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In vitro bioaccesibility and release kinetics of phenolic compounds from guava (*Psidium guajava* L.) and soursop (*Annona muricata* L.) pulp

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Abstract

Tropical fruits are known as healthy, guava and soursop, are considered as a source of phenolic compounds (PC), and are generally consumed fresh or in pulp. The beneficial effect attributed to fruit consumption is related not only to the amount, but rather to how much of these PC can be bioaccesible in the organism. Hence the aim of this study was to evaluate the bioaccesibility of PC of the guava and soursop pulp. During *in vitro* digestion process was observed that the highest release occurred during intestinal stage, which could be due to the partial release of PC associated with the cell wall material of the pulps. PC bioaccessibility values were 79.93% for guava and 83.91% for soursop, gallic and chlorogenic acids were mainly detected in both samples, although caffeic acid was detected only in soursop pulp. On the other hand, the release kinetics of PC from guava and soursop pulp shows a similar release rate in both, indicating that a large part of the PC present in these pulps are potentially bioaccessible and can be available to be absorbed by the small intestine.

Key words: bioaccesibility, *in vitro* digestion, polyphenols, *Psidium guajava* L., *Annona muricata* L.

Bioaccesibilidad y cinética de liberación *in vitro* de compuestos fenólicos en pulpas de guayaba (*Psidium guajava* L.) y guanábana (*Annona muricata* L.)

Resumen

Las frutas tropicales son conocidas como saludables, la guayaba y guanábana, se consideran ricos en compuestos fenólicos (CF), y generalmente se consumen frescos o en pulpa. El efecto beneficioso atribuido al consumo de frutas se relaciona con la cantidad de CF que pueden ser bioaccesibles en el organismo. El objetivo de este estudio fue evaluar la bioaccesibilidad de los CF de las pulpas de guayaba y guanábana. Durante el proceso de digestión *in vitro*, se observó que la liberación más alta se produjo durante la etapa intestinal, esto podría deberse a la liberación parcial de los CF asociada con el material de la pared celular de las pulpas. Los valores de bioaccesibilidad de los CF fueron 79.93% para guayaba y 83.91% para guanábana, los ácidos gálico y clorogénico fueron detectados como los principales CF presentes en ambas muestras, aunque el ácido cafeico fue detectado solo en la pulpa de guanábana, por su parte, la cinética de liberación de los CF en las pulpas mostraron una tasa de liberación similar en ambas muestras, lo que indica que una gran parte de los CF presentes en estas pulpas son potencialmente bioaccesibles y pueden estar disponibles para absorberse en el intestino delgado.

Palabras clave: bioaccesibilidad, digestión *in vitro*, polifenoles, *Psidium guajava* L., *Annona muricata* L.

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Introduction

Diets rich in tropical fruits have been associated with reduce develop of some diseases. An essential part of a healthy diet is the consumption of fruits, because it has been demonstrated that fruits can be an important source of some compounds known as bioactive compounds (BC) such as phenolic compounds (PC). Guava (*Psidium guajava*) and soursop (*Annona muricata*) are two tropical fruits that are a source of dietary fiber and PC, and the combination of these components may have an additive and/or synergistic effect in healthy properties (Ajila & Prasada Rao, 2013; Liu, 2003; Quirós-Sauceda *et al*., 2014). Guava and soursop are mainly consumed fresh, but also they are consumed as processed products, mainly as purees due to the consumers increase the demand of processed products that are easily accessible, ready to eat and provide health benefits (Pérez-Beltrán *et al*., 2017). Most studies are focused only in the quantification of PC from plant foods; while other studies highlight the effect of the combination or addition of fruit ingredients to processed foods to increase the content of BC that improve their functional properties (Blancas-Benítez, de Jesús Avena-Bustillos, Montalvo-González, Sáyago-Ayerdi & McHugh, 2015a; Kristl, Slekovec, Tojnko, & Unuk, 2011).

However, despite of the fact that the PC have shown important biological properties, including their strong *in vitro* antioxidant capacity (Abderrahim *et al*., 2015; Tang *et al*., 2015), their simple chemical identification and *in vitro* assessments can not necessarily is taken as a direct prediction of their real potential effect on human health. The simple quantification of PC do not include an assessment of the bioaccessibility of BC (Rein *et al*., 2013), since a compound can only exert health benefits if it remains available for absorption after all the phases involved in the gastrointestinal digestion process have taken place (Espín, García-Conesa, & Tomás-Barberán, 2007; Rein *et al*., 2013). The scientific literature contains several studies where the BC content of guava and soursop have been determined (Coria-Téllez, Montalvo-Gónzalez, Yahia, & Obledo-Vázquez, 2018; Jiménez-Escrig, Rincón, Pulido, & Saura-Calixto, 2001; Onyechi, Ibeanu, Nkiruka, Eme, & Madubike, 2012; Rojas-Garbanzo, Zimmermann, Schulze-Kaysers, & Schieber, 2017; Soares, Pereira, Marques, & Monteiro, 2007). However, there are no reports on the bioaccessibility of the PC presents in processed fruit products like, purees. Thus, the aim of this work was to determine, using an *in vitro* digestion procedure, the *in vitro* bioaccessibility and release kinetics of PC from guava and soursop pulp.

Materials and methods

Sample preparation

Guava (*Psidium guajava* L.) and soursop (*Annona muricata* L.) fruits were acquired from a local market in Tepic, Nayarit. The fruits were used in maturity stage they were transported to the Laboratorio Integral de Investigación en Alimentos, and were processed immediately to produce the purees. The guava was used only seedless, while soursop fruit was used the pulp without peel and seeds. The samples were homogenized (Ultraturrax, T25, IKAworks, Wilmington, NC) and were freeze-dried (Labconco Model 77522020, Kansas, USA) ground, sieved and stored hermetically for later use in analysis.

In vitro digestion model and bioaccessibility percentage (%) in guava and soursop pulp

Freeze-dried guava and soursop pulps were submitted to an *in vitro* digestion model adapted from the methodology proposed by Saura-Calixto, García-Alonso, Goñi, & Bravo (2000) with some modifications (Blancas-Benítez, Pérez-Jiménez, Montalvo-González, González-Aguilar & Sáyago-Ayerdi, 2018) (Figure 1). Firstly, the samples were subjected to an enzymatic hydrolysis process with pepsin (300 mg/mL, P-7000, Sigma Aldrich) (37 °C, 1 h) (**Step 1**, gastric fraction-GasF), pancreatin (5 mg/mL, P-1750, Sigma Aldrich) (37 °C, 6 h) and α-amylase (120 mg/mL, A-6255, Sigma Aldrich) (37 °C, 16 h) (**Step 2**, intestinal fraction-IntF). After the hydrolysis, samples were centrifuged (**Step 3**) to separate the soluble and insoluble indigestible fractions. The supernatants were dialyzed (D9652, 12–14 KDa, Sigma Aldrich, 48 h) to simulate passive absorption (**Step 4**). After dialysis, the PC associated with the soluble indigestible fraction (SIF) was determined (**Step 5**). The residue, after samples were centrifuged 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 correspond to the non-bioaccesible PC fraction. The total soluble polyphenols (TSP) content of the different fractions GasF, IntF, SIF, and IIF were determined, and the samples were analyzed by HPLC-DAD as described below. The *in vitro* bioaccessibility percentage (%BA) of PC was determined using Eq. 1:

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

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

In vitro release kinetics of PC in guava and soursop pulp

The in vitro released kinetics of PC from guava and soursop pulp were determined according to an *in vitro* digestion method (Blancas-Benítez *et al.,* 2015b) 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). The solution was incubated at 37 °C for 1 h, afterwards, phosphate buffer

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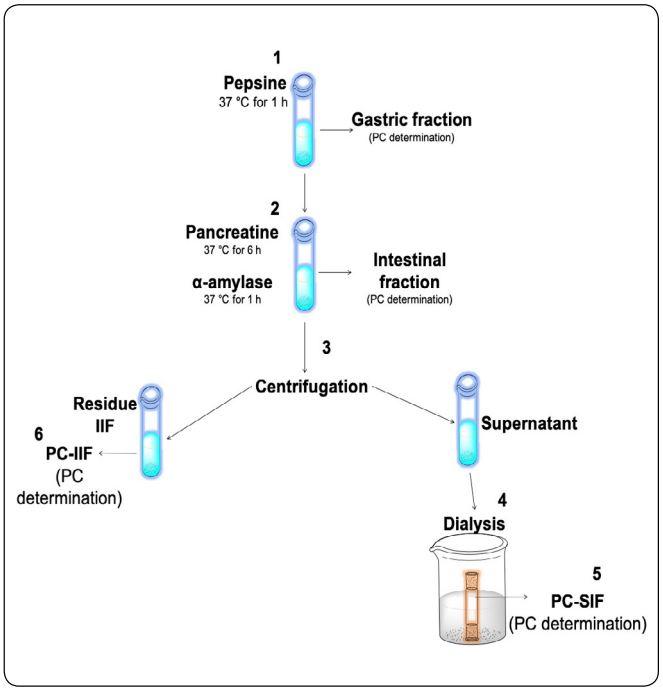

Figure 1. Bioaccesibility of phenolic compounds in guava and soursop pulp samples: Step 1 gastric fraction, Gas-F (Pepsine), Step 2 intestinal fraction, Int-F (Pancreatine and amylase), Step 3 centrifugation to separate supernatants and residues, Step 4 dialysis 24-48 h, Step 5 non-bioaccesible PC, associated to soluble indigestible fraction (SIF), Step 6 non-bioaccesible PC, associated to insoluble indigestible fraction (IIF).

(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 extract of the liquid containing the dialyzed compounds were taken and used for the analysis of the TSP using HPLC-DAD, 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:

$$Vf = \sum\left(\frac{\Delta C}{\Delta t}\right) \qquad (2)$$

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).

Total soluble polyphenols (TSP) content in the guava and soursop pulp digestion fractions

TSP contents were quantified in all digested fractions of guava and soursop 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, 250 µL aliquots of each fraction (*in vitro* digestion or kinetics assay) were mixed with 1000 µL of sodium carbonate (7.5% w/v) and 1250 µL 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 were expressed as mg of gallic acid equivalents (g GAE/100 g DW).

Identification of phenolic compounds (PC) by HPLC-DAD analysis on digested fractions of guava and soursop pulp

The identification of the PC was carried out using an HPLC Agilent 1260 series (Agilent Technologies, Santa Clara, CA, USA) equipped with a UV–Vis Diode Array Detector (DAD). Samples were injected (10 µL, flow rate 0.4 mL/min in a Poroshell 120 EC-C18 (4.6 mm × 150 mm, particle size 2.7 µm) (Agilent Technologies). The gradient elution was carried out using water containing 0.1% trifluoracetic acid (Sigma Aldrich) as solvent A and acetonitrile (Sigma Aldrich) as solvent B 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. DAD detection was performed at 280-320 nm. 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.

Antioxidant capacity (AOX)

All analyses of AOX were slightly modified to adjust in a microplate reader. Ferric reducing antioxidant power (FRAP) assay was performed as was described by Benzie & Strain (1996), modified by Álvarez-Parrilla, de la Rosa, Amarowicz & Shahidi (2010). FRAP solution 10:1:1 (v/v/v) dissolved in a sodium acetate buffer (0.3 M; pH 3.6), TPTZ-HCl (10 mM, 40 mM), and ferric chloride hexahydrated (20 mM) was warmed to 37 °C before mixing it with the samples. Briefly, 24 µL of sample from the aqueous-organic extraction was mixed with 180 µL of FRAP solution and the absorbance was measured at 595 nm after 30 min using microplate reader (Biotek, Synergy HT). Results were expressed as millimol of trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic) equivalents per gram DW (mmoL TE/g DW). Radical-scavenging activity was determined by 2,2′-azinobis- 3-ethylbenzotiazoline-6-sulphonic acid (ABTS) radical assay as was described by Re *et al.*, (1999); with some modifications. For this determination, ABTS (7 mM) was dissolved in potassium persulphate (2.42 mM) and kept in the dark at room temperature for 14 h. The

solution was adjusted with phosphate buffer at an absorbance of 0.70 (±0.02). Trolox was used as a standard and methanol as a blank. Samples of 10 µL of extract were added in a microplate reader (Biotek, Synergy HT, Winooski, VT, USA) of 300 µL of capacity and 280 µL of ABTS radical was added. Then, the mixture was incubated at 37°C in the dark and the absorbance was measured after 6 min, at 734 nm. A calibration curve was prepared using an aqueous solution of trolox as standard. The results are reported in mmoL TE/g DW.

Statistical analysis

All analyses were performed in triplicate; means values and standard deviations from each determination were calculated. Statistical significance between guava and soursop pulp was analyzed by t-student. Data were analyzed using the software Statistic 8.0 Release for Windows (Stat Soft. Inc., Tulsa, OK, USA) with a significance level of α=0.05.

Results and discussion

***In vitro* digestion and bioaccessibility percentage (%) in guava and soursop pulp**

Table I shows that the TSP of guava and soursop pulp in gastric and intestinal stages of an *in vitro* digestion. The PC in Gas-F from soursop pulp were higher than guava pulp. Nevertheless in the IntF of the digestion an increase in the content of TSP respect the GasF was observed from both pulps. This could be due to the partial release of PP bound to the cell wall material of the plant food (Chandrasekara & Shahidi, 2012). It has been reported that some PC present in plant foods, can be associated mainly with carbohydrates which can decrease their bioaccesibility (Saura-Calixto, 2010). During intestinal digestion, a series of enzymes, mainly some hydrolases responsible for the hydrolysis of carbohydrates, could react with some links or interactions that exist between carbohydrates and PC (González-Aguilar, Blancas-Benítez, & Sáyago-Ayerdi, 2017; Blancas-Benítez *et al.*, 2015b), which would result in an increase in the concentration of PC in the samples, after intestinal hydrolysis, a process that would occur when consuming any plant food, and that can be observed in the guava and soursop pulps analyzed in this study.

The PC profile of guava and soursop pulp found on *in vitro* digestion showed that gallic acid and chlorogenic acid were mainly detected in both samples, although caffeic acid was detected only in soursop pulp. Also, gallic acid and chlorogenic acid were the PC that was mostly found in SIF and IIF, respectively from guava and soursop pulp. Although these two main compounds are potentially absorbed in the intestine, a substantial percentage of them were also found on the indigestible fractions, which can reach the colon. In the colon this phenolic acids, because of its simple structure, may still be absorbed on the large intestine, it has been previously documented on various *in vitro* and *in vivo* models (Rui *et al*., 2014; Vetrani *et al*., 2016). All these compounds have been previously identified in both guava and soursop fruit, and they have been attributed beneficial effects for health (Coria-Téllez, Montalvo-González, Yahia & Obledo Vázquez, 2018; Jiménez-Escrig, Rincón, Pulido & Saura-Calixto, 2001; Onyechi, Ibeanu, Nkiruka, Eme & Madubike, 2012; Rojas-Garbanzo, Zimmermann, Schulze-Kaysers & Schieber, 2017; Soares, Pereira, Marques & Monteiro., 2007).

For the bioaccessibility determination was considerate the overall distribution of the TSP content, the data showed that 67.69% of the TSP from the guava pulp are bioaccessible, while 71.30% of TSP from soursop pulp. It is apparent that soursop pulp has a higher bioaccessibility of PC than the guava pulp. This suggests that soursop pulp PC are not bound to the food matrix, making them more available for intestinal absorption than PC present in guava pulp (Bohn, 2014; Cuervo *et al*., 2014).

***In vitro* release kinetics of PC in guava and soursop pulp**

Figure 2 shows the release kinetics of PC from guava and soursop pulp. The PC released did not showed statistical difference with similar rate in the soursop pulp (0.10 mg GAe/min) and the guava pulp (0.09 mg GAe/min), during the first 30 min of digestion. The release rate was similar after 150 min of digestion, without subsequent changes in either pulps, These results may be related to those from Table I, where a higher bioaccessibility was found for soursop pulp, it is apparent that the soursop pulp PC might have a low interaction with the food matrix and they could be highly bioaccessible than the PC presents in guava pulp.

Figure 3 shows the different PC that was identified through time of the *in vitro* digestion on each fruit pulp. Gallic acid was the main compound found in the guava and soursop pulp. The PC from both pulps remained constant throughout the 180 min of digestion. As previously mentioned, gallic

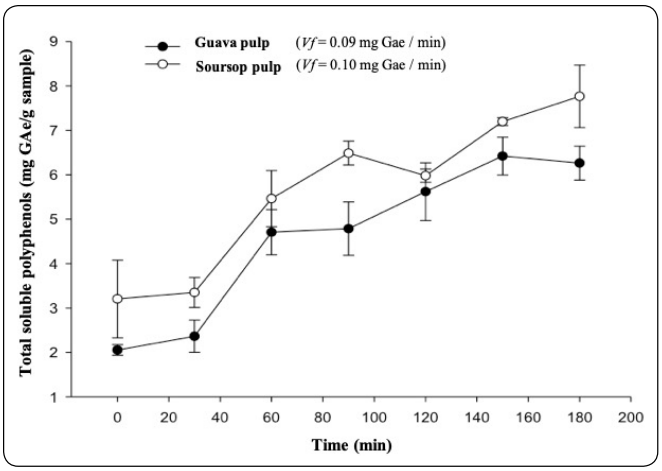

Figure 2. *In vitro* release kinetics of total soluble polyphenols of guava and soursop pulps

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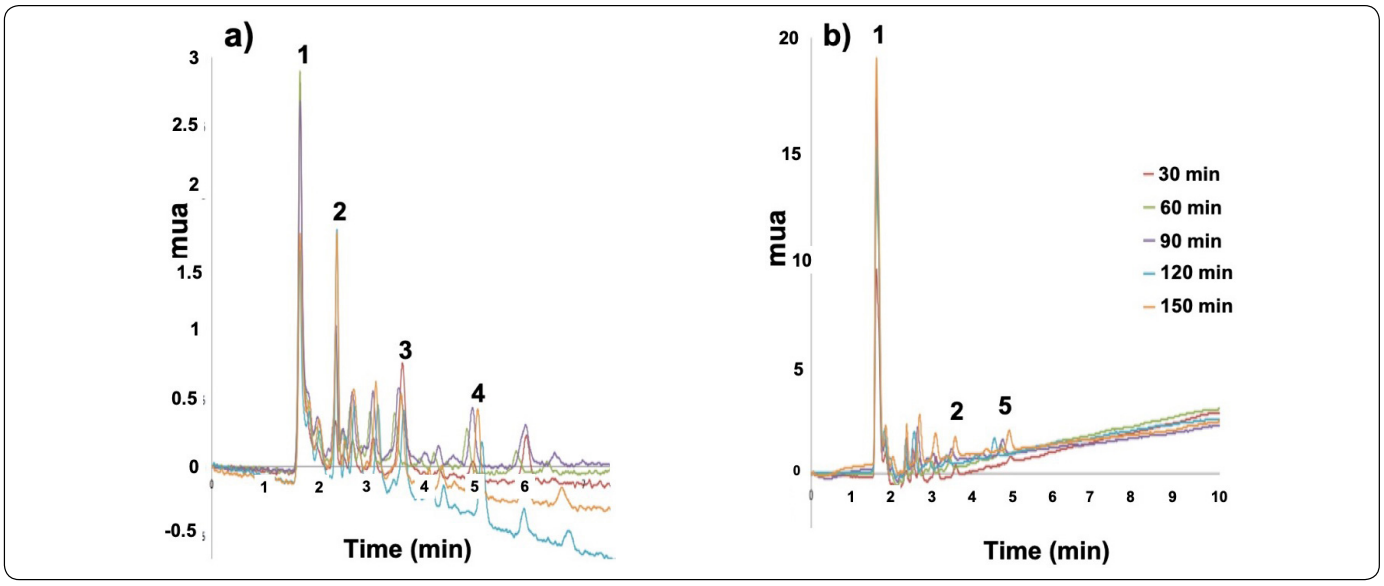

Figure 3 Phenolic compounds profile (HPLC-DAD) of guava (a) and soursop (b) pulp digestion fractions. a) Gastric fraction, b) Intestinal fraction c) After dialysis process Peak annotations; 1: Gallic acid, 2: Chlorogenic acid, 3: Coumaric acid, 4: Hydroxycinnamic acid, 5: Caffeic acid.

Table I Polyphenols released during each of the stages of the *in vitro* digestion, and bioaccesibility of polyphenols of guava and soursop pulp.[1]

	Guava pulp	Soursop pulp
TSP (g GAE per 100 g sample)		
Gastric fraction	14.33 ± 0.17[b]	17.15 ± 0.28[a]
Intestinal fraction	28.88 ± 0.59[a]	29.83 ± 1.0[a]
Phenolic compounds profile (%)		
Gallic acid	77.02 ± 0.31[b]	83.5 ± 0.43[a]
Chlorogenic acid	12.5 ± 0.21[a]	6.4 ± 0.11[b]
Coumaric acid	5.5 ± 0.51	n.d.
Hydroxycinnamic acid	4.6 ± 0.12	n.d
Caffeic acid	n.d.	10.1 ± 0.10
Non-bioaccesible PC (SIF)	8.16 ± 0.17[a]	6.77 ± 0.10[b]
Phenolic compounds profile (%)		
Gallic acid	59.5 ± 0.05[b]	30.94 ± 0.18[a]
Chlorogenic acid	40.2 ± 0.23[b]	60.80 ± 0.11[a]
Caffeic acid	n.d.	9.83 ± 0.08
Non-bioaccesible PC (IIF)	1.73 ± 0.67[a]	2.51 ± 0.34[a]
Gallic acid	10.45 ± 0.22[a]	3.42 ± 0.31[b]
Chlorogenic acid	87.50 ± 2.3[a]	78.93 ± 1.4[a]
Caffeic acid	n.d.	17.45 ± 0.46
Bioaccesible PC (%)	67.69[b]	71.30[a]

[1]Data are means of three repetitions ± standar deviation. Different lowercase letters in the same row indicate significant difference ($p<0.05$); n.d.: not detected, %Bioaccesibility= $(\frac{(PC\text{-}IntF)-(PC\text{-}SIF)}{(PC\text{-}IntF)+(PC\text{-}IIF)})$ x 100, PRFM = PP released from the food matrix PASIF = CF associated with SIF.

acid was the main compound detected, and its percentage of detection increased during the *in vitro* digestion. The same behavior was observed for chlorogenic, coumaric and hydroxycinnamic acids in guava pulp; and for chlorogenic and caffeic acids in soursop pulp. These results not only revealed the potential bioaccessibility of PC, but the ratio with which these compounds could be released and absorbed in the small intestine (Blancas-Benítez *et al*., 2015b).

Conclusions

The bioaccessibility of the PC was higher in the soursop pulp (71.30 %) than in the guava pulp (67.69 %). The main PC identified were gallic acid in the guava pulp, and chlorogenic acid in the soursop pulp. The release kinetics rate of PC was higher in the soursop pulp than on the guava pulp. These results highlight that although the guava pulp has a higher PC content than soursop pulp, not all of these PC will be bioaccessible. The importance of the bioavailability analysis lies to indicate the functional potential that fruit pulps can have. However further studies are required to establish their *in vivo* effects after intake of ready to eat foods like fruit pulp.

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