

## Study of the Specificity of Cross-Povidone (PVPP) as Binding Agent in the Quantification of Polyphenolic Compounds

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O polímero polivinilpirrolidona (PVPP) proporciona uma alternativa analítica ao invés do pó-de-pele e caseína, como um agente complexante na quantificação de taninos. Neste trabalho foi estudado a especificidade do PVPP em complexar com compostos polifenólicos, na presença de rutina. Os ácidos gálico e tânico, catequina e pirogalol foram utilizados como substâncias de referência (PRS), junto com o extrato aquoso das folhas do *Psidium guajava* L. A especificidade da complexação foi avaliada pelo método espectrofotométrico e por cromatografia líquida de alta eficiência (CLAE) com arranjo de diodos (DAD). As análises para a mistura das PRS e rutina, demonstraram claramente que a complexação com PVPP não é uma reação específica e independe da quantidade de polímero utilizada. As análises por CLAE-DAD do extrato de *Psidium guajava* revelaram picos característicos de flavonóides, além de catequina e ácido gálico. Todos os picos destes flavonóides, catequina e ácido gálico decresceram quando adicionou-se o PVPP. Isto confirma a falta de especificidade. Desde que a ligação PVPP-polifenóis depende de características estruturais particulares, a extensão desses resultados a outras espécies vegetais deve ser evitada.

Cross-povidone, or polyvinylpyrrolidone (PVPP) affords an analytical alternative instead of hide-powder and casein as binding agent in the content assay of vegetable tannins. In this work we studied the specificity of PVPP to bind polyphenolics in the presence of flavonoids. Gallic acid, tannic acid, catechin and pyrogallol were used as polyphenolic reference substances (PRS), along with an aqueous extract from *Psidium guajava* L. leaves. The binding specificity was assayed by UV-Vis and High Performance Liquid Chromatography-Photodiode array (HPLC-PDA) methods. The analyses of PRS-rutin mixtures showed clearly that PVPP binding is a non-specific reaction, unrelated to the amount of PVPP used. HPLC-PDA analysis revealed peaks which could be characterized as flavonoids in the *Psidium guajava* extract, beside catechin and gallic acid. All these flavonoids, catechin and gallic acid peaks decreased as PVPP was added; this confirms the lack of specificity. Since the polyphenolic-PVPP reaction depends on specific structural features, any extensive conclusion should be avoided.

**Keywords:** PVPP, tannins, flavonoids, specificity, HPLC-PDA

### Introduction

Almost all of the assays of polyphenolics and tannin content in current chemical Codexes and Pharmacopoeias are based on polyphenol-protein binding. For that purpose hide-powder and casein are normally used as protein substrates.<sup>1-5</sup> On a following step the assays are accomplished with a spectrophotometric quantitation by the Folin-Ciocalteu method.<sup>6,7</sup> It was recently demonstrated that methods using hide-powder and casein are non-specific when flavonoids are also present in the reaction milieu.<sup>5,8</sup>

The lack of specificity was also related to other methods intended for the tannin assay, including gravimetric and HPLC-UV methods.<sup>9,10</sup>

The capacity of insoluble cross-linked povidone (PVPP) to bind polyphenols arises in this context as a seldom explored analytical alternative.<sup>5,11,12</sup> One example of this is the FAO/IAEA method for the *Quantification of Tannins in Tree Foliage* Monograph.<sup>7</sup> The capacity of PVPP to bind polyphenolics was earlier ascribed to the structural likeness between the pyrrolidone group and the amino acid proline.<sup>13</sup> Other main factors determining the polyphenolics-PVPP reaction include chemical features of the polyphenol molecule (number of hydroxyl groups, isomers and conformation features)

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as well as the pH, temperature and ionic strength of the reaction milieu.<sup>5,11,12,14-16</sup> This work was designed to evaluate systematically analytical variables related to the binding reaction between PVPP and the reference substances, gallic acid, tannic acid, catechin and pyrogallol. Complementary, an aqueous extract from *Psidium guajava* leaves was included as a model plant extract. *Psidium guajava* is a plant that provides a large amount of polyphenols, among them gallic acid and catechin. Flavonoids are reported for *P. guajava* such as quercetin and its glycosilates derivatives.<sup>17-21</sup>

## Experimental

### Chemicals

Standard substances gallic acid, catechin, tannic acid and rutin were purchased from Sigma (St. Louis, MO). Pyrogallol, anhydrous sodium carbonate, and HPLC solvents were purchased from Merck (Darmstadt, Germany). PVPP was purchased from Sigma and purified by acidic washing before its use.

### Plant material

*P. guajava* L. leaves were harvested in Porto Alegre County (march, 2004), dried in an air-circulating oven (Memmert, TV 60 UL, Germany) at 40 °C for five days. The dried material was comminuted in a cutter mill (SK1 Retsch, Germany). The powder fraction with a particle size of 180 µm was stored in glass vessels, protected from light. The water content was determined using the Loss-on-drying Assay of the German Pharmacopoeia.<sup>2</sup>

### Extract preparation

A mass of 0.5 g of plant material was extracted with 150.0 mL of water by heating at 100 °C on a water-bath for 30 min. The extract was cooled at room temperature, transferred quantitatively to a 250 mL volumetric flask and diluted up to 250.0 mL with water. The solution was filtered through a Whatmann paper filter discarding the first 50 mL of filtrate. The remaining filtrate was freeze-dried and stored adequately until it was used.

### Preparation of standard solutions

Standard solutions were freshly prepared daily with purified water so that final concentrations of 0.4 mg mL<sup>-1</sup> (tannic acid and gallic acid), 1.4 mg mL<sup>-1</sup> (catechin) and 3.0 mg mL<sup>-1</sup> (pyrogallol) were obtained and used immediately. All preparations were protected from light full-time.

### Preparation of PVPP dispersions

Four samples of PVPP were accurately weighted and magnetically stirred with purified water during 24 h so that final concentrations of 0.5, 2.5, 7.5 and 15 mg mL<sup>-1</sup> were obtained. After this hydration procedure, the preparations were used at once.

### Comparison of PVPP-PRS complexes

A 5.0 mL aliquot of each polyphenolic reference substances (PRS) solution was added to each one of four PVPP dispersions. The mixture was stirred magnetically during 30 min. The mixture was centrifuged at 3000 rpm (2.01 g) for 30 min (Fanem, Brazil), and the supernatant liquid then decanted and retained. A 5.0 mL aliquot of each preparation was diluted up to 25.0 mL with water. The absorption was measured at 270 (for gallic acid and tannic acid), 280 (for catechin), and 267 nm (for pyrogallol) using water as a blank and a double beam spectrophotometer (Hewlett Packard, HP8452A, USA). Three replicate procedures were carried out with each PRS solution.

### Influence of the pH on the catechin fraction bound

Eight 700 µg mL<sup>-1</sup> solutions of catechin were mixed separately with 20 mL of a 15 mg mL<sup>-1</sup> PVPP dispersion (prepared as described above for *PVPP dispersions*). The pH was adjusted at 2.94 and 3.36 using HCl 0.1 mol L<sup>-1</sup>; at 6.99 and 7.98 with phosphate buffer, and at 8.43, 8.99, and 9.39 with borate buffer.<sup>5</sup> The mixture at pH 6.0 contained water alone. After that, each preparation was treated as described in Comparison of PVPP-PRS complexes, above item. The absorption was measured at 280 nm (acidic pH) or 290 nm (alkaline pH), using purified water as a blank. The results were expressed as catechin bound fraction (CBF) and represent the mean value of at least three replications.

### HPLC-assay for the PVPP binding specificity

Solutions containing 80 µg mL<sup>-1</sup> of gallic acid, catechin, pyrogallol and rutin were prepared separately using an acetonitrile:water (1:3 v/v) mixture as a solvent. From each solution, 10.0 mL aliquots were mixed with 10.0, 50.0, 150.0, and 300.0 mg of PVPP and afterward stirred, centrifuged and decanted as described for Comparison of PVPP-PRS complexes, above item. Appropriate aliquots from the decanted liquid were filtered through a 0.45 µm polyvinylidene difluoride (PVDF) filter. The injection volume was 20 µL.

For the *P. guajava* extract analysis, a 100.0 mg sample of freeze-dried extract was dissolved in water, filtered through a 0.45  $\mu\text{m}$  PVDF syringe filter and injected. Aliquots of 10.0 mL of this solution were treated with 10.0; 50.0; 150.0 and 300.0 mg of PVPP, stirred by 30 min magnetically, filtered in the same way and injected. Each result represents the mean value of at least three replicate.

The samples were analyzed with a Waters Alliance HPLC system (Mod. 2695, USA) equipped with a model 2695 solvent delivery system, a model 2487 programmable UV/Vis detector (Waters, 2487) and a photodiode-array (PDA) detector (Waters, 996); the system was controlled by Waters Empower software. The samples were loaded onto a reversed phase C<sub>18</sub> Gemini column (Phenomenex, 250 mm  $\times$  4.60 mm, 5  $\mu\text{m}$  particle size) preceded by a C<sub>18</sub> guard cartridge (Shimadzu, 10mm  $\times$  4 mm), packed with Bondapack C<sub>18</sub> 125 A, 37-55  $\mu\text{m}$ . The temperature was kept at 20-25  $^{\circ}\text{C}$ . For quantification purposes dual wavelength detection of the standards was performed at 280 and 352 nm. HPLC analysis at 352 nm was used for detection and characterization of rutin and *P. guajava* flavonoids. The detection sensitivity was set to 0.5 U.A. The mobile phases consisted of two mixtures: aqueous 0.5% phosphoric acid (A) and acetonitrile + 0.5% phosphoric acid (60:40 m/m) (B). The mobile phase gradient in weight ratio was as follows: 13 to 25% B over 25 min (step 1); 25% B, isocratic over 5 min (Step 2); 25 to 33% B over 7 min (step 3); 33% B, isocratic over 3 min (step 4); 33 to 40% B over 7 min (step 5); 40% B, isocratic over 3 min (step 6); 40 to 43% B over 3 min (step 7); 43% B, isocratic over 2 min (step 8); 43 to 13% B, over 15 min (column clean up). The flow rate was 0.8 mL min<sup>-1</sup>.

#### UV-assay for the PVPP binding specificity

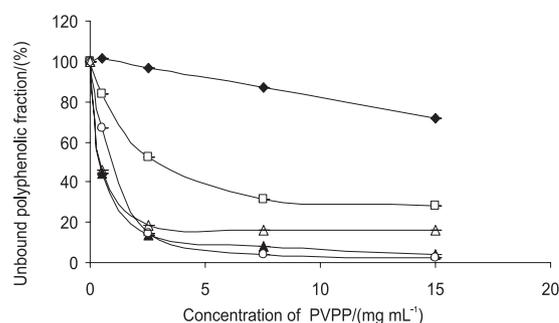
The quantitation of PRS was carried out spectrophotometrically in a similar way as described for Comparison of PVPP-PRS complexes, above item. In this case, 100.0 mL samples of catechin and pyrogallol standard solutions were spiked with 20.0 mg of rutin separately. Both spiked solutions were treated as described above for Comparison of PVPP-PRS complexes, above item. The analysis was performed at 280 (catechin), 267 (pyrogallol), and 352 nm (rutin).

## Results and Discussion

In the current work, one of the objectives was the comparison of the binding of PVPP to four standard substances frequently used to express tannin or polyphenols content, namely gallic acid, tannic acid, catechin and

pyrogallol. Together with the standard substances, an extract from *P. guajava* leaves was included as a model plant extract, owing its tannin, flavonoids and other polyphenolics richness.<sup>17-21</sup> Rutin was chosen as the model glycosidic flavonoid because of its ubiquitous distribution in higher plant species. The analyses of polyphenolic reference substances (PRS), rutin and *P. guajava* extract were performed by spectrophotometry and HPLC-PDA. The HPLC-PDA method was particularly developed for the detection and quantification of the PRS *P. guajava* extract and the flavonoid rutin.

The simple comparison of the free polyphenolic fraction curves exposed clear binding differences in terms of polyphenolic-PVPP complex formation (Figure 1).



**Figure 1.** Complexation of gallic acid ( $\square$ ), tannic acid ( $\blacktriangle$ ), catechin ( $\circ$ ), pyrogallol ( $\blacklozenge$ ) and *P. guajava* extract ( $\triangle$ ) with PVPP by UV detection in characteristic wavelength of each PRS.

The foremost drive mechanism linked to the PVPP binding is associated to its extremely hydrophilic structure, which implies intermolecular hydrogen bonding.<sup>22</sup> Earlier studies showed that affinity to PVPP was generally increased with the number of phenolic hydroxy groups available for hydrogen bonding.<sup>14</sup> This should explain the strong interaction between PVPP and tannic acid, which contains many hydroxyl groups in its numerous galloyl groups.<sup>11,23</sup> Similar results were related earlier for tannic acid and catechin studies using gelatin and trypsin as protein substrates.<sup>24,25</sup> These authors ascribed the better complexation of tannic acid to its higher molecular weight and hydroxyl density.

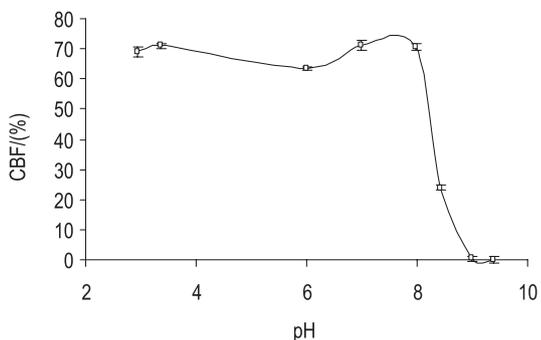
The PVPP-gallic acid interaction was higher than that observed for pyrogallol, owing probably the more reactive carboxylic group attached to gallic acid molecule. Previous studies with PVPP and several drugs revealed that the carboxylic groups were more effective than the hydroxylic groups in complexation with the PVPP.<sup>11</sup>

In the PVPP-catechin case, the complexation involves the five hydroxyls attached to C3, C5, C7, C3' and C4' sites, plus the hydrophobic bonding contribution. When the catechin bound fraction (CBF) curve is compared to the gallic acid ones, it becomes evident that the five catechin hydroxyl

surpasses the total effect due to the three hydroxyl groups and one carboxylic acid attached to the gallic acid molecule.

Besides being an important tannin fraction, *P. guajava* leaves contain flavonoids and other polyphenolics.<sup>16,18,19,21,26,27</sup> The binding behavior of the *P. guajava* extract was comparable to tannic acid and catechin, as observed in Figure 1.

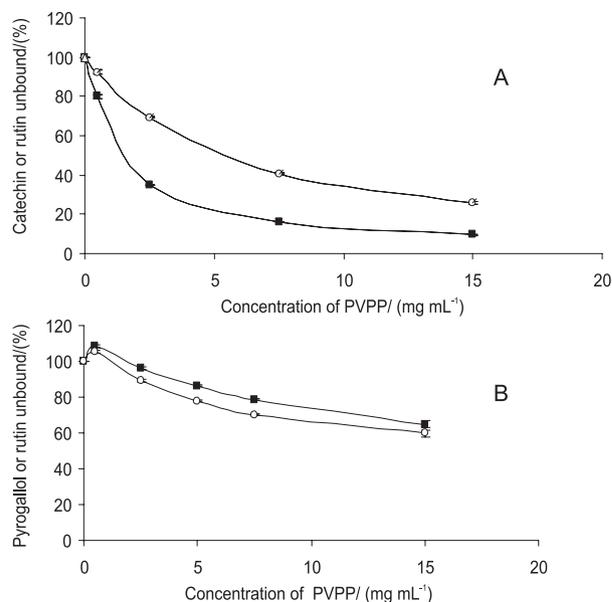
The influence of the pH on the binding effectiveness of PVPP is well-documented in the literature.<sup>5,14,15,27</sup> Catechin was chosen to evaluate the pH influence on the binding capacity because its interaction was greater than that with pyrogallol or gallic acid (tannic acid was excluded because of its molecular complexity). The maximal level of CBF was observed in acidic pH range, namely; at pH values lower than about 7.5-8.0 where phenols would be un-ionized. Above this pH range the free (unbound) catechin fraction increased rapidly (Figure 2). It confirms that the suppression of the phenolics ionization (*i.e.*, hydrogen bonds between PVPP and catechin become stronger) increase the PVPP binding.<sup>5,11,14,15,25</sup>



**Figure 2.** Effect of pH on the catechin bound fraction (CBF) to PVPP determined by UV detection at 280 nm.

In a former work we asked whether casein and hide-powder were able to bind *P. guajava* tannins in the presence of flavonoids in a specific way.<sup>8</sup> This question can be extended to PVPP because some of the main features of the casein-tannin and hide-powder-tannin binding are also noticeable in PVPP cases. Alike these protein substrates, the influence of hydrogen bonds and hydrophobic interactions are also manifested in the PVPP molecule, namely, in the pyrrolidone moiety and in the vinyl chain, respectively.<sup>28-30</sup> It is also worth mentioning that pyrrolidone structure closely resembles some of the proline ones.<sup>13</sup> So we can compare them and consider that factors such pH, ionic strength and temperature influence the protein binding effectiveness to polyphenol.<sup>5,28-31,34</sup> In our experiments the pH was acidic (6.0-5.5) and the temperature was always  $25 \pm 2$  °C.

In this context we studied by UV-Vis and HPLC-PDA techniques the specificity of PVPP to bind catechin and pyrogallol after the addition of rutin.



**Figure 3.** (A) Complexation of catechin and rutin with PVPP; (■) 280 nm, (○) 352 nm. (B) Complexation of pyrogallol and rutin with PVPP; (■) 267 nm, (○) 352 nm. Degrees of complexation were determined by UV method.

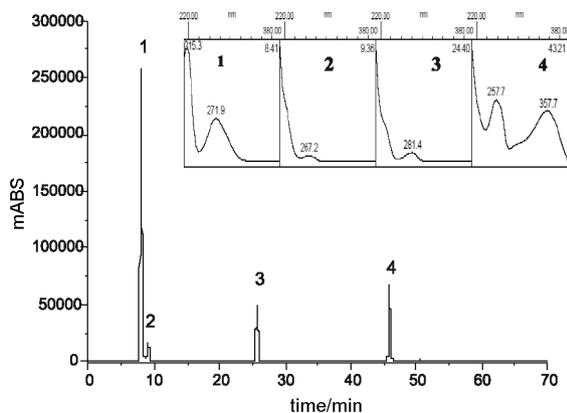
Comparison of the unbound fractions of rutin and catechin (Figure 3) shows that PVPP is able to bind both substances, but to a different extent.

From the difference between the absorption measured at 280 and 352 nm it can be seen which compound was bound to PVPP more efficiently. This reasoning is based on the postulation that rutin and catechin absorb strongly at 280 nm, however, at 352 nm rutin absorbs still strongly while catechin is practically transparent. As the absorption decreases at 280 nm (due to the mixture of the two substances), it was more marked than that at 352 nm (Figure 3); it can be inferred that PVPP bound to catechin more readily than to rutin. Both substances have the same number of non-substituted hydroxyl, so the absorption difference is probably due to the steric hindrance caused by the rutoside attached at the rutin C-3 position.<sup>23</sup> Following a similar train of logic, one can state that PVPP bound to pyrogallol and to rutin in a similar extent.

These results were compared with that obtained by HPLC. A typical HPLC separation of gallic acid, catechin, pyrogallol and rutin is illustrated in Figure 4. Owing its complexity tannic acid was excluded once again from this analysis.

The results are in agreement to the UV-analyses. Significant amounts of all the PRS and rutin were bound to PVPP even with as little as 10.0 mg of PVPP used (Table 1).

The inclusion of a *P. guajava* extract and its analysis by HPLC-PDA represent an approach to evaluate the interference of flavonoids with the binding of other



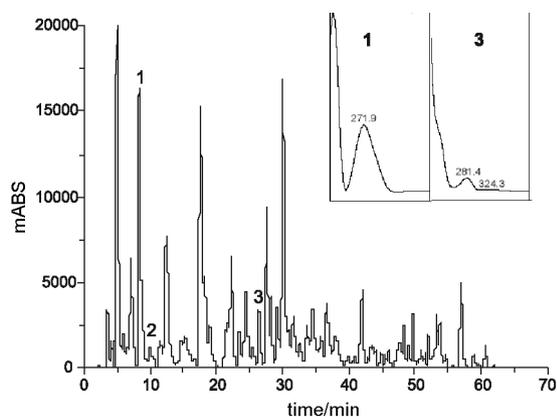
**Figure 4.** HPLC-PDA-chromatogram of the polyphenolic reference substances: gallic acid (1), pyrogallol (2), catechin (3), and rutin (4) with detection at 280 nm. The insert shows the respective UV spectra.

**Table 1.** Percentages of bound fraction, determined by HPLC method, for gallic acid, pyrogallol, catechin and rutin after complexation with different amounts of PVPP

Amount of PVPP added/(mg)	Bound fraction/(%)		
	gallic acid + pyrogallol	catechin	rutin
10	20.13 (0.04) <sup>a</sup>	73.98 (0.27) <sup>a</sup>	15.78 (0.04) <sup>a</sup>
50	56.19 (0.27) <sup>a</sup>	96.56 (0.25) <sup>a</sup>	49.70 (0.41) <sup>a</sup>
150	57.67 (0.01) <sup>a</sup>	98.95 (6.40) <sup>a</sup>	75.18 (0.32) <sup>a</sup>
300	51.76 (0.30) <sup>a</sup>	99.71 (7.59) <sup>a</sup>	86.43 (0.68) <sup>a</sup>

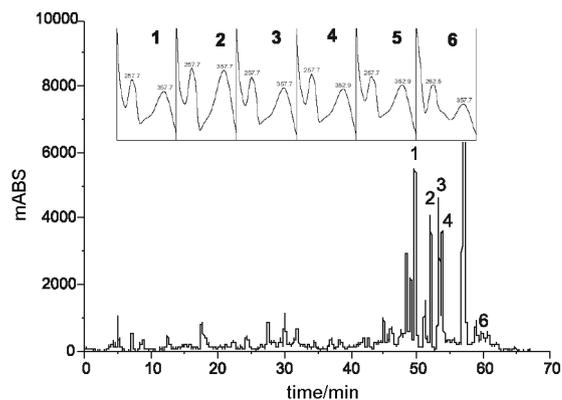
<sup>a</sup>Coefficient variation.

polyphenolics in more complex matrix. The HPLC-PDA analysis of the *P. guajava* extract along with the co-elution of PRS revealed the presence gallic acid and catechin in this extract (compare Figures 5 and 6 with 4). The occurrence of both polyphenolics was earlier related for this species, together with epicatechin, elagic acid and tannin.<sup>17,19,26,27,30</sup> Conversely, no peak of the extract HPLC-PDA chromatogram could be assigned to rutin.



**Figure 5.** HPLC-PDA-chromatogram of the *P. guajava* extract showing the presence of gallic acid (1) and catechin (3) after detection at 280 nm. The insert shows the respective UV spectra.

The flavonoid occurrence in *P. guajava* leaves was extensively related.<sup>16-21,27</sup> This flavonoid fraction became evident in the HPLC chromatogram after a 45 min retention time and by the detection at a wavelength of 352 nm, at which neither catechin, gallic acid nor pyrogallol exhibits absorption (Figure 6).



**Figure 6.** HPLC-PDA-chromatogram of *P. guajava* extract at a wavelength detection of 352 nm. The peaks coded as 1, 2, 3, 4, 5, 6 were characterized as flavonoids. The insert shows the respective UV spectra.

The peaks coded as 1 to 6 showed UV-spectra that are characteristic of flavonoids, more specifically, flavones.<sup>35</sup> Five of the HPLC peaks had absorption maxima near 257 and 357 nm, with a shoulder located at about 300 nm (this resembles that of peak 4 in Figure 4). Those UV-spectra resemble closely the spectra related for some flavonoids isolated from *P. guajava* leaves, among them quercetin and its glycosyl derivatives 3-*L*-4-arabinefuranosid- (avicularine), 3-*L*-4-piranosid- and 3-*O*- $\beta$ -glycosylquercetin.<sup>16-21,27</sup> The peak number 6 differs from the other because of their absorbance maxima at 262.5 and 357.7 nm, with a shoulder positioned at about 295 nm, notwithstanding, its flavonoid character can be easily ascribed.<sup>35</sup>

The influence of the increasing treatment with PVPP on the *P. guajava* flavonoid HPLC-fingerprint is summarized in Table 2. As one can observe, the addition of 50.0 mg de PVPP was already capable to bind catechin completely and most of the flavonoid fraction. Moreover, after the addition of 150.0 and 300.0 mg of PVPP all flavonoids and polyphenolics were bound to PVPP in a large extent, with a noteworthy exception of gallic acid.

The results as a whole showed that PVPP was able to bind the test polyphenolics as catechin, tannic acid, pyrogallol and, in a minor extent, gallic acid. However, there was evidence of a lack of specificity when some of the compounds were mixed with rutin. The HPLC-PDA analysis carried out using a *P. guajava* extract led to similar conclusions. Therefore, the results make us discard any possibility of method validation, intended specifically for *P. guajava* leaves.

**Table 2.** Percentages of bound fraction of gallic acid, catechin, and flavonoids of *P. guajava* after complexation with different amounts of PVPP

Amount of PVPP added/mg	Polyphenolic detection at 280 nm				Flavonoid detection at 352 nm			
	gallic acid	catechin	peak 1	peak 2	peak 3	peak 4	peak 5	peak 6
10.0	28.64 (2.26) <sup>a</sup>	76.53 (1.94) <sup>a</sup>	36.24 (3.16) <sup>a</sup>	71.68 (1.43) <sup>a</sup>	79.40 (1.00) <sup>a</sup>	65.00 (0.25) <sup>a</sup>	55.19 (0.14) <sup>a</sup>	84.00 (1.17) <sup>a</sup>
20.0	30.89 (0.25) <sup>a</sup>	100	37.80 (2.84) <sup>a</sup>	84.30 (1.19) <sup>a</sup>	90.67 (1.36) <sup>a</sup>	77.62 (1.41) <sup>a</sup>	69.73 (0.36) <sup>a</sup>	85.35 (6.64) <sup>a</sup>
30.0	42.69 (4.76) <sup>a</sup>	100	46.79 (1.53) <sup>a</sup>	91.21 (3.82) <sup>a</sup>	94.94 (2.04) <sup>a</sup>	86.03 (0.67) <sup>a</sup>	82.41 (2.84) <sup>a</sup>	86.34 (4.96) <sup>a</sup>
40.0	47.38 (4.40) <sup>a</sup>	100	53.90 (1.33) <sup>a</sup>	93.83 (1.72) <sup>a</sup>	96.47 (3.49) <sup>a</sup>	89.78 (2.54) <sup>a</sup>	87.68 (0.99) <sup>a</sup>	89.13 (8.83) <sup>a</sup>
50.0	57.59 (1.62) <sup>a</sup>	100	68.09 (0.64) <sup>a</sup>	95.96 (3.18) <sup>a</sup>	97.72 (5.09) <sup>a</sup>	92.97 (2.20) <sup>a</sup>	92.87 (1.57) <sup>a</sup>	91.35 (4.34) <sup>a</sup>
150.0	63.34 (0.97) <sup>a</sup>	100	88.76 (2.56) <sup>a</sup>	100	100	97.81 (5.20) <sup>a</sup>	98.24 (1.76) <sup>a</sup>	91.95 (4.01) <sup>a</sup>
300.0	69.79 (0.32) <sup>a</sup>	100	95.38 (8.81) <sup>a</sup>	100	100	100	99.44 (21.98) <sup>a</sup>	91.77 (9.94) <sup>a</sup>

<sup>a</sup>CV%: coefficient of variation.

Studies on flavonoid-PVPP structure-affinity relationships had evidenced the influence of other relevant factors, as hydroxyl number, methyl- and glycosyl-substitution patterns and coplanarity of the flavonoid ring C. Thus, highly substituted flavonoids and several isoflavones show little of none interaction with PVPP.<sup>22</sup> Therefore, the ability of PVPP to bind rutin must be regarded specifically avoiding any oversimplification. The use of PVPP as binding agent aiming the assay of polyphenolics content in vegetable matrixes should be evaluated case by case and further studies are needed.

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