***In vitro* evaluation of the kinetics of the release of phenolic compounds from
guava (*Psidium guajava* L.) fruit**

Short title: Bioaccessibility of phenolic compounds from *Psidium guajava* L

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ABSTRACT

Psidium guajava L. is a source of phenolic compounds (PC). The aim of this study was to evaluate the bioaccessibility and kinetics of the release of PC from guava fruit by using an *in vitro* digestion model. Similar percentages of bioaccessibility were observed in whole guava (WG) (64.79%) and seedless guava (SG) (67.69%), and the release rates of PC were 10.55 and 8.70 mg/min, respectively. Gallocatechin was the major compound identified in the bioaccessible fraction, and guavinoside B was the major component in the non-bioaccessible fraction. Procyanidin B and guajaverin were detected for the first time in WG and SG. These data show that PC are significantly released from the food-matrix in both samples, allowing them to be absorbed in the small intestine. Higher proportion of certain PC (guavinoside A or guavinoside B) was observed in the non-bioaccessible fraction of WG, which could be related with different biological effects.

KEYWORDS: Phenolic compounds, bioaccessibility, *in vitro* digestion, release kinetics, *Psidium guajava* L.

1. Introduction

Currently, the consumption of tropical fruits is increasing due to their desirable sensorial attributes and nutritional qualities. Indeed, they are an important source of both dietary fiber and several bioactive compounds that have been associated with different health-related properties (Maria do Socorro et al., 2010). Such is the case of guava (*Psidium guajava* L). The peel and pulp of this fruit are an excellent source of vitamins (A, C, thiamine, niacin, and riboflavin), minerals (phosphorus, calcium, and iron) (Medina & Pagano, 2003), dietary fiber (Martínez et al., 2012) and phenolic compounds (PC) (Mahattanatawee et al., 2006), including gallic acid, ellagic acid, and quercetin (Jiménez-Escrig, Rincón, Pulido, & Saura-Calixto, 2001). Most of the PC identified in guava pulp have also been detected in guava leaves; such compounds include the hydrolyzable tannins, guavin A, guavin B and quercetin (Díaz-de-Cerio, Gómez-Caravaca, Verardo, Fernández-Gutiérrez, & Segura-Carretero, 2016). Guava is consumed fresh and it is common to remove its seeds, which may constitute up to 10% of total weight. This may affect the amount and type of PC ingested.

Moreover, to show *in vivo* health effects, the PC present in guava first need to be bioaccessible. Bioaccessibility is defined as the amount of a food component that can cross the intestinal barrier as a result of its release from the food matrix (Shim, Ferruzzi, Kim, Janle, & Santerre, 2009). The interactions of PC with the food matrix are one of the factors that can delay or decrease their release during digestion, affecting their potential bioavailability (Sengul, Surek, & Nilufer-Erdil, 2014). It should be remarked that discarding seeds from guava also implies the removal of a fraction of the connective tissue around the seeds, mainly composed of soluble

polysaccharides such as galactomannans (Soukoulis, Gaiani & Hoffman, 2018). Since there are evidences of the interactions between carbohydrates present in the food matrix and PC, affecting the metabolic fate of the latter (Bouayed, Deußner, Hoffmann, & Bohn, 2012; Chandrasekara & Shahidi, 2012), the consumption of guava with or without seeds may affect not only the amount and type of PC but also their bioaccessibility during the different digestion steps.

Several *in vitro* digestion models have been suggested for emulating the physiological conditions of digestion; they provide a useful alternative to animal and human models by rapidly screening food ingredients (Alminger et al; 2014). Although they are a previous step for *in vivo* assays, there are good correlations between results for PC release obtained by *in vitro* digestion models or by *in vivo* assays (Bohn et al., *in press*). The use of these systems has revealed different molecular interactions affecting the bioaccessibility of PC in different matrices, such as complete and residues of decoction of *Hibiscus* calyces, red grapes, mango byproducts, strawberry or juçara fruit (*Euterpe edulis* Martius) (Tagliazucchi et al., 2010; Blancas-Benitez et al., 2015; Mercado-Mercado et al., 2015; Ariza et al., 2016; Schulz et al., 2017). However, the bioaccessibility of the PC present in guava fruit has not been explored in detail.

Hence, the aim of this study was to use an *in vitro* digestion procedure to determine the bioaccessibility and kinetics release of PC from whole and seedless guava fruit, including the identification of individual PC, in order to elucidate whether the mode of consuming this fruit could affect the profile or the bioaccessibility of PC.

2. Materials and methods

2.1 Sample preparation

Guava fruits (*Psidium guajava* L.) were obtained from a local market in Tepic, Nayarit, Mexico and were divided into two batches of 30 fruits. The first batch corresponded to whole guava (WG) (pulp, peel and seeds), and the second one to seedless guava (SG) (pulp and peel). In order to ensure sample homogeneity, both samples groups were freeze-dried (LABCONCO, Freezone, USA), ground (Nutribullet, NBR-0804B, USA), sieved (0.5 microns), and then stored in sealed bags at -20 °C until analysis. Therefore, the freeze-dried samples of whole guava contain all parts of the fruit, including, pulp, peel and seed, while freeze-dried samples of seedless guava contain only pulp and peel of guava.

2.2 In vitro digestion model and bioaccessibility percentage (%) in whole guava and seedless guava

Lyophilized samples (WG and SG) were submitted to an *in vitro* digestion model adapted from the methodology proposed by Saura-Calixto, Garcia-Alonso, Goñi, and Bravo (2000) (Figure 1). First, the samples were subjected to an enzymatic hydrolysis process with pepsin (300 mg/ml, P-7000, Sigma Aldrich) at a temperature of 37 °C for 1 h (step 1); the PC released to the supernatant at this stage were considered as the PC present in the gastric fraction (GasF). Subsequently, the samples were hydrolyzed with pancreatin (5 mg/mL, P-1750, Sigma Aldrich) for 6 h and α -amylase (120 mg/mL, A-6255, Sigma Aldrich) for 16 h, both at 37 °C (step 2). The PC released at this stage of digestion are considered as the PC present in the intestinal fraction (IntF). After the triple hydrolysis, samples were centrifuged (step 3) to separate the soluble and insoluble indigestible fractions. The supernatant was dialyzed (D9652, 12–14 KDa, Sigma

Aldrich) for 48 h to simulate passive absorption (step 4). After dialysis, the PC associated with the **soluble indigestible fraction (SIF)** was determined (step 5). The pellet formed during centrifugation, which contained the PC that was non-bioaccessible in the small intestine, was used to determine the PC associated with **insoluble indigestible fraction (IIF)** after an organic extraction (Pérez-Jiménez et al., 2008) (step 6). Both the PC associated with the SIF and IIF corresponded to the fraction of PC that was not bioaccessible in the small intestine. **In this study, we focused on PC released during the gastric and small intestine stages, and due to the lower release of these compounds at mouth stage, it was not considered (Williamson & Clifford, 2017).**

The **total soluble polyphenol (TSP)** contents of the different fractions GasF, IntF, SIF, and IIF were determined, and the samples were analyzed by HPLC/MS as described below. The *in vitro* **bioaccessibility percentage (%BA)** of PC was determined using Eq. 1:

$$\% \text{ BA} = \frac{(\text{PC-IntF}) - (\text{PC-SIF}) \times 100}{(\text{PC-IntF}) + (\text{PC-IIF})} \quad (1)$$

Where PC-IntF= the PC released on the intestinal fraction, PC-SIF= the PC associated with the soluble indigestible fraction, and PC-IIF= the PC associated with the insoluble indigestible fraction.

2.3 *In vitro* kinetics of the release of phenolic compounds (PC) in whole guava (WG) and seedless guava (SG)

The *in vitro* kinetics of PC release from WG and SG were determined according to an *in vitro* digestion method (Blancas-Benitez et al., 2015). Briefly, 300 mg of dried sample was weighed and combined with 10 mL of phosphate buffer (0.05 M, pH

1.5) and 0.2 mL of pepsin solution (300 mg/mL, P-7000, ≥250 units/mg, Sigma Aldrich), and the solution was incubated at 37 °C for 1 h. Afterwards, phosphate buffer (4.5 mL, 0.05 M, pH 6.9) was added, and the samples were transferred to cellulose dialysis bags (D9652, 12–14 KDa, Sigma Aldrich). One milliliter of pancreatic α-amylase (120 mg/mL, A-6255, 110 units/mg, A6255, Sigma) was added to each dialysis bag, the samples were adjusted to a volume of 30 mL, and the dialysis tubes were sealed. The tubes were placed in a glass vessel with 200 mL of phosphate buffer (0.05 M, pH 6.9) that had previously been stabilized at 37 °C. The samples were incubated for 3 h with continuous stirring. At 30 min intervals, 1 mL extracts of the liquid containing the dialyzed compounds were taken and used for the analysis of the TSP using HPLC/MS, as described in the following section. To calculate the kinetic parameters of PC release during the *in vitro* digestion, the **final rate (Vf)** of PC release was determined according to Eq. 2:

$$Vf = \Sigma \left(\frac{\Delta C}{\Delta t} \right) \quad (2)$$

Where ΔC is the difference in concentration between the final and the initial PC concentration, Δt is the time difference between a specific time and the initial time, and Vf is the final rate of PC release during the *in vitro* digestion (mg GAE/min).

2.3.1 Total soluble polyphenols (TSP) content in the digested guava fractions

TSP contents were quantified in all digested fractions of WG and SG from the *in vitro* digestion assay and at each point in the release kinetics assay; this analysis was conducted according to the method described by Montreau (1972) with slight modifications. Next, 250 μL aliquots of each fraction (*in vitro* digestion or kinetics

assay) were mixed with 1000 μ L of sodium carbonate (7.5% w/v) and 1250 μ L of Folin-Ciocalteu's reagent. Afterward, the absorbance of each sample was measured at 750 nm using a 96-well microplate reader (Bio-Tek®, Synergy HT, Winooski, VT, USA) with Gen5 software. Gallic acid was used as the standard (0.0125–0.2 mg/mL, $R^2 \geq 0.9997$), and the results are expressed as mg of gallic acid equivalents (g GAE/100 g DW).

2.3.2 Identification of phenolic compounds (PC) by HPLC-DAD-MS analysis in digested guava fractions.

The identification of the PC was carried out using an HPLC Agilent 1260 series system (Agilent Technologies, Santa Clara, CA, USA) equipped with a UV–Vis diode array detector (DAD). Samples were injected (10 μ L, flow rate 0.4 mL/min) into a Poroshell 120 EC-C18 column (4.6 mm $\times$ 150 mm, particle size 2.7 μ m) (Agilent Technologies). The gradient elution was carried out using water containing 0.1% trifluoroacetic acid (Sigma Aldrich) as solvent A and acetonitrile (Sigma Aldrich) as solvent B and was applied as follows: 0 min, 5% B; 10 min, 23% B; 15 min, 50% B; 20 min, 50% B; 23 min, 100% B; 25 min, 100% B; 27 min, 5% B, 30 min, 5% B. Detection was performed at 280–320 nm. For the MS analysis, a 6120 Agilent Quadrupole LC/MS equipped with an electrospray ionization interface was used in negative ionization mode with the following conditions: drying gas flow (N_2), 13.0 L /min; nebulizer pressure, 40 psi; gas drying temperature, 350 $^{\circ}$ C; and capillary voltage, 3500 V. The data analysis was performed using OpenLab CDS ChemStation Edition software (Agilent Technologies, Santa Clara, CA, USA). Characterization of the PC was based on retention time and mass spectrometric data. The compounds were first detected using a single MS scan in the 100 to

1100 *m/z* range followed by a targeted search based on the peaks showing major signals in the UV–Vis chromatograms.

2.4 Statistical Analysis

A completely randomized experimental design was used, in order to compare WG and SG fruit samples. All analyses were performed in triplicate, and the mean and standard deviation from each determination were calculated. Data were analyzed using the Student's T-test and the Statistic 8.0 Release software for Windows (Stat Soft. Inc., Tulsa, OK, USA) with a significance level of $\alpha=0.05$.

3. Results and discussion

3.1 *In vitro* digestion and bioaccessibility percentage (%) in WG and SG

PC released from food matrices is a key aspect of the biological relevance of polyphenols. In this study, PC *in vitro* release of PC was evaluated followed by the determination of TSP and PC profile at each digestion stage. Previous studies reported a general agreement between results obtained by such approaches and those from *in vivo* studies (Bohn et al., 2017). The PC released during the digestion of the WG and SG samples in the present study are shown in Table 1. No significant differences ($p>0.05$) were observed between the samples during gastric digestion. During the *in vitro* digestion process, a significant release of PC in the GasF was observed, and values up to 15 g GAE/100 g DW were seen. This result could be due to the partial release of PC bound to the cell walls of the fruit matrix (Bouayed, Hoffmann, & Bohn, 2011). At this stage, pepsin digestion in conjunction with the low pH in the stomach and the peristaltic movements, significantly favor reducing the particle size of the food matrix and enhancing the release and solubilization of the PC (Chen et al., 2014).

In contrast, in the IntF, significant differences ($p < 0.05$) in the amount of TSP released from the food matrix were observed between WG and SG, and the highest values were observed for SG (Table 1). The release of PC in this stage, which mimics the small intestine are affected by changes in pH and the action of the pancreatine and α -amylase enzymes. Both factors are known to enhance breakdown of macromolecules weakening the interactions between carbohydrate and PC, increasing its bioaccessibility (Pekkinen et al., 2014). The *in vitro* digestion model used on this study does not consider the interaction of the food matrix with the microbiota present in the upper digestive tube. This was considered as a valid approach since PC is known to be transformed mostly by the colonic microbiota. Indeed, although *in vitro* studies using ileostomy fluid suggested some biotransformations by enteric bacteria (Schantz, Erk & Richling, 2010), they may be considered as marginal as compared with colonic transformations (Williamson & Clifford, 2017).

The non-bioaccessible fraction comprises the SIF and IIF. After the dialysis process, the contents of PC in the SIF were not significantly different between the WG and SG samples ($p > 0.05$); the difference was approximately 5%. SIF corresponds to the PC that was not able to cross the dialysis membrane; these compounds can be associated with dietary fiber from the food matrix or are PC with high molecular weights (González-Aguilar, Blancas-Benítez, & Sáyago-Ayerdi, 2017). These values were higher than those observed in the IIF, which was 1.73% for WG and 1.51% for SG. The IIF corresponds to the residue of the food matrix and contains insoluble dietary fiber, hydrolyzable polyphenols and other insoluble compounds (Saura-Calixto, 2011) (Table 1). Nevertheless, this fraction of PC,

which are non-bioaccessible in the small intestine in the system tested here, could become more bioaccessible in two different ways: through release due to the action of the intestinal enzymes on glycosides that releases the corresponding aglycones that can be taken up by passive diffusion into the enterocytes (Chandrasekara & Shahidi, 2012) or through further transformation by colonic microbiota or the release of absorbable metabolites (Williamson & Clifford, 2017). The lowest PC values were detected in the IIFs in both samples. These polyphenols were not bioaccessible at all in the small intestine. However, they could become bioaccessible in the large intestine after fermentation by the microbiota, which is a key step in the metabolic fate of polyphenols (Williamson & Clifford, 2017). The study of each of the indigestible fractions, both soluble and insoluble, is of great importance because the compounds associated with these indigestible fractions are not absorbed in the small intestine, reaching the colon where is fermented by the microbiota producing short chain fatty acids such as acetic, propionic and butyric acids and other that may exert different beneficial effects (Saura-Calixto, 2011, Sáyago-Ayerdi, Zamora-Gasga & Venema, *in press*). Based on the release of PC in the different stages of *in vitro* gastrointestinal digestion and considering the PC associated with SIF and IIF, the bioaccessibility was calculated between 64-68% in both samples (Table 1). This result seems to indicate that the presence of seeds in guava does not affect PC bioaccessibility. Similarly, food matrix differences in mango peel and pulp did not affect the bioaccessibility of PC (Blancas-Benitez et al., 2015). While in other products, such as *Hibiscus*, the bioaccessibility of PC in decoction residues was 2.7-fold higher than that in complete calyces (Mercado-Mercado et al., 2015). Interestingly, of

these examples, the bioaccessibility values of the PC were similar to those found in this study for guava only in the decoction residue; in the other examples, the bioaccessibilities were much lower.

3.2 Evaluation of the bioaccessibility of individual PC in WG and SG

A fundamental part of the study of the bioaccessibility of bioactive compounds involves not only quantifying the total amounts of the compounds but also determining the types of PC that are bioaccessible. Table 2 shows the PC profiles determined at each stage of the *in vitro* digestion process for whole and seedless guava fruit. The major PC identified in all stages of the *in vitro* digestion were flavonoids and tannins. In particular, the following PC were identified in the GasF and IntF both in WG and SG: quercetin, guavinoside C and guajaverin (quercetin derivatives), gallocatechin, the ellagitannin pedunculagin, prodelphinidin dimer and procyanidin B. These compounds have been related with several health-related properties, such as gallocatechin or ellagitannins (Cerdá, Tomás-Barberán, & Espín, 2005; Holt, Heiss, Kelm, & Keen, 2012) reported in guava fruits. In this study, procyanidin B and guajaverin were identified by the first time in guava fruit. Procyanidins are one of the classes of PC with the highest evidences of cardiometabolic beneficial effects, including clinical trials (González-Abuín, Pinent, Casanova-Martí, Arola, Blay, & Ardévol, 2015). Interestingly, procyanidin B was recently identified in sour guava (*Psidium friedrichsthalianum*) by the first time (Cuadrado-Silva, Pozo-Bayón & Osorio, 2017) and guajaverin (quercetin 3-arabinoside) previously identified in leaves and stems of the guava tree (Díaz-de-Cerio et al., 2016), which was reported to be an antiplaque agent (Prabu, Gnamani & Sadulla, 2006).

Regarding the differences between the PC profile in GasF and IntF, it was found that gallic acid, guavinoside A and guavinoside B were only found in the GasF, while naringenin appeared mostly in the IntF. This indicates that, while gallic acid could be completely released from the food matrix at the gastric step. Other PC needs the action of intestinal enzymes in order to be released. Recent studies employing *in vitro* digestions in samples such as strawberry or the tropical juçara fruit, showed that PC profile varies during the different digestion steps (Ariza et al., 2016; Schulz et al., 2017). Also, it has been reported that some PC such as anthocyanins and other flavonoids are unstable in intestinal conditions, and their contents may decline by up to 43% during digestion (Bermúdez-Soto et al., 2007). Interestingly, the hydrolyzable tannin guavinoside A, here released in the intestinal step, has been reported to show hypoglycemic and hepatoprotective activity (Eidenberger, Selg, & Krennhuber, 2013). This property attributed to this compound can be added to the list of health benefits that have another PC present in guava fruit.

Overall, all these PC were present in both WG and SG, although there were also some compounds specific of one of the samples. For instance, naringenin and geraniin were not detected in the GasF of WG, but they were present in the other samples. Geraniin is a dehydroellagitannin that may be involved in the induction of apoptosis in human melanoma cells (Lee et al., 2008). While naringenin is a flavanone that can promote moderate cytostatic activity against cancer cell lines (Kang, Youn, Hong, & Kim, 2011).

Regarding the non-bioaccessible fraction, guajaverin and procyanidin B, both at high concentrations were detected in WG and SG, indicating that a major fraction

of these compounds would reach the colon. Additionally, guavinoside A and B were detected in WG, while pedunculagin was identified in SG. The non-bioaccessible PC reach the colon where they may exert additional health effects either by increasing the antioxidant content of the intestinal lumen or by serving as a fermentable substrate for the colonic microbiota and subsequently releasing absorbable and beneficial metabolites (Dall'Asta et al., 2012).

Overall, the differences observed among the samples analyzed indicate that the potential effects of guava consumption with or without seeds can depended more on the PC that can be released in each stage of digestion and on the type of non-bioaccessible compounds potentially biotransformed by the microbiota. Than on the different types of PC present on the sample. WG samples exhibited a relevant content of PC in the GasF and the IntF, but a higher proportion of certain PC (guavinoside A or guavinoside B) in the non-bioaccessible fraction, which could indicate potentially higher health-related effects.

3.3 *In vitro* kinetics of the release of PC in WG and SG

To get more information on the metabolic fate of PC present in guava, their kinetics release was assessed. This evaluation, complementary to bioaccessibility assessment, provides information on how long PC are expected to be released from food matrix after intake, being potentially absorbed and circulating in the bloodstream. Previous *in vitro* studies have obtained relevant information regarding this parameter (Blancas-Benítez et al., 2015) that may be used to evaluate potential absorption peaks in further *in vivo* assays.

No significant differences ($p>0.05$) in the release of PC from either WG or SG were observed (Figure 3). In both cases, a constant release of PC during the digestion

process was observed. The total release rates of PC in WG and SG were 10.55 mg/min and 8.70 mg/min, respectively (Figure 3). PC present in the fruit and released from the food matrix could potentially be absorbed throughout the gastrointestinal digestion processes.

The PC identified during the release kinetics assay were pedunculagin, an isomer of geranin, procyanidin B, guavinoside B, and guavin B (Table 3). Only guavinoside B was detected at each step of digestion in both samples. These results agree with the detection of these compounds during the *in vitro* evaluation of bioaccessibility, which confirms the release of these compounds from food matrices during digestion and subsequent intestinal absorption. The same types of PC were observed in each of the aliquots analyzed during the release kinetics studies; however, as the digestion process progresses and the kinetics change, the profiles of the identified PC change between each of the analyzed aliquots, which would indicate a release and potential gradual absorption of the compounds identified in the kinetics studies.

4. CONCLUSIONS

In this study, guava samples with or without seeds showed, by *in vitro* digestion experiments had a gradual kinetic release of PC and a similar bioaccessibility in the small intestine (about 70%). Nevertheless, there was a higher proportion of certain PC (guavinoside A or guavinoside B) in the non-bioaccessible fraction of WG that could indicate differential biological effects. Finally, the compounds procyanidin B and guajaverine, previously identified in leaves and stems of the guava tree, were detected in guava fruit by the first time indicating an additional benefit for the consumption of this fruit. The results obtained in this research indicate that the

consumption of the guava fruit, both with seed and without seed, can contribute to increase the consumption and assimilation of PC in the diet, and represents a basis for further studies on the effects of non-bioaccessible PC, and their potential health benefits on the colon.

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