and shows the diversity of structural
1015 transformations occurring in the colon prior to absorption (Aura 2008; Selma and others
1016 2009). Colonic metabolism of phenolic compounds starts with the transient appearance
1017 of aglycones and the subsequent formation of hydroxylated aromatic compounds and
1018 phenolic acids (Rechner and others 2004; Aura 2008). . Flavones, flavanones, flavanols,
1019 proanthocyanidins, and phenolic acids share hydroxyphenylpropionic acid metabolites
1020 (Rechner and others 2004; Aura 2008), whereas flavonols (quercetin, myricetin) and
1021 ferulic acid dimers share hydroxylated phenylacetic acid metabolites (Aura and others
1022 2002, Braune and others 2009). Moreover, flavanols also yield hydroxyphenylvaleric
1023 acids and corresponding valerolactone derivatives (Aura and others 2008; Sanchez-
1024 Patan and others 2012). Anthocyanins yield benzoic acids, hydroxylated benzaldehydes,
1025 and acetaldehydes (Aura and others 2005; Fleschhut and others 2006; Czank and others
1026 2013). Complex microbial metabolites, such as lactones formed from plant lignans or

1027 ellagitannins (Heinonen and others 2001; Cerda and others 2004), are re-absorbed from
1028 the colon and are subject again to liver metabolism and the conjugate derivatives are
1029 excreted via urine (Adlercreutz and others 1995). Thus plasma and urine excretions
1030 reflect both the hepatic and colonic metabolism of polyphenols (Table 4).

1031 Limitations of *in vitro* colonic models include that they may not fully represent the
1032 microbiota present in the colonic lumen and mucosa and that the combined rates of
1033 catabolism and absorption that occur *in vivo* are not reproduced. However, the use of
1034 colonic models provides information on the types of microbial metabolites formed
1035 (Table 4) and helps to elucidate the pathways involved. Batch models are of particular
1036 interest for a first assessment of colonic metabolism of phenolic compounds, which is
1037 characterized by a high inter-individual variability (Gross and others 2010), or for
1038 comparison of different sources or doses of compounds (Bolca and others 2009). The
1039 anaerobic batch colonic model developed by Barry and others (1995), which uses
1040 pooled human feces from several healthy donors, has been particularly suitable as
1041 coupled with a metabolomics platform to investigate the effects of structure and dose of
1042 fruit proanthocyanidin fractions on the efficiency of microbial metabolism and structure
1043 of flavanol monomers (Aura and others 2012; Aura and others 2008).

1044 Dynamic, multi-compartment colonic models are useful for long-term experiments
1045 needed to evaluate the spatial and temporal adaptation of the colonic microbiota to
1046 dietary phenolic compounds and the microbial metabolism of these phytochemicals.
1047 These models are designed to and should harbor a reproducible microbial community
1048 that should be stable upon inoculation, colon region-specific, and relevant to *in vivo*
1049 conditions (Macfarlane and others 1998; Van den Abbeele and others 2010). Dynamic
1050 colonic models have shown that microbial metabolism of black tea and red wine (Van
1051 Dorsten and others 2012) and cocoa (Fogliano and others 2011) is dependent on colon

1052 location. In addition, dynamic models may be used to enrich the colonic microbiota
1053 with polyphenol-converting species such as *Eubacterium limosum* to increase the
1054 production of 8-prenylnaringenin from hop extracts (Possemiers and others 2008).
1055 Dynamic colonic simulators have integrated new tools to improve modeling the
1056 physiological colonic conditions, such as the incorporation of a mucosal environment
1057 (Macfarlane and others 2005; Van den Abbeele and others 2012) and a mucus layer
1058 combined with epithelial cells (Marzorati and others 2011). The models can
1059 differentiate between the luminal microbiota with a large metabolic degradation
1060 capacity and the mucosa-associated microbiota able to closely interact with the host.
1061 An important element to be considered for designing colonic model experiments is
1062 the use of one or multiple fecal donors in terms of diversity of the microbiota
1063 population, as high-and low-polyphenol metabolizing phenotypes can skew the extent
1064 of metabolism of certain compounds (Selma and others 2009; Bolca and others 2012).
1065 Meanwhile, comparison of human gut metagenomes has suggested the classification of
1066 individuals into three distinc enterotypes (Arumugan and others 2011). The
1067 maintenance of anaerobic conditions during stool processing and inoculation to the
1068 models is crucial for microbial and enzymatic activities. Another important matter to be
1069 considered is the pH adjustment needed to avoid suppression of particularly minor
1070 conversion activities, for example slow enterolactone formation (Aura 2008). In
1071 summary, *in vitro* colonic models are the preferred choice to study mechanisms of
1072 polyphenol microbial metabolism as well as the polyphenol-induced modulation of gut
1073 microbiota. However, the ability of colonic models to simulate the *in vivo* conditions is
1074 limited by the lack of studies involving the formation of microbial biofilms adhering to
1075 the colonic epithelium. The simulation of intestinal absorption to remove end products

1076 of microbial metabolism is also relevant to prevent inhibition of the colonic microbiota
1077 during *in vitro* studies.
1078
1079 **Determination of bioaccessible fraction and further coupling techniques following**
1080 **digestion and/or colonic fermentation**
1081
1082 During the past few years *in vitro* digestion model systems have been used to analyze
1083 the structural and chemical changes that occur in different foods under simulated
1084 gastrointestinal conditions. These methods either simulate either disintegration, food
1085 matrix and digestion processes only (for bioaccessibility) or both digestion and
1086 absorption processes (for bioavailability estimates). According to the desired endpoints
1087 of the studies, there are considerable differences in the type of experimental parameters
1088 measured after digestion. These include chemical changes (such as hydrolysis of
1089 macronutrients), gastric solubilization of drugs, nutrient availability, release of
1090 encapsulated components, studying competitive processes, and structural changes (such
1091 as break-down of specific structures), aggregation, droplet coalescence, or droplet
1092 disruption (Chen and others 2011). Thus, samples obtained by *in vitro* digestion, either
1093 following small intestinal digestion or following further colonic fermentation *in vitro*,
1094 have been used in a variety of ways. In addition, the obtained fractions have been
1095 coupled to further investigation prodcedures, allowing for example the estimation of
1096 uptake into or transport through the intestinal epithelium.
1097
1098 *Estimation of bioaccessibility*
1099 The estimation of the bioaccessibility of non-polar food constituents such as
1100 carotenoids has been made both by measuring the transfer of carotenoids from the food

1101 matrix to the aqueous layer obtained after *in vitro* digestion and centrifugation (Hedrén
1102 and others 2002; Bengtsson and others 2009) or by filtering the aqueous fraction
1103 through a 0.22 µm membrane to obtain micelles (Reboul and others 2006; Huo and
1104 others 2007), or both. Since the micellarized carotenoids are considered to be the form
1105 in which these compounds will ultimately be absorbed by the intestinal cells, it has been
1106 suggested that assessment of carotenoid bioaccessibility must include the isolation,
1107 extraction and measurement of carotenoids in micelles (Etcheverry and others 2012).
1108 Reboul and others (2006) showed a high correlation (r= 0.90) of the *in vitro*
1109 bioaccessibility of α- and γ-tocopherol, β-carotene, and lycopene with the *in vivo* values
1110 measured in the micellar phase from human duodenum during digestion of a carotenoid-
1111 rich meal. Their findings suggest that estimation of carotenoid micellarization *in vitro*
1112 can be indicative of the amount available for uptake in the gastrointestinal tract *in vivo*.
1113 For polyphenols, Bouayed and others (2011, 2012) studied bioaccessibility following
1114 simulated gastric and intestinal *in vitro* digestion of fresh apple. They used a cellulose
1115 semi-permeable membrane, chosen as a simplified mechanical model for the epithelial
1116 barrier to identify dialyzable polyphenols after intestinal digestion. They suggested that
1117 dialyzable polyphenols in the intestinal phase could potentially be taken up by the
1118 enterocytes and suggested it may be a practical step prior to coupling to cellular
1119 methods due to increased purity of the dialysate, preventing negative impacts on cell
1120 viability. Similar studies were performed by other researchers (Liang and others 2012,
1121 Rodriguez-Roque and others 2013). At the same time, it is difficult to study the *in vivo*
1122 changes and digestive stability of different food constituents during their passage
1123 through the digestive tract, albeit some approaches, such as studying ileostomists, have
1124 allowed some comparisons to *in vitro* small intestinal digestion (Walsh and others 2007;
1125 Erk and others 2012).

1126

1127 *Bioaccessibility following colonic fermentation*

1128 *In vitro* digestion procedures have also been employed to produce berry samples that
1129 are characteristic of components that survive digestion, and therefore more
1130 physiologically relevant, for studies on bioactivities relevant to colon cancer models
1131 (Brown and others 2012).

1132 Due to the limited sampling possibilities (and intra- and inter-individual variations),
1133 the function and the composition of ileal microbiota is hard to study *in vivo*. The effect
1134 of small intestinal microflora on the enzymatic hydrolysis of phenol glycosides was
1135 studied in an *ex vivo* ileostomy model (Knaup and others 2007). Ileostomy effluents
1136 from 3 healthy subjects were used for incubation with synthetic quercetin and p-
1137 nitrophenol glycosides. The conclusion was that the hydrolysis of phenol glycosides is
1138 influenced both by the structural components of the phenols and the microflora in the
1139 small intestine. Schantz, Erk and Richling (2010) have also reported evidence of
1140 degradation of polyphenols in the small intestine, using an ex vivo ilostomy model to
1141 study the microbial metabolism and chemical stability of green tea cathechins and gallic
1142 acid.According to studies in ileostomy patients, the ileal microbiota is restored 6 months
1143 after surgery (Hove and Mortensen, 1996) which may resemble the reflux situation
1144 occurring in subjects with a healthy colon, or even take the role of colon fermentation to
1145 some extent in ileostomy patients.

1146 Phenolic microbial metabolites are relevant in terms of human health because they
1147 appear in plasma and are excreted in urine (Aura 2008). Pharmacokinetic studies show
1148 that microbial metabolite concentrations are elevated for up to 24-48 h in the
1149 bloodstream after a single dose of their precursors before returning to baseline values
1150 (Sawai and others 1987; Gross and others 1996; Kuijsten and others 2005).

1151 Enterolactone, enterodiol, and urolithins are excreted via urine as hepatic conjugates
1152 (Heinonen and others 2001; Cerda and others 2004), whereas microbial phenolic acid
1153 metabiltes appear in urine mainly in a free form in contrast to beverage- derived
1154 phenolic acids which are excreted mainly as sulphates and glucoronides (Sawai and
1155 others 1987; Stalmach and others 2009). In a recent work, Ludwig and others (2013)
1156 show that after ingestion of coffee, the main colon-derived metabolites found in plasma
1157 and/or in urine were dihydrocaffeic acid, dihydroferulic acid, and their sulfated and
1158 glucuronidated metabolites. As the metabolites described above and their hepatic
1159 conjugates are found in plasma and urine, therefore they circulate through the body and
1160 may exhibit both local and systemic effects. Phenolic metabolite levels in plasma range
1161 from low to high *nano* molar concentrations (Sawai and others Ando 1987; Kilkkinen
1162 and others 2001; Kern and others 2003; Johnsen and others 2004; Kuijsten and others
1163 2006), whereas urinary levels are at the *micro* molar range. In peripheral tissues, the
1164 concentrations can be anticipated to be even lower.

1165 A good example of studies including *in vitro* digestion models and colon conversion
1166 and pharmacokinetic studies in human volunteers was performed by Mateo Anson and
1167 others (2009, 2011). The group showed that bioprocessing of wheat bran with enzymes
1168 (xylanase, cellulose, β-glucanase, and feruloyl esterase) and yeast enhanced the
1169 bioaccessibility of ferulic acid, *para*-coumaric acid, and sinapic acid from white wheat
1170 bread matrix in the *in vitro* gastrointestinal models TIM-1 and TIM-2 by 5-fold. Since
1171 the release of *para*-coumaric acid and sinapic acid occurred mainly in the TIM-1 model
1172 simulating the upper intestine, the microbial conversion products (3-(3'-hydroxyphenyl)
1173 propionic acid and 3-phenylpropionic acid) from the TIM-2 colon model were shown to
1174 be related to matrix bound ferulic acid content (Mateo Anson and others 2009). In a
1175 subsequent pharmacokinetic *in vivo* study volunteers consumed 300 g white wheat

1176 bread samples fortified with either native or bioprocessed wheat bran, and then phenolic
1177 acids and their metabolites were followed for 24 hours. The release and conversion of
1178 microbial metabolites were enhanced by bioprocessing of bran by 2- to 3-fold and their
1179 time course profiles in plasma were altered by bioprocessing of bran (Mateo Anson and
1180 others 2011).

1181

1182 *Coupling digesta to uptake and transport models of the intestinal epithelium*

1183 More recently, human enterocyte cell culture models (such as Caco-2 cells) was
1184 coupled with a simulated gastric model. Small intestinal digestive processes or
1185 following further colonic fermentation have been widely used as a predictive tool for
1186 the absorption of bioactive components from foods (Chitchumroonchokchai and Failla
1187 2006). Caco-2 is a cell line originating from human colonic carcinoma that exhibits
1188 some morphological and functional characteristics similar to those of differentiated
1189 epithelial cells that line the intestinal mucosa (Sambruy and others 2001). The *in vitro*
1190 digestion/Caco-2 cell culture model developed by Glahn and others (1998) offers a
1191 rapid, low-cost method for screening foods and food combinations for iron uptake
1192 before more definitive human trials (Hur and others 2011). Most Caco-2 cell model
1193 studies were carried out to model iron uptake and many researchers reported that the
1194 estimation of iron bioavailability, but also that of other phytochemicals such as
1195 carotenoids from the *in vitro* digestion/Caco-2 cell culture model has been well
1196 correlated, qualitatively and quantitatively, with human data (Garrett and others 1999;
1197 Mahler and others 2009). Caco-2 cells have also been applied to a number of uptake and
1198 transport studies for both hydrophilic constituents (such as polyphenols) and lipophilic
1199 compounds (such as carotenoids). Garret and others (1999, 2000) developed a coupled
1200 digestion/Caco-2 human intestinal cell system to examine cellular acquisition of

1201 micellarized carotenoids and other lipophilic components from digested foods,
1202 supplements, and meals. While the majority of studies have focused on simple uptake
1203 employing a biphasic model with the apical membrane and the cell layer, uptake models
1204 including also an additional basolateral compartment are also available to allow the
1205 study of fluxes and, therefore, kinetic parameters through the cell layer (Reboul and
1206 others 2006; Manzano and Williamson 2010; Biehler and others 2011a). However, the
1207 latter requires transwell inserts, which are more costly, and the concentrations to be
1208 determined are usually lower and may require more sophisticated analytical instruments
1209 for detection, such as mass spectrometry, and may not be feasible for easily studying
1210 minor food constituents. More detailed discussion on characteristics and limitations of
1211 standard *in vitro* digestion methods coupled with a Caco-2 cell model can be found in
1212 review articles by Failla and others (2008a) and by Biehler and others (2011a). More
1213 recently, the Caco-2 cell model has been extended by adding a layer of mucus-
1214 producing cells (such as HT-29 MTX cells) on top of the Caco-2 cells. However, only
1215 preliminary data are available on how this system performs compared to Caco-2 cells
1216 alone, although this may represent a more realistic approach, which may further hamper
1217 uptake of more lipophilic constituents due to the additional mechanical barrier
1218 (Nollevaux and others 2006). Also, Ussing chambers are used, in order to obtain a better
1219 understanding of the transepithelial transport processes on a molecular basis. This is a
1220 model that simulates the mucosa and its luminal/apical side (Bergmann H and others
1221 2009; Clarke LL 2009). For example, Deusser H and others (2013) have used the
1222 Ussing chamber to evaluate apple polyphenol transport and their effect on mucosal
1223 integrity.
1224
1225 **Conclusions and Summary**

1226

1227 Many considerations have to be taken into account when determining
1228 bioaccessibility of phytochemicals by means of *in vitro* digestion models. Two
1229 important criteria are whether the focus of research is on biochemical transformation of
1230 food components and metabolomics, favoring the metabolic batch models, or if the
1231 close simulation of dynamic physiological conditions and changes in microbial
1232 population are the primary aims, the use of continuous models such as the TIM models
1233 can be recommended. An additional criterion is the lipophilicity of the phytochemicals
1234 of interest. While for hydrophilic compounds such as for polyphenols, often associated
1235 with fiber or complex carbohydrates, amylase digestion and perhaps particle size appear
1236 to play predominant roles. Whereas, for lipophilic compounds, (such as carotenoids)
1237 emulsifying agents, (presence of dietary fats, bile salts, and sufficient lipolytic activity),
1238 appear crucial, thus their use during digestion should be well considered and
1239 standardized. This includes adjusting pH values and sufficient digestion times to allow
1240 for optimal enzyme functioning comparable to the *in vivo* situation. The suggested
1241 conditions for static digestion models are outlined in Table 5. In addition, lipophilic
1242 phytochemicals require separation of the micellar fraction prior to further investigation,
1243 such as via ultracentrifugation (static model), filtration, or employing a membrane
1244 (dynamic model). Coupling the cell-based uptake model with large intestinal digestion
1245 model is a comparatively novel but important completion of modeling digestion. This
1246 may especially be suitable for compounds such as polyphenols, which are metabolized
1247 and taken up from the colon.

1248 Until now, the lacking of consensus values for the different digestion parameters has
1249 hampered the possibility to compare results across different studies. The suggested
1250 conditions are based in relevant *in vivo* data, yet further studies should be done to

1251 validate its use and limitations in phytochemicals digestion. While still having their
1252 limitations, much insightful information has been gained from applying *in vitro*
1253 digestion models to phytochemical research. The recent improvements in our
1254 understanding and the advances in the technology warrant continuous research in the
1255 important area of bioavailability.
1256
1257
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1270
1271 **Conflicts of Interest**
1272 The authors report no conflicts of interest. The authors alone are responsible for the
1273 content and writing of the paper.
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2017 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**

Type of study	α-Amylase activity (U/mL)*	pH of digestion	Time of digestion (min)	Temperature (º C)	Study/reference
Digestion of grape seed flavonoids (human saliva)	Not specified	6.9	10	37	(Laurent and others2007)
Digestibility of soya bean, cowpea and maize	ca. 1	7	30	37	(Kiers and others2000)
Bioactivity of wheat bread; changes in the antioxidant activities of vegetables	200	6.75	10	37	(Gawlik-Dziki, 2009, 2012)
Developing digestion procedure with mammalian enzymes	25-125	7	15	37	(Lebet and others1998)
ß -Carotene micellarization	900	6.5 ±0.2	10	37	(Thakkar and others 2007)
ß -Carotenoid bioacessibility	300	6.7-6.8	10-15	37	(Bengtsson and others 2009, 2010
ß -Carotene bioaccessibility (human saliva from n=9)	12.5[e]	6.7-6-9	10	37	(Schweiggert and others 2012)
ß-Carotene bioaccessibility from sweet potato	35	7.0	10	37	(Poulaert and others 2012)
Polyphenol release during digestion	150	6.9	10	37	(Tagliazucchi and others 2012)

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B - Human studies (*in vivo*)

Type of study	α-Amylase activity (U/L)**	Time of digestion (min)	pH	Study/reference
Studying impact of saliva process on lipophilic polyphenol availability	Not specified	0.5	nd	(Ginsburg and others 2012)
Physiology of human saliva including mucin and electrolytes	Not specified	nd	7.0	(Aps and Martens, 2005)
Human salivary α-amylase activity	4-1653, mean 284[e]	nd	nd	(Suska and others2012)
List of reference values	60-282, mean 170[f]	nd	nd	(Jakob, 2008; Kopf-Bolanz and others 2012)
Stress and alpha-amylase	220-500 between 8am. and 20pm.[f]	nd	nd	(Nater and others 2010)
Oral digestion of cereals by humans	52-77(basal) 58-66(with cereals)[e]	5	7.1±0.1	(Hoebler and others 1998)
Saliva activity measurements	190[f]	nd	nd	(Rohleder and Nater, 2009)

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2036 *"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).
2037 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
2038 from DNS to IFCC is x 2.5/342 = x 0.0073.
2039 ** 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
2040 EPS and Phabedas) yield comparable results
2041 [e]: Phabedas (Magle AB, Lund, Sweden) test: blue color from starch breakdown measured at 620 nm. Conversion from µkat to U according to
2042 http://www.phadebas.com/data/phadebas/files/document/Instructions_Phadebas_Amylase_Test.pdf, 60U=1µkat
2043 [f] releases 1 µmol/min p-nitrophenol from 4,6-ethyliden-G7-p-nitrophenol-D-maltoheptasoid (*ethylidene*-G7PNP , measured at 405 nm. 60 U=1 µkat (IFCC EPS method, Ethylen protected
2044 substrate)
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2052 Table 2: Typical concentrations of gastric enzymes in human studies and *in vitro* experiments.

A - *In vitro* studies

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

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2060 Table 2 cont.

B- Human studies (*in vivo*)

Fluid investigated and type of study	Pepsin (U/mL)	Gastric residence time (h)	pH	Study/reference
Digestion of adults	942[$] (1207)[*](basal)	nd	nd	(Armand and others1995)
Digestion of infants	ca. 85[$] (109)[*] (pp) 190[$] (243)[*] (basal)	nd	nd	(Armand and others1996a)
Helicobacter pylori impact on stomach	47[b] (174)[*]	nd	1.41 (basal)	(Feldman and others 1998)
18 individuals, fasting juice	37±21 [7-70][a] (3700)[*]	nd	1-4, median 2 (basal)	(Ulleberg and others 2011)
Pepsin inhibitors in humans	20-260 µg/mL[&]	nd	nd	(Pearson and Roberts, 2001)
Measurement gastric secretion	nd	nd	1.1 (basal); 3.5 (60 min. pp) 2.0(120 min. pp)	(Gardner and others 2002)
Characterization of digestive fluids	110-220 µg/mL (basal)[&] 260-580 µg/mL (pp)	nd	2 (basal) 6 (60 min. pp) 5 (120 min. pp)	(Kalantzi and others 2006)
Helicobacter pylori impact on pepsin	114 to 1030 µg/mL[&]	nd	2.4 (basal)	(Newton and others 2004)
Gastric residence time, solid meal	nd	3.5± 0.7	nd	(Mojaverian and others 1988)
Gastric residence time of capsule	nd	1.2± 0.45	1.5±0.04 (basal)	(Mojaverian, 1996)
Gastric passage time of capsule	nd	1.0	*nd*	(Worsoe and others 2011)
Digestability of rice pudding	nd	65% com-plete (1.5h)	nd	(Darwiche and others 1999)

2061 *"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,
2062 with hemoglobin as substrate.
2063 [$] *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
2064 solution. 1 unit equivalent to approx. 1.28 "Sigma units" (http://www.worthington-biochem.com/pm/assay.html).
2065 [a] 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
2066 as substrate. 1 unit equivalent to approx. 100 "Sigma units".
2067 [b] measured as international units, with 1 IU=3.7 Anson units.
2068 nd= no data; pp=post-prandial; [&] µg enzyme/mL
2069 Table 3: Concentrations of intestinal enzymes and bile salts in humans studies and *in vitro* experiments.

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A - *In vitro* studies

Type of study	Bile salts+ (mmol/L)	Pancreatin* concentration, ca. (mg/L)	Minimum pancreatin activity (U/mL)*	pH	Digestion time (min)	Study/reference
Bioaccessibility of iron	ca. 4 (2 g/L)	300	2.4	7.5	150	(Miller and others 1981)
Bioaccessibility of carotenoids	ca. 8.6 (4.3 g/L)	720	5.8	7-7.5	120	(Biehler and others 2011b)
Bioaccessibility of carotenoids	12 (6 g/L)	2500	20	7.5	120	(Yonekura and others 2009)
Bioaccessibility of carotenoids	4.4 (2.1 g/L)	390	3.1	7.5	120	(Garrett and others 1999)
Bioaccessibility of carotenoids	7.5 (3.75 g/L)	600	4.8	7.5	30	(Hedrén and others 2002)
Bioaccessibility of carotenoids	2.8 (1.44 g/L)	240	2.0	6.0	30	(Dhuique-Mayer and others 2007)
Bioaccessibility of carotenoids	3.0 (1.5 g/L)	250	2.0	ca. 7	120	(Liu and others 2004)
Bioaccessibility of polyphenols	3.0 (1.5 g/L)	250	2.0	5 to 7.5	120	(Gil-Izquierdo and others 2002)
Bioaccessibility of polyphenols	4.3 (2.2 g/L)	360	2.9	6.5→7.0-7.5	165	(Bouayed and others 2011)
Bioaccessibility of polyphenols	44 (22 g/L)	3,600	29	6.5	120	(Cilla and others 2011)
Bioaccessibility of polyphenols	10 (5 g/L)	800	6.4	7.5	120	(Tagliazucchi and others2012)
Bioavailability of polyphenols	10 (5 g/L)	800	6.4	nd	120	(McDougall et al., 2005a, b)

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2077 Table 3 cont.

B - Human studies (*in vivo*)

Fluid investigated and type of study	pH	Bile salts (mmol/L)	Lipase activity (U/mL)	Study/reference
Duodenal fluids; jejunal fluids;	7.0±0.4 6.8±0.4	0.6 - 5.5 (fasted)	n.d.	(Perez de la Cruz Moreno and others 2006)
Duodenal fluids	nd	3.8–11.8 (fed) (2-6 g/L)	n.d.	(Van Deest and others 1968)
Duodenal fluids (standard meal)	nd.	5 – 10 (fed)	n.d.	(Tabaqchali and others 1968)
Duodenal fluids (standard meal)	5.5-8.0, mean 6.5	20 (fed) (2–18 g/L)	15-120 (fed) (mean 50)[&]	(Borgstrom and others 1957)
Review article	nd	4 – 20 (fasted)	n.d.	(Garidel and others 2007)
Orlistat and enzyme activity	6-6.5	n.d.	1000 (fed)[e] (0.6 g/L)	(Sternby and others 2002)
Pancreatic enzyme examinations.	nd	n.d.	70-1000, mean 300[&]	(Braganza and others1978)
Duodenal fluids (after regular diet)	n.d.	n.d.	10 (fasted), 130 (fed)[&]	(Dukehart and others 1989)
Review lipolysis	n.d.	3-7 (fasted) 5-15 (fed)	150-300[&]	(Patton and Carey 1981; Zangenberg and others 2001a, b)
Fasting 18 individuals	5-9, mean 7	2.7±1.3 (fasted)	units not comparable to other tests	(Ulleberg and others, 2011)
Duodenal fluids 6-14 individuals (median)	6.2 (fasted) 5.2-6.6 (fed)	2.6 (fasted) 11.2 (fed 30 min) 5.2 (fed 180 min)	n.d.	(Kalantzi and others2006)
Duodenal fluids Test meal	6.0-7.0	5.9±1.8 (fasted) 6.7-13.4 (fed)	600 (fasted)[e] 1200-1400 (fed)[e]	(Armand and others1996b)
Duodenal juices Meal - Review	n.d.	n.d.	80-7000[e]	(Armand, 2007)
Small intestinal transit time (min)		90		(Kim, 1968)
GI passage times (min)		197		(Degen and Phillips, 1996)
GI passage times (min)		199		(Yu and others1996)

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2079 *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
2080 unit liberates at least 1 µmole acid from olive oil/triolein per minute at 37 ^{o}C and pH 9 (http://www.pharmacopeia.cn/v29240/usp29nf24s0_m60320.html). Definition
2081 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

2082 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
2083 umol of glycosic linkage is hydrolyzed per min at pH 6.8 (and conditions further described for the amylase assay,
2084 http://www.pharmacopeia.cn/v29240/usp29nf24s0_m60320.htm).
2085 [&] same as USP units.
2086 [e] Tributyrin units: 1 TBU (lipase unit) is the amount of enzyme which releases 1 mmol titratable butyric acid per min at 40 ^{o}C, pH 7.5. Yields comparable results to triolein units when
2087 expressed at same unit of molarity (McCoy and others 2002).
2088 [+] values calculated from weight assuming a molecular weight of 500 g/mol and 100% purity.
2089 [$]postprandial

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

Food	Metabolites *in vivo*	Reference	Metabolites *in vitro*	Colonic model	Reference
Tea	1.3-Dihydroxyphenyl-2-*O*-sulfate 5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone 5-(3′,4′,5′-Trihydroxyphenyl)-γ-valerolactone Hippuric acid 1,3-Dihydroxyphenyl-2-*O*-sulphate	(Daykin and others 2005)	3-Phenylpropionic acid 3-(3’,4’-Dihydroxyphenyl) propionic acid 3-(3-Hydroxyphenyl)propionic acid 3-Hydroxyphenylacetic acid 2,6-Dihydroxybenzoic acid 1,2,3-Trihydroxyphenol	Batch Time: <72 h pH: 7.15±0.07 (start), 6.92 ±0.26 (end)	(Gross and others 2010)
			3-Phenylpropionic acid 5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone 3-(3’,4’-Dihydroxyphenyl) propionic acid 3(4-Hydroxyphenyl)propionic acid	SHIME[1] Stomach, small intestine and 3-colonic vessels dynamic model (pH 5.6-5.9, 6.1-6.4 and 6.6-6.9). Time: 2 weeks (continuous)	(Van Dorsten and others 2012)
Red wine, grapes	3-(3-Hydroxyphenyl)-propionic acid 3-Hydroxyphenylacetic acid 3,5-Dimethoxy-4-hydroxybenzoic acid 3-Hydroxyhippuric acid Hippuric acid 4-Hydroxyphenylacetic acid	(Jacobs and others 2012)	3-(3-Hydroxyphenyl)-propionic acid 3- and 4-Hydroxyphenylacetic acid 5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone γ-Valerolactone	Batch pH: 6.8 Time: 48 h	(Sanchez-Patan and others 2012)
			3-(3’,4’-Dihydroxyphenyl) propionic acid 2-(3’,4’-Dihydroxyphenyl) acetic acid 5-(3’-Hydroxyphenyl) pentanoic acid 3,5-Dimethoxy-4-hydroxybenzoic acid 3-Methoxy-4-hydroxybenzoic acid	Batch pH: monitored at each time point Time: 0, 2, 4, 6, 8, and 24h	(Aura and others 2012; Gross and others 2010)
Chocolate, cocoa	3-(3-Hydroxyphenyl)-propionic acid 5-(3,4-Dihydroxyphenyl) valerolactone and conjugates 5-(3,4-Dihydroxyphenyl) valerate conjugates 4-Hydroxy-5-(3,4-dihydroxyphenyl)valeric acid Phenylvalerolactone derivatives	(Llorach and others 2009)	3-(3-Hydroxyphenyl)-propionic acid 3-Hydroxyphenylacetic acid 3,4-Dihydroxybenzoic acid	3-Colonic vessels dynamic model pH: 5.5, 6.2 and 6.8 Time: 36 h	(Fogliano and others 2011)
Soy	*O*-Demethylangolensin, Equol Dihydrogenistein	(Joannou and others 1995)	*O*-Demethylangolensin, Equol	Batch Time: 72 h	(Possemiers 2007)

			3-Methoxy–4-hydroxyphenylacetic acid 4-Hydroxyphenyl acetic acid	TIM[2]-2 colonic dynamic model pH: 5.8, 6.4 and 7.0 time: <28 h	(Gao and others 2006)
Berries, nuts	4’-Hydroxymandelic acid, 3’,4’-Dihydroxyphenylacetic acid 3-(4’-hydroxyphenyl)lactic acid 4’-Hydroxyhippuric acid Hippuric acid Urolithins	(González-Barrio and others2011)	4-Hydroxybenzoic acid 3,4-Dihydroxybenzoic acid 3-(3’-Hydroxyphenyl)propionic acid 3-(3’,4’-Dihydroxyphenyl)propionic acid 3-(4’-Hydroxyphenyl)lactic acid Urolithins	Batch Time: <72 h pH: 7.2 (start), 6.2 (end)	(González-Barrio and others2011)
	4-Hydroxy-5-(phenyl)valeric acid conjugates Vanillic acid glucuronide Hydroxyhippuric acid Ferulic acid glucuronide 1,3-Dihydroxyphenyl-2-*O*-sulfate Urolithin A and B conjugates	(Tulipani and others2011)			
Citrus fruits	4-Hydroxy-phenylpropionic glucuronide 4-Hydroxy-benzoic acid glucuronide 3-Methoxy-4-hydroxy-phenylacetic glucuronide 3- and 4-Hydroxyphenylacetic glucuronide Hippuric acid glucuronide	(Vallejo and others 2010)	3-Methoxy–4-hydroxyphenylacetic acid 4-Hydroxyphenyl acetic acid 3,4-Dihydroxyphenylacetic acid 3-(3-Hydroxyphenyl) propionic acid 3-(4-Hydroxy–3-methoxyphenyl) propionic acid 3-Hydroxyphenyl acetic acid Hippuric acid.	TIM-2 colonic dynamic model pH 5.8, 6.4 and 7.0. Time: <28 h	(Gao and others 2006)

[1]SHIME, Simulator of the Human Intestinal Microbial Ecosystem (Molly and others 1993).
[2]TIM, TNO Intestinal Model (Minekus 1995).

1 Table 5: Summarized conditions for simulated digestion under static conditions, based
2 on common *in vitro* conditions applied, feasibility, and their similarity to *in vivo*
3 conditions
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Phase of digestion	Common *in vitro* values*	Common *in vivo* values*	Tentatively suggested**
Oral phase			
-α-amylase (U/mL)[a]	110	26	25-200
-time (min)	10	0.5-5	1-5
-pH	6.9	7.1±0.1	7.0±0.2
Gastric phase			
-pepsin (U/mL) [b,d]	1,400-4,300	170-1200; 0.1-0.2 g/L	5,000-10,000
-time (min)	60	60-72;140-210[c]	60; 120[c]
-pH	2.0	2 (fasted); 3.5 (120 min (fed)[+])	3.5±0.5
Small intestine			
-lipase[$]	4.0 (0.5g/L)	70-1000 (fed); 10 (fasted)	20-200
-bile salts (mmol/L)	7.5 (3.8 g/L)	5 (fasted) 10 (fed)[+]	10
-time (min)	120	200	120-200
-pH	7-7.5	6.8±0.4	7±0.2
Large intestine			
-time (min)	42 (24-72)	35±2.1	35-45
-pH	6.6 (5.5-7.2, start) 6.6 (end)	6.2 (5.7-6.7)	6.2-6.6

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6 *median value taken from Tables 1, 2, 3, and 4
7 **taking into account human trials (Tables 1, 2, 3 and 4) and herein reported physiological values
8 [a] "Sigma units"(see Table 2). For conversion into IFCC units x 0.0073 (i.e. 140 units = 1.02 IFCC units).
9 [b] "Sigma units:" Pepsin typically used: 800 – 2500 units/mg such as by Sigma-Aldrich. 1 unit: One unit will produce
10 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
11 table 2 for conversion factors.
12 [c] liquid and solid meals, respectively
13 [d] gastric lipase not required for water soluble compounds, however for lipophilic compounds such as carotenoids a
14 concentration of 40-80 U/mL is recommended (Armand 1999, 2007). Gastric lipase (tributyrin units): 10-65 (mean
15 40, Armand 1999); 60-80 (Armand 2007). 1 TBU (lipase unit) is the amount of enzyme (g) which releases 1 µmol
16 titratable butyric acid per minute under the given standard conditions.
17 [+] post-prandial
18 [$]1 unit liberates at least 1 µmole of acid from olive oil/triolein per minute at 37 °C and pH 9. Comparable to tributyrin
19 units when expressed at same molarity.

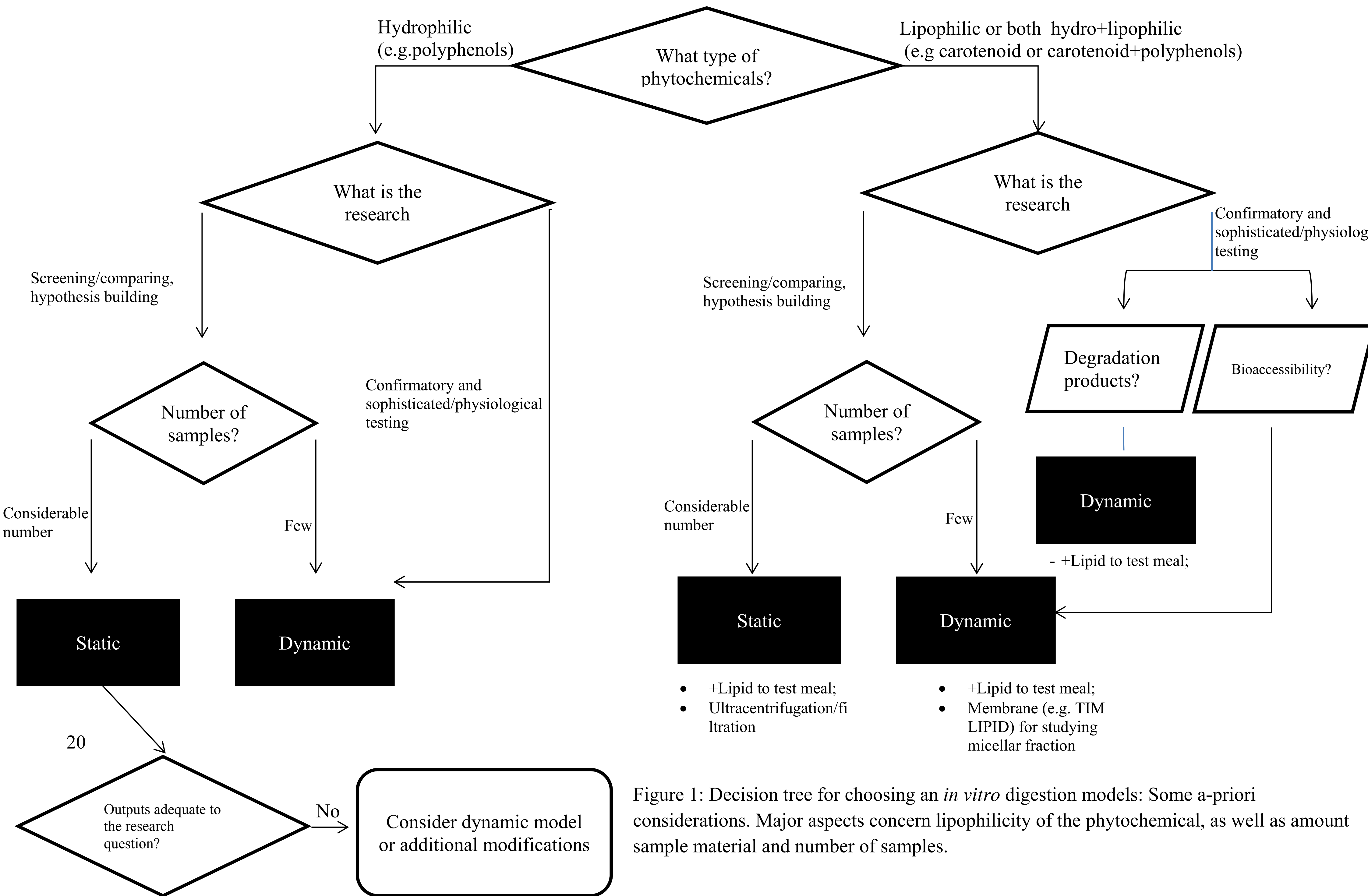

Figure 1: Decision tree for choosing an *in vitro* digestion models: Some a-priori considerations. Major aspects concern lipophilicity of the phytochemical, as well as amount sample material and number of samples.