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Methodology for the determination of polyphenol bioaccessibility

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ABSTRACT

Polyphenols are secondary metabolites of plants, commonly present in the human diet. Since they exhibit a wide range of bioactivities, polyphenols are extensively studied in the fields of nutrition and human health. Current studies have shown a high interest in determining the bioaccessibility of polyphenols, the amount of polyphenols that becomes available for absorption in the digestive tract. Bioaccessibility can be determined with the help of *in vitro* static gastrointestinal (GI) digestion models. In such a methodology, food samples containing polyphenols are subjected to a series of conditions that mimic the human gastrointestinal tract, with associated parameters. A high number of GI models with slightly different parameters were published. The purpose of this paper is to review the literature, focusing on the determination of polyphenol bioaccessibility and the parameters used in these GI digestion models, such as time, temperature, and pH of digestion, as well as enzyme concentrations. Gastrointestinal digestion models consist of oral, gastric and small intestine phases. These models provide a simple and reliable methodology which enables insight into the amount of bioaccessible polyphenols.

Introduction

Polyphenols are natural bioactive compounds commonly present in human nutrition, since they are widespread in plants where they are produced as secondary metabolites (Abbas et al., 2017). The main role of polyphenols in plants is the protection against pathogens and herbivores. In addition, they contribute to the colour and the taste of the plants, which attracts insects for pollination and seed dispersal (Juadjur and Winterhalter, 2012). There are over 8000 phenolic compounds that have been identified in plants (Lewandowska et al., 2013) and those can generally be divided into flavonoids (flavonols, flavan-3-ols, flavones, isoflavones, flavanones, and anthocyanidins) and nonflavonoids (Etxeberria et al., 2013).

Polyphenols exert various potential bioactivities in the human body. Bioactivity represents all the events that a bioactive compound undergoes from the moment of intake to the final physiological responses it causes (Fernández-García et al., 2009). These include the transport of the bioactive compound to the target tissue, the interaction with macromolecules, the potential metabolism or biotransformation, and the physiological response in an active site (Fernández-García et al., 2009). Although there is much to be proven, some studies have shown that nutrition rich in polyphenols, or certain polyphenol groups, is associated with a potential reduction of the risk of chronic diseases such as cardiovascular disease (Mendonça et al., 2018), specific cancers (Link et al., 2010, Stagos et al., 2012), and diabetes (Hanhineva et al., 2010). Further studies are needed to support these theses. However, in order to show positive effects on the human body, polyphenols must be released from the food matrix during digestion and absorbed in a certain amount (Jakobek, 2015). Here we come to the

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problem of bioaccessibility of polyphenols, which has been the topic of many research papers (Bouayed et al., 2011; Gil-Izquierdo et al., 2001; Gil-Izquierdo et al., 2003; Tagliazucchi et al., 2011).

Bioaccessibility can be defined as the amount of ingested compounds that is available for absorption in the digestive tract (Palafox-Carlos et al., 2011). The amount of bioaccessible polyphenols may differ greatly from the amount of polyphenols present in the consumed food. On the other hand, bioavailability is defined as the rate and the extent to which bioactive substances are absorbed and become available at the site of action (Palafox-Carlos et al., 2011; Parada and Aguilera, 2007). It can be seen from the previous two definitions that the bioavailability of a bioactive compound largely depends on its bioaccessibility. The bioavailability of isolated food components is often examined, although the bioavailability of these components incorporated into the food matrix could be significantly different (Saura-Calixto et al., 2007). To avoid such cases, *in vitro* simulated digestion models have been applied to evaluate the bioaccessibility of dietary polyphenols. Since the first model developed to evaluate the bioaccessibility of food iron (Miller et al., 1981), to date, many models have been published using different approaches and parameters (Bouayed et al., 2011; Gil-Izquierdo et al., 2001; Gil-Izquierdo et al., 2003; Tagliazucchi et al., 2011).

The aim of this paper was to shortly present the parameters of *in vitro* static models that can be used to evaluate the bioaccessibility of polyphenols, which includes individual digestive phases (oral, gastric, and intestine), and the conditions of pH, temperature, time, and enzyme amounts in *in vitro* simulated digestion.

Polyphenols

With over 8000 different compounds, polyphenols are one of the most numerous and widespread groups of natural products in the plant kingdom. From a chemical point of view, it is a group of compounds possessing phenolic structural elements, which consist of aromatic rings to which one or more hydroxyl groups are attached (Belščak-Cvitanović et al., 2018). The division of polyphenols by chemical structure is based on the number of phenolic rings that a particular compound possesses and how these rings are linked. There are several different approaches to classify polyphenols in this way, resulting in small differences in the number of classes. However, the most commonly used classification of polyphenols divides them into 5 major classes: phenolic acids, flavonoids, stilbens, lignans, and others (Fig. 1) (Belščak-Cvitanović et al., 2018; Manach et al., 2004). *Flavonoids* represent the most abundant group of polyphenols identified in the plant world. They consist of two aromatic rings linked together by three carbon bridges. There are over 4000 different flavonoids known, which can be further subdivided into several categories, with flavonols, flavones, flavan-3-ols, anthocyanidins, flavanones, and isoflavones being the most prevalent in the human diet. Flavonoids may appear in nature as aglycones, but most flavonoids have sugar attached to their initial structure, that is, in glycosidic form (Belščak-Cvitanović et al., 2018; Crozier et al., 2009). *Phenolic acids* account for about one-third of phenolic compounds in the human diet (Yang et al., 2001). They are characterized by a benzene ring with an attached carboxyl group and one or more hydroxy or methoxy groups (Belščak-Cvitanović et al., 2018). Phenolic acids can be divided into two main groups: benzoic acid and cinnamic acid derivatives. *Lignans* are formed from two units of phenylpropane. Because they are partially integrated into the lignin polymer, they are widely distributed in the human diet (Belščak-Cvitanović et al., 2018; Moreno-Franco et al., 2011). The richest dietary source of lignans are flax seeds (Belščak-Cvitanović et al., 2018; Moreno-Franco et al., 2011). *Stilbenes* are polyphenols produced by plants in response to disease, injury, or stress. They are represented in small amounts in the human diet and the main representative of stilbenes is resveratrol found in red wine (Crozier et al., 2009).

Bioactivity

Polyphenols exhibit a wide range of properties such as solubility in organic solvents, absorption of ultraviolet light, protection of plants against pathogens and stress, and pigmentation and odorization of plants. However, the two physiochemical properties for which polyphenols are most extensively studied in the field of human nutrition are the reducing activities and the binding properties of polyphenols (Belščak-Cvitanović et al., 2018).

A diet rich in polyphenols is thought to increase the chance of cardiovascular safety (Abbas et al., 2017; Mendonça et al., 2018) although these effects are not entirely clear. They have also shown anti-atherosclerotic potential (Santhakumar et al., 2018). Their suggested protective role still requires further studies. A number of studies have linked a diet rich in polyphenols to a reduced risk of cancer (Costa et al., 2017; Link et al., 2010; Stagos et al., 2012). All of those potentially positive bioactivities need to be confirmed by further studies. It is believed that polyphenols can improve glycemic control by different mechanisms, thereby reducing the risk of diabetes (Hanhineva et al., 2010).

As mentioned before, in order to exert potential health benefits, polyphenols must be absorbed in a certain amount. A small percentage of polyphenols enter the blood circulation by crossing the epithelial cells in the small intestine, while the majority of polyphenols reach the large intestine (Kawabata et al., 2019; Ozdal et al., 2016). The microbiota present in the large intestine, having an ecosystem of around 10^{13}- 10^{14} bacterial cells, represents the highest concertation of microorganisms in the human body (Ozdal et al., 2016). By modifying the structure of aglycones, glycosides, and conjugates, the gut microbiota could affect polyphenol bioavailability. In 2019, Kawabata et al. reviewed the role of microbiota in bioavailability and physiological functions of dietary polyphenols. They concluded that gut microbiota catabolizes polyphenols, either by the action of intestinal bacteria or enzymes present in the microbiota, such as hydrolase and dioxygenase. Faeces excrete the resulting catabolites, but some might be absorbed through epithelial cells in the large intestine. It is proposed that these catabolites might be significant contributors to the overall health benefits of polyphenols (Kawabata et al., 2019).

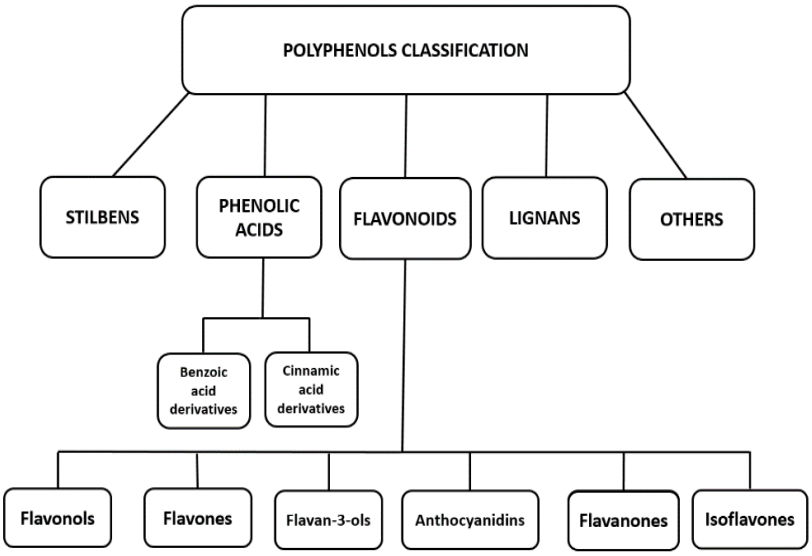

Fig. 1. Classification of dietary polyphenols

Bioaccessibility

The *in vitro* static gastrointestinal (GI) method is the most common method for determining the bioaccessibility of polyphenols, which is well described in the review paper (Carbonell-Capella et al., 2014). In 1981, Miller et al. developed a simulation of the digestive process for better insight into the digestion and absorption of dietary iron (Miller et al., 1981), which was later adapted by Gil-Izquierdo et al. (2001) for the study of phenolic compound release. In summary, this method consisted of pepsin-HCl digestion that simulated the gastric phase, and pancreatin digestion with bile salts to simulate digestion in the small intestine (Gil-Izquierdo et al., 2001). Later studies have introduced some modifications, such as the introduction of the oral phase (Gwalik-Dziki, 2012; Shim 2011; Tagliazuchi et al., 2011) or the termination of enzymatic activity using crushed ice (Villanueva-Carvajal et al., 2013), but the basis of the method has remained the same.

Oral phase simulation

Many of the developed methods of simulating human digestion do not include the oral phase. This is due to the relatively short duration of this phase (seconds to minutes) and to the saliva pH being close to neutral (6.2 - 7.6), so no significant release of the compounds is expected at this stage (Alegria et al., 2015). However, there are certain chemical, biochemical and physical processes that occur in the oral cavity, especially for solid samples, due to longer retention times compared to liquid samples. At this stage, nutrients and bioactive compounds are exposed to changes in pH, ionic strength, and temperature. In addition, there is an interaction with digestive enzymes (lingual lipase, amylase, and protease) and saliva biopolymers (mucin), as well as a reduction in the size of the bolus particles due to chewing (mastication). It has been suggested that these factors must be taken into account in the *in vitro* simulation of the oral phase (McClements and Li, 2010).

The process of oral digestion consists of several steps. Parameters and conditions for conducting those steps are shown in Table 1. The first step is usually the homogenization of the sample, which can be carried out in a laboratory blender. The purpose of this step is to simulate mastication and it is carried out in the presence of a simulated saliva solution. The pH of this solution varies in the range of 6.75 – 7.5, depending on the buffer used (Gawlik-Dziki et al., 2012; Laurent et al., 2007; Shim et al., 2012; Quatrin et al., 2020). Then, α-amylase is added to this solution. The activity of α-amylase is mostly expressed in units per millilitre (U/mL), where one unit represents the amount of amylase that will liberate 1 mg of maltose from starch under the given conditions (3 minutes, 20 °C, and pH 6.9) (Alminger et al., 2014). Different authors, depending on the substrate and model they worked on, used different amounts of α-amylase and the values ranged from 75 to 200 U/mL (Gawlik-Dziki, 2012; Lin et al., 2019). The next step is the incubation of the solution at 37 °C, and the incubation period (digestion) studied in literature ranges from 0.5 to 10 minutes (Bergantin et al., 2017; Gawlik-Dziki, 2012; Laurent et al., 2007; Shim, 2012; Tagliazucchi et al., 2012).

The influence of the oral phase saliva on polyphenols was investigated by Ginsburg et al. (2012). Saliva has been shown to have an effect on the dissolution of polyphenols present in fruit and fruit drinks, resulting in an increased availability of lipophilic polyphenols and their longer retention in the mouth (Ginsburg et al., 2012). It is also possible that salivary proteins could potentially serve as a screening mechanism that allows the absorption of flavonoids and at the same time neutralizes the less desirable effects of tannins (Bennick, 2002).

Gastric phase simulation

Food digestion in the stomach is a complex process that involves the mechanical degradation of food as well as enzymatic digestion, and this phase is crucial for evaluating the bioaccessibility of polyphenols. Gastric juice is secreted in large quantities (approximately 2 litres per day) by the human stomach. It is mainly composed of water (99%), hydrochloric acid, enzymes, and mucoproteins (Blanco et al., 2017).

Prior to digestion in the stomach, solids should be reduced to the optimum size (1-2 mm), which is done by peristaltic waves originating from the stomach (Kong and Singh, 2010). Upon arrival in the stomach, the food increases the pH of the stomach from an initial 1.3 - 2.5 to over 4.5, depending on the buffering capacity of the food. In the next 3-4 hours (the usual time food remains in the stomach) the pH drops to 1.8 - 2.9 (Alminger et al., 2014). The time required for gastric emptying depends on several factors such as volume, viscosity, and pH. Liquid food leaves the stomach in proportion to its volume, while solid food stays in the stomach for a longer time (Schulze, 2006). Parameters and conditions of the gastric phase in the simulated digestion process are shown in Table 2. Gastric digestion is simulated by pepsin, which causes the hydrolysis of homogenized samples over a period of time. Homogenization of the sample is carried out in a laboratory blender (Bouayed et al., 2011; Tagliazucchi et al., 2011), unless oral phase simulation has been performed previously. The homogenization was followed by the addition of simulated gastric fluid, and the amount varied depending on the amount of substrate and the model used (Bouayed et al., 2011; Cilla et al., 2011; Gawlik-Dziki, 2012; Gil-Izquierdo et al., 2003; McDougall et al., 2005b; Tagliazucchi et al., 2012). The simulated gastric fluid was mostly composed of pepsin, while NaCl was used in some studies as well (Gawlik-Dziki, 2012; Tagliazucchi et al., 2011; Bouayed et al., 2011). Most commonly reported values for pepsin concentrations were around 300 U/mL of simulated fluid (Gawlik-Dziki, 2012; Tagliazucchi et al., 2011; Cilla et al., 2011; McDougall et al., 2005b; Bermudez-Soto et al., 2007) (one unit will cause a change in absorbance of 0.001 at 280 nm and 37 °C, in one minute and pH 2.0, with haemoglobin as substrate (Alminger et al., 2014)), while NaCl values ranged from 1.75 g/L – 7.25 g/L (Bouayed et al., 2011; Gawlik-Dziki, 2012). The targeted pH depends on the model and ranges from 1.2 to 3 (Bergantin et al., 2017; Bouayed et al., 2011; Cilla et al., 2011; Gawlik-Dziki, 2012; Gil-Izquierdo et al., 2003; McDougall et al., 2005b; Tagliazucchi et al., 2011), which corresponds to the human stomach in a fasting state (Bouayed et al., 2011; Cilla et al., 2011; Gil-Izquierdo et al., 2003) and is achieved by the addition of concentrated hydrochloric acid. Some authors have considered an "intermediate step" of digestion and raised the pH to 4 (Dhuique-Mayer et al., 2007; Reboul et al., 2006). The samples were then incubated at 37 °C. The period of incubation is determined according to the time required for gastric emptying and ranges from 1 to 2 hours (Bouayed et al., 2011; Cilla et al., 2011.).

Table 1. Parameters and conditions in oral phase simulated digestion

Polyphenol source	Sample amount	pH	Salivary fluid (mL)	α-amylase	Other components	Time (min)	Temperature (°C)	References
grape seed flavonoids	NS*	6.9	NS	1000-1500 U/mg protein	NU	10	37	Laurent et al., 2007
peach, plumes, prunes, walnuts, tomatoes	10 g	6.9	5	150 U/mL	1.336 mM $CaCl_2$ 0.174 mM $MgSO_4$ 12.8 mM KH_2PO_4 23.8 mM $NaHCO_3$ Casein 2 g/L	10	37	Tagliazucchi et al., 2011
tomato, onion, garlic, lettuce	10 g	6.75	5	200 U/mL	16.76 mM Na_2HPO_4 1.396 mM KH_2PO_4 136.89 mM NaCl Mucin 100 mg/L	10	37	Gawlik-Dziki, 2012
Smilax china (wild vine shrub)	0.5 g	6.9	NS	NS	NU	5	37	Shim, 2011
red chicory	3 g	NS	3.5	150 U/mL	$CaCl_2$	0.5	37	Bergantin et al., 2017
red grapes and corresponding wines	1g / 2 mL	NS	2	NU	NU	0.5	NS	Lingua et al., 2018
quercetin-fortified bread products	5g	NS	4	75 U/mL	7.5 mM CaC_2	2	37	Lin et al., 2019
jaboticaba fruit peel	5 g	7.5	NS	75 U/mL	NU	2	37	Quatrin et al., 2020

NS- Not specified; NU - Not used

Table 2. Parameters and conditions in gastric phase simulated digestion

Sample	Sample amount	pH	Gastric fluid (mL)	Pepsin	NaCl (g/L)	Time (h)/ Temperature (°C)	References
Polyphenol source							
orange juice	100 mL	2	NS	315 U/mL	NU	2 / 37	Gil-Izquierdo et al. 2001.
pomegranate juice	50 mL	2	NS	320 U/mL	NU	2 / 37	Pèrez-Vicente et al., 2002
tomato, onion, garlic and lettuce	10 g	1.2	15	300 U/mL	1.75	1 / 37	Gawlik-Dziki, 2012
peach, plums, prunes, walnut, tomatoes	10 g	2	15	315 U/mL	2	2 / 37	Tagliazucchi et al., 2011
apple varieties	10 g	2-2.5	62	2.5 mg/mL	7.25	1 / 37	Bouayed et al., 2011
Hibiscus sabdariffa L	1 g	1.5 - 2	225	403.15 U/g	NU	2 / 37-40	Villanueva-Carvajal et al., 2013
grape seed extract	1 mL	2	0.05	1125 U/mL	NU	1	Laurent et al., 2007
8 fruit beverages	80 g	2	NS	243.75 U/g	NU	2 / 37	Cilla et al., 2011
red wine anthocyanins	20 mL	1.7	NS	315 U/mL	NU	2 / 37	McDougall et al., 2005b
green tea	20 mL	2	3	5.2 mg/mL	NU	1 / 37	Green et al., 2007
chokeberry extract	50 mL	2	NS	315 U/mL	NU	2	Bermudez-Soto et al., 2007.
raspberry anthocyanins	20 mL	1.75	NS	315 U/mL	NU	2 / 37	McDougall et al., 2005a
red chicory	3 g	3	7.5	2666 U/mL	NU	2 / 37	Bergantin et al., 2017
red grapes and corresponding wines	1g / 2 mL	2	NU	450 U/g/mL	NU	2 / 37	Lingua et al., 2018
quercetin-fortified bread products	3 g	3	8	2000 U/mL	NU	2 / 37	Lin et al., 2019
jaboticaba fruit peel	5 g	3	NS	2000 U/mL	NU	2 /37	Quatrin et al., 2020
Carotenoid source							
citrus juice	30 ml	4	2	40 mg/mL	NU	0.5 / 37	Dhuique-Mayer et al., 2007
main dietary sources	NS	4	2	40 mg/mL	NU	0.5 / 37	Reboul et al., 2006

NS- Not specified; NU - Not used

Small intestine phase simulation

Most of the enzymatic digestion and absorption of nutrients from the ingested food takes place in the small intestine. The acidic chyme, a bolus mixed with gastric juices, enters the small intestine where it needs to be neutralized for optimal pancreatic enzyme activities. For this purpose, the pancreas secretes sodium bicarbonate ($NaHCO_3$), which causes the pH to increase to that of neutral. Most enzymes that work in the small intestine are secreted by the pancreas and liver, and reach the small intestine via the pancreatic duct. Pancreatic enzymes (proteases, lipases, and amylases) work together with other digestive enzymes (such as maltase, lactase, and peptidase) produced by the brush border (a microvillus membrane on the surface of the small intestine) in the degradation of nutrients. The liver, apart from enzymes, secretes bile that is stored in the gallbladder. The role of bile is to emulsify triglycerides, which are hydrophobic, to make lipase, which is hydrophilic, available (Alminger et al., 2014; Blanco et al., 2017). The key parameters for *in vitro* simulation of digestion in the small intestine are the pH, temperature, time, and the amount and composition of simulated small intestine fluids (electrolytes, bile, and enzymes). Those parameters are shown in Table 3. Basically, all the models are similar, with slight modifications regarding the pH values, the composition of simulated small intestine fluid (Bouayed et al. 2011; Cilla et al. 2011; Gil-Izquierdo et al. 2003; McDougall et al. 2005a; Tagliazucchi et al. 2012), and whether cellulose dialysis tubing is implemented to simulate intestinal absorption (Bouayed et al., 2011; Gil-Izquierdo et al., 2001; Pérez-Vicente et al., 2002). The first step of the simulation itself is the neutralization of the results of gastric digestion. This is done by adding sodium bicarbonate ($NaHCO_3$) or sodium hydroxide (NaOH). After the pH raises to the desired value (ranging between 5 – 7.5), simulated small intestine fluid can be added (Bouayed et al., 2011; Cilla et al., 2011; Gil-Izquierdo et al., 2003; Lingua et al., 2018; McDougall et al., 2005a; Tagliazucchi et al., 2012). Simulated small intestine fluid contains a mixture of pancreatin and bile extract, while a few authors used NaCl and KCl salts (Bouayed et al., 2011; Gawlik-Dziki, 2012; Laurent et al., 2007). Pancreatin values ranged between 0.16 g/L – 4 g/L (Bermudez-Soto et al., 2007; Villanueva-Carvajal et al., 2013), while most common bile values were 12 and 25 g/L of simulated small intestine fluid (Bermudez-Soto et al., 2007; Bouayed et al., 2011; Gil-Izquierdo et al., 2001; Laurent et al., 2007). Incubation is carried out at 37 °C (Bouayed et al., 2011; Cilla et al., 2011; Gil-Izquierdo et al., 2003; McDougall et al., 2005a; Tagliazucchi et al., 2012). The final pH ranges between 7 and 7.5.

The main factor affecting the stability of polyphenols under intestinal conditions is pH. Most *in vitro* models have almost neutral pH and oxygen is present, and such conditions favour the degradation of certain phenolic compounds via non-enzymatic oxidation (Bergmann et al., 2009). Anthocyanins, which showed a high rate of disappearance in the intestinal phase, are particularly sensitive (Bermudez-Soto et al., 2007; McDougall et al., 2005a; Tagliazucchi et al., 2012). A study conducted on anthocyanins from pomegranate showed that recovery from intestinal digestion is significantly less if the acidification of digestion results is not carried out (18% vs. 70%) (Perez-Vicente et al., 2002). Therefore, anthocyanin analysis at a pH lower than 2 should be favoured.

It is worth mentioning that some authors used *in vitro* colonic fermentation to assess the bioaccessibility of polyphenols in the large intestine. Essentially, this method consists of mixing residues from intestinal digestion with certain volumes of faecal slurry and culture medium, and incubating at 37 °C for 24 or 48 hours. The faecal slurry is prepared by diluting faeces from healthy donors in phosphate or carbonate buffer (Chait et al., 2020; Quatrin et al., 2019).

In general, oral, gastric, and small intestine phases can be included in the determination of the bioaccessibility of polyphenols. The flow chart describing each step in the determination of polyphenol bioaccessibility is shown in Fig. 2. The oral phase is important, since the availability of polyphenols could potentially be increased at this stage. The most common duration of the oral phase was 10 minutes, but under *in vivo* conditions, this phase is significantly shorter (from seconds to a minute). Therefore, the duration of the oral phase could be reduced to 5 minutes or even less. As for the gastric phase, the pH values ranged around 2. However, the arrival of food in the stomach causes an increase in pH, so the simulation of the gastric phase could be carried out at slightly higher pH values, which could provide more realistic conditions. Gastric emptying depends on the form of the sample, so the gastric phase could last 60 minutes for liquid samples and 120 minutes for solid ones. The parameters for the small intestine phase simulation are fairly uniform. However, the neutral pH of this phase could cause difficulties in the identification of some polyphenols after digestion. The example are anthocyanins, which are usually identified in acidic pH. So, the acidification of digestion results could enable better quantification of anthocyanins after digestion. The temperature at all stages of the digestion simulation should be 37 °C, which corresponds to the temperature of the human body.

Table 3. Parameters and conditions in small intestine phase simulated digestion

Polyphenols source	sample amount	Dialyse tube	Initial pH/ final pH	pH increasing compound	small intestine fluid (mL)	Pancreatin concentration	Bile	Salts	Time (h)/ Temperature (°C)	Reference
orange juice	100 mL	Yes	5/7.5	$NaHCO_3$	5	4 g/L	25 g/L	NU	2 / 37	Gil-Izquierdo et al., 2001
pomegranate juice	20 mL	Yes	5/7.5	$NaHCO_3$	5	4 g/L	Not used	NU	2 / 37	Pèrez-Vicente et al., 2002
tomato, onion, garlic and lettuce	10 g	No	6/7	$NaHCO_3$ NaOH	15	1.43 g/L	8.57 g/L	5 mL of 120 mM NaCl 5 mL of 120 mM KCl	2	Gawlik-Dziki, 2012
peach, plums, prunes, walnut, tomatoes	10 g	No	7.5	$NaHCO_3$	NS	0.8 g/L	5 g/L	NU	2 / 37	Tagliazucchi et al., 2011
apple varieties	10 g	Yes	6.5/7-7.5	$NaHCO_3$	1	2 g/L	12 g/L	5.5 mL of 150 mM NaCl	2 / 37	Bouayed et al., 2011
Hibiscus sabdariffa L	1 g	No	6.5 - 7	$NaHCO_3$	25	0.16 g/L	1 g/L	NU	2/ 37	Villanueva-Carvajal et al., 2013
grape seed extract	1 mL	No	6 / 7	$NaHCO_3$ NaOH	0.25	2 g/L	12 g/L	120 mM NaCl 5 mM KCl	2	Laurent et al., 2007
8 fruit beverages	80 g	No	6.5 / 7.2	$NaHCO_3$ NaOH	NS	0.4 g	2.4 g	NU	2	Cilla et al., 2011
red wine anthocyanins	20 mL	Yes	7	$NaHCO_3$	4.5	4 g/L	25 g/L	NU	2 / 37	McDougall et al., 2005b
green tea	20 mL	No	5.3 / 7.2	$NaHCO_3$ NaOH	9	2 mg/L	12 g/L	NU	2 / 37	Green et al., 2007
chokeberry extract	20 mL	No	7	$NaHCO_3$	5	4 g/L	25 g/L	NU	2	Bermudez-Soto et al., 2007
raspberry anthocyanins	NS	Yes	7	$NaHCO_3$	4.5	4 g/L	25 g/L	NU	2 / 37	McDougall et al., 2005a
red chicory	3 g	No	7	NaOH	11	1.14 g/L	0,142 g/L	NU	2 / 37	Bergantin et al., 2017
red grapes and corresponding wines	1g / 2 mL	Yes	7.5	$NaHCO_3$	NU	1.2 mg/g/mL	5.6 mg/g/L	NU	2 / 37	Lingua et al., 2018
quercetin-fortified bread products	3 g	Yes	7	NaOH	NS	100 U/mL	10 mM	NU	6 / 37	Lin et al., 2019
jaboticaba fruit peel	5 g	Yes	7	NaOH	NS	100 U/mL	10 mM	NU	2/37	Quatrin et al., 2020

NS- Not specified; NU - Not used

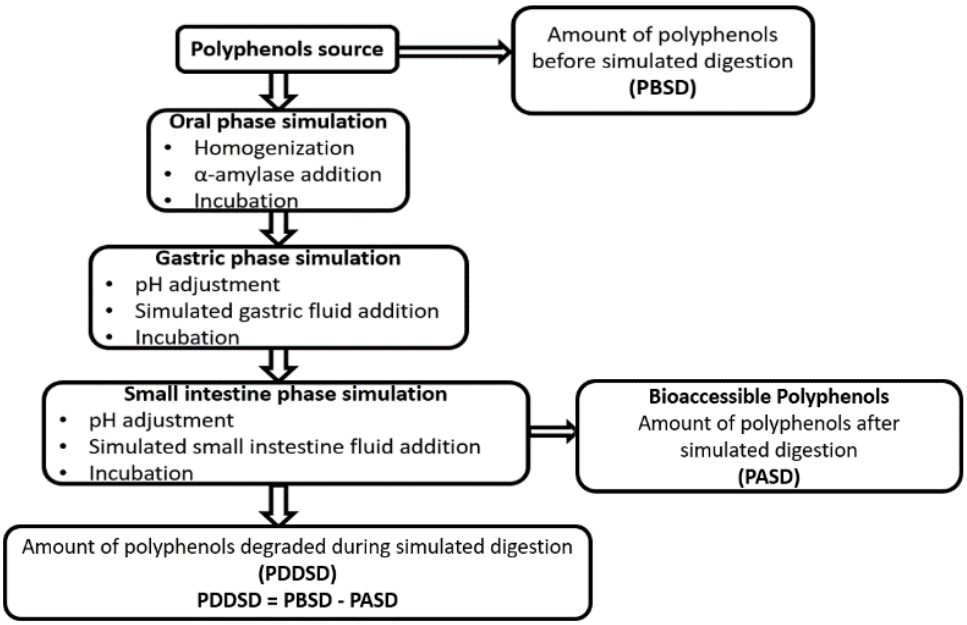

Fig. 2. Flow chart for the determination of bioaccessible polyphenols

However, the major disadvantage of the *in vitro* static GI method is its inability to reproduce the dynamic environment of the intestine. The complexity of the human digestion system, as well as the effect of the individual's physiological state, nutritional status, age, and similar, cannot be taken into account by this method (Alegría et al., 2015). In a recent review, Bhon et al. proposed some concerns that could hinder the predictability of the bioavailability of *in vivo* polyphenols using *in vitro* digestion models. For instance, a very small number of models use the colonic fermentation step where polyphenols are heavily metabolized. Furthermore, covalently bound polyphenols are not extractable by chemical means and may not be released in the gastric/small intestine phase (Bohn et al., 2018). Nevertheless, *in vitro* static GI methods can be useful for the assessment of the influence of certain factors like food structure, food composition, interactions between food components, and food processing upon polyphenol bioaccessibility.

Conclusion

The *in vitro* static gastrointestinal method represents a reliable, practical, and economical way to simulate digestion and to determine bioaccessible polyphenols. It consists of oral, gastric, and small intestine phases. In the oral phase, the main steps are the homogenization of the sample, the addition of simulated saliva solution with α-amylase (pH 6.75 – 6.9), and incubation. The gastric phase consists of the addition of simulated gastric fluid which contains pepsin and a period of incubation at pH 1.2 – 2.5. The small intestine phase includes neutralization, the addition of simulated small intestine fluid which contains pancreatin and a bile salt mixture, and incubation (initial pH 5 – 6-5, final pH 6.5 – 7.5). This methodology is rapid and simple, and its major advantage is its reproducibility, since precise control of digestion conditions is possible.

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