Binding of Flavonoids by Polyvinylpolypyrrolidone

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A binding assay was developed with which to monitor the binding capacity of polyvinylpolypyrrolidone (PVPP) for individual flavonoids. The assay was conducted by shaking dilute solutions of flavonoid in suspensions of PVPP in methanol/10 mM citrate buffer (1:3) at pH 6.0. The molar ratio of PVPP/flavonoid was 100:1, based on the molecular weight of the monomer unit of PVPP. Binding was rapid, and it was observed that those compounds which possess the greater number of hydroxy groups generally bind most efficiently. When comparing flavonoids from different classes but with identical hydroxylation patterns, it was noted that flavones bind better than isoflavones, which in turn bind better than flavonones and dihydroflavonols. In light of the favorable stoichiometry and rates of binding, the incorporation of small quantities of PVPP into plant and microbial culture systems will be useful for serving to trap signal flavonoids which plants exude through their roots to communicate with microbes.

INTRODUCTION

Flavonoids occur in several structural forms, but the nucleus of all consists of two C_6 aromatic rings linked by a C_3 unit. In flavones, flavonols, dihydroflavonols, flavanones, and isoflavones, the central C_3 unit forms part of a third central ring, but in chalcones the central ring is not formed. Flavonoids are most often hydroxylated to various degrees, and hydroxyl groups are sometimes glycosylated or methylated. Structures and trivial names of compounds which represent various flavonoid categories are shown in Figure 1, along with the carbon atom numbering system for the A, B, and C rings.

Flavonoids are known to be ubiquitous among higher plants, and now it is known that they are involved in some important physiological processes. Certain of them have been recently found to act as inducers of rhizobial (Long, 1989) symbiosis or otherwise to serve as signal compounds to soil microbes [for a comprehensive review, see Phillips (1992)]. Earlier, a variety of flavonoids (mainly isoflavonoids) were shown to function in legumes as plant defensive compounds (phytoalexins) in response to infection (Van Etten et al., 1989).

It has been known for some time that flavonoids bind to polyvinylpolypyrrolidone (PVPP), a commercially available material produced by cross-linking polyvinylpyrrolidone (PVP). PVPP is water-insoluble but extremely hydrophilic, binding water through its carbonyl functionalities by hydrogen bonding. Hydrogen bonding is the dominant mechanism by which PVPP binds flavonoids and a variety of other phenolic compounds, and this binding capacity has been utilized for removing such compounds from tissue homogenates during the isolation of plant enzymes (Loomis and Battaile, 1966). Hydrogen bonding has also provided the basis for the use of PVPP as an HPLC stationary phase for separating a variety of compounds, including gibberellins (Glenn et al., 1972) and indole-3-acetic amino acids (Percival, 1986). Mixed layers of PVPP and cellulose were used for TLC separation of anthocyanins (Wrolstad, 1968).

In developing a method to determine total phenols in tobacco leaf, Andersen and Sowers (1968) examined the binding by PVPP of several phenolic compounds, including the flavonoids quercetin and rutin. They observed that binding was optimized when pH was sufficiently low to suppress ionization. In addition, affinity to PVPP was found to generally increase with the number of phenolic hydroxy groups available for hydrogen bonding, confirming earlier findings (Loomis and Battaile, 1966).

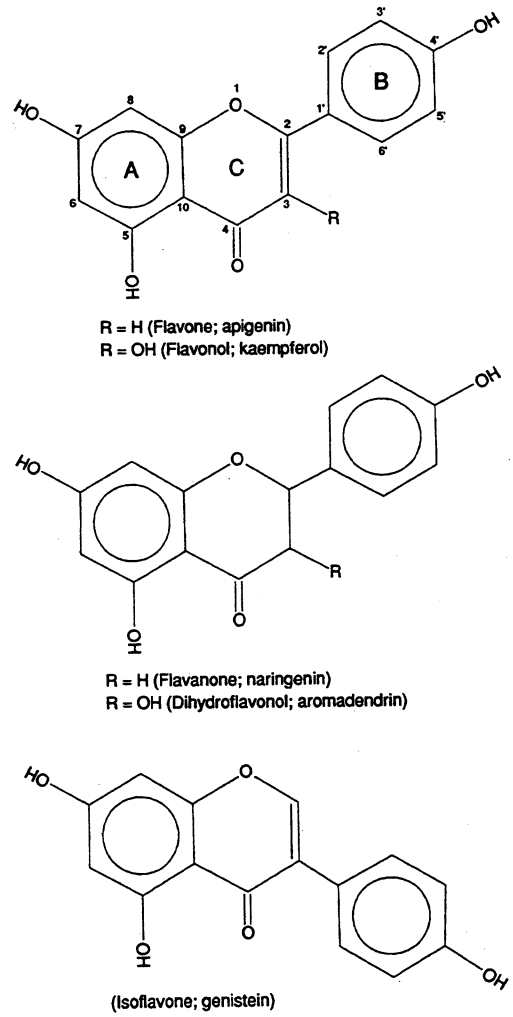


Figure 1. Structures and carbon atom numbering system of compounds representing various classes of flavonoids.

Evidence suggests that flavonoids exuded by plant roots serve as signal compounds to initiate the symbiotic relationship between vesicular-arbuscular mycorrhizal

fungi and higher plants (Tsao and Phillips, 1991; Siqueira et al., 1991; Bécard et al., 1992). We are studying this relationship using root organ cultures in gellan medium. The objective of the present study is to determine for the first time the relative binding to PVPP of a wide variety of flavonoids and to establish optimal conditions for binding. The results suggest the potential utility of adding PVPP to plant root organ cultures and to pot cultures, to trap flavonoids and other phenolics exuded from roots. This will faciliate their isolation from complex matrices for subsequent characterization and will allow determination of the effects of the presence of PVPP on the various plant–microbe associations.

MATERIALS AND METHODS

Materials. The mono- and dihydroxyflavones were purchased from Indofine Chemical Co., Somerville, NJ. Remaining flavonoids were purchased either from Indofine, Sigma (St. Louis, MO), or Aldrich (Milwaukee, WI). Isoliquiritigenin was synthesized by slightly modifying the procedure of Kape et al. (1992).

Preparation of Stock Solutions. *Flavonoid stock solutions* were prepared by dissolving each compound in methanol at the 1.0 mg/mL level. In some cases it was necessary to sonicate the mixture to achieve total dissolution. Stock solutions were stored in the dark. It was necessary to purify polyvinylpyrrolidone (PVPP, Sigma) prior to use to remove traces of H_2O_2, PVPP fines, and any soluble PVP that may have been present. Purification was done essentially as described earlier (Loomis and Battaile, 1966) by stirring 300 g of PVPP in boiling 10% HCl (3 L) for 10 min. The mixture was then allowed to cool, during which time most of the PVPP sedimented. The supernatant was decanted and the PVPP stirred into water (about 5 L); the process of settling and decantation was repeated several times. Finally, after stirring the PVPP into acetone for 1 h and removing the acetone by vacuum filtration, the purified PVPP was dried overnight in a vacuum oven. A yield of 54.7 g of purified PVPP was obtained, the particles of which ranged in diameter from 100 to 400 μm. Purification resulted in the removal of a sizable quantity of particles of less than 100 μm which had been present in commercial PVPP.

The PVPP stock solution was prepared by suspending purified PVPP in deionized and distilled water at the 25 mg/mL level. To assure complete PVPP hydration, this solution was prepared at least 24 h before use.

Preparation of Methanol/Water Mixtures at Several pH Values. Citrate buffers (10 mM) were used for studies at pH 3.0, 4.5, and 6.0, and trizma buffer was used for the pH 7.2 study. It is essential that pH values be readjusted after methanol addition, because the addition results in elevation of pH by 0.2–0.4 units.

Standard Binding Assay for Flavonoids. Twelve milliliters of 50 μM flavonoid solutions was prepared by diluting appropriate volumes of each stock solution (1.0 mg/mL methanol) with a 25% solution (50% for monohydroxyflavones) of methanol in 10 mM citrate buffer, pH 6.0. For example, 0.203 mL of quercetin stock solution was diluted with 11.80 mL of the 25% methanol in citrate buffer solution. The assay was then conducted in triplicate by adding 3.0 mL of 50 μM quercetin solution to each of three glass vials (7.0 mL). The remaining solution was retained for T_0 measurement. To each of the three vials was added 66 μL of the PVPP stock solution (25 mg/mL). This was withdrawn with a micropipet during rapid stirring of the suspension, to ensure addition of reproducible quantities. A 100:1 molar ratio of PVPP to flavonoid resulted (on the basis of formula weight of PVPP monomer unit of 111.1). Mixtures were then shaken for 60 min, after which time PVPP was allowed to settle out over about 5 min. Samples were withdrawn from the supernatant and added to cuvettes for absorbance measurement. The T_0 samples were prepared by adding water (22 μL) to 1.0-mL aliquots of the portions of flavonoid solution (above) which had not been exposed to PVPP. Absorbance values were measured at T_0 and T_{60} at the λ_{max} value of each flavonoid, using a Shimadzu UV160U UV–visible spectrophotometer. From the mean value of the triplicate samples, fraction bound (FB) was calculated according to FB = $(A_{t0} - A_{t60})/A_{t0}$.

Data Analysis. To estimate the fraction of guest flavonoids bound to PVPP at an infinite [PVPP]:[guest] ratio as well as the association constant, K (the reciprocal of which is provided in Table II as [PVPP]:[guest] at half-maximal binding), data were fit to a quadratic equation utilizing a modified Gauss–Newton spreadsheet developed in this laboratory. For these calculations we have assumed

$$\text{PVPP} + \text{guest} \rightleftharpoons [\text{P}\cdot\text{G}] \tag{1}$$

where

$$K = \frac{[\text{P}\cdot\text{G}]}{[\text{P}][\text{G}]} = \frac{[\text{P}\cdot\text{G}]}{\{[\text{P}]_0 - [\text{P}\cdot\text{G}]\}\{[\text{G}]_0 - [\text{P}\cdot\text{G}]\}} \cong \frac{\dfrac{[\text{G}]_0\text{FB}_i}{\text{FB}_{max}}}{\left\{[\text{P}]_0 - \dfrac{[\text{G}]_0\text{FB}_i}{\text{FB}_{max}}\right\}\left\{[\text{G}]_0 - \dfrac{[\text{G}]_0\text{FB}_i}{\text{FB}_{max}}\right\}} \tag{2}$$

$[\text{P}]_0$ and $[\text{G}]_0$ represent the initial concentrations of PVPP and guest, respectively. FB_i denotes the fraction of guest bound at any particular level of variable $[\text{P}]_0$ ($[\text{G}]_0$ constant), and FB_{max} is the value at inifinite $[\text{P}]_0$. Solving for FB_i gives

$$\text{FB}_i = \text{FB}_{max}\,(1 + [\text{P}]_0K + [\text{G}]_0K - (1 + 2[\text{P}]_0K + 2[\text{G}]_0K + [\text{P}]_0{}^2K^2 - 2[\text{P}]_0[\text{G}]_0K^2 + [\text{G}]_0{}^2K^2)^{1/2}\big/2[\text{G}]_0K \tag{3}$$

To determine how fast the above reaction approaches an equilibrium, absorbance at 370 nm was plotted as a function of time and fit to an appropriate exponential equation

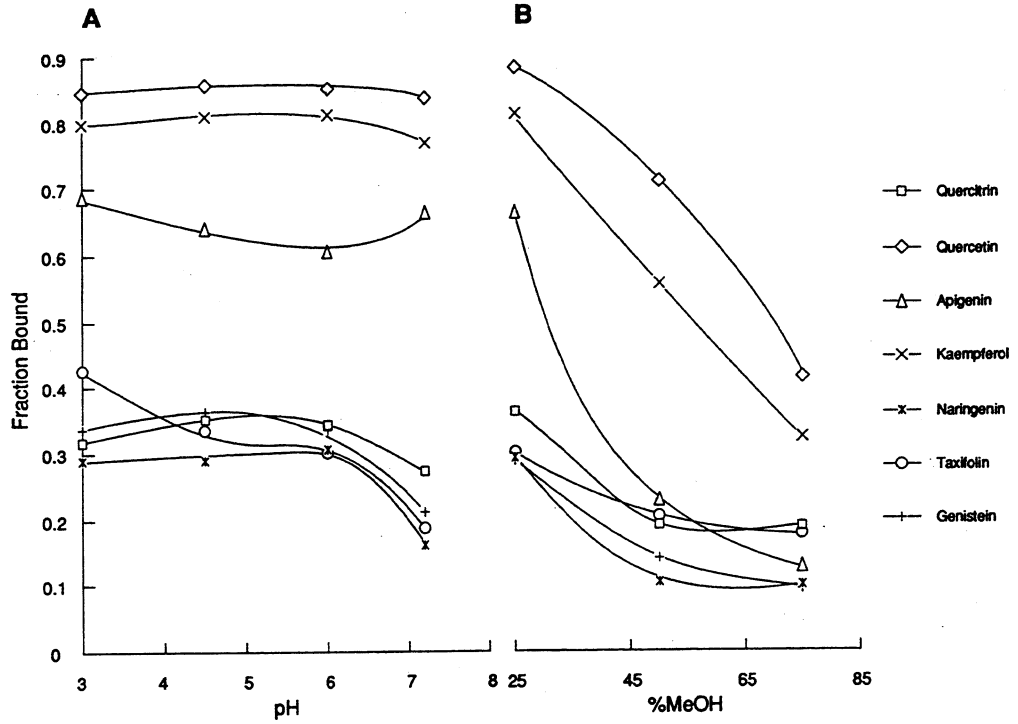
$$A_{370\text{nm}} = A_0e^{-\tau k} + A_\infty \tag{4}$$

using a modified Gauss–Newton curve-fitting procedure. In this relationship A_0 is the initial absorbance at 370 nm, k is the exponential rate constant (in units of τ^{-1}), τ is time (minutes) in solution, and A_∞ is the infinite time absorbance asymptote. We define the solution half-life of the flavonoid as

$$\tau_{1/2} = \ln\{2\}/k \tag{5}$$

RESULTS AND DISCUSSION

The binding capacity of PVPP for each flavonoid was measured after optimization of spectrophotometric assay conditions. The compounds depicted in Figure 1 represent several flavonoid categories and were used for this purpose. In Figure 2, parts A and B, are shown, respectively, plots of fraction bound (FB) vs both pH and methanol concentration. It has been observed in more limited studies (Loomis and Battaile, 1966; Andersen and Sowers, 1968) that binding is highest in pure water and at pH values sufficiently low to suppress ionization of phenolic hydroxyl groups. Some of the less substituted flavonoids were insoluble in pure water, so 25% methanol in citrate buffer solution was used. PVPP was found to bind each compound more effectively there than in solutions containing more methanol. A pH value of 6.0 was used, since below this value binding by PVPP was relatively constant, and this pH will be useful for subsequent use of PVPP in root organ culture systems. Flavonoid concentrations were chosen so that absorbance measurements at T_0 would be in the range 0.500–1.000 absorbance unit. A molar ratio of PVPP to flavonoid (based on molecular weight of monomer unit of PVPP) of 100:1 was selected, on the basis of the results shown in Figure 3, where the binding of those compounds (guests) depicted in Figure 1 was examined using different ratios. Significant binding to PVPP is approached by most flavonoids at 100:1 ratio,



re 2. Effect of pH and methanol concentration on binding to PVPP of representatives of various classes of flavonoids, under ard assay conditions.

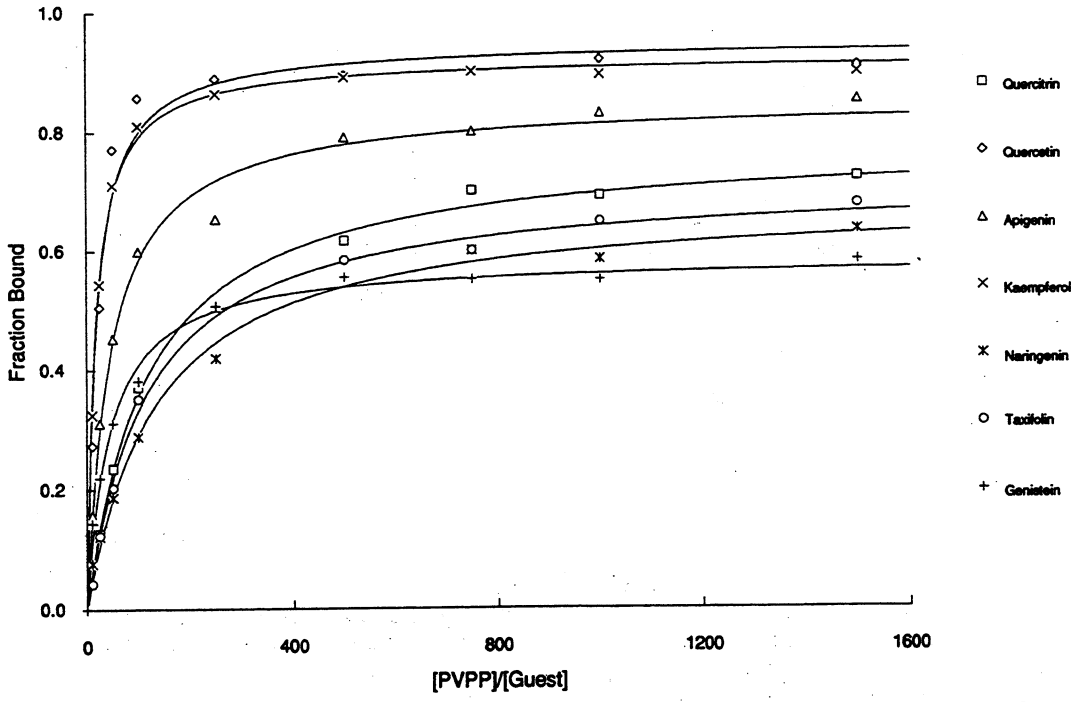


Figure 3. Effect of PVPP/flavonoid ratio on binding to PVPP by representatives of various classes of flavonoids, under standard assay conditions except for the variable PVPP levels.

and ranges in fraction bound among compounds is greater than at higher ratios.

A summary of the standard binding assay of individual flavonoids by PVPP is listed in Table I and is reported as fraction bound (FB). Indicated within each flavonoid class is the substitution pattern in order of increasing hydroxyl group substitution. Some flavonoids were additionally substituted with methoxyl (formononetin, biochanin A, hesperetin, hesperidin) or glycosyl (quercitrin, rutin, apigenin 7-glucoside, luteolin 7-glucoside, naringenin, hesperidin) groups. Within each class of flavonoids, binding increases with number of hydroxyl groups, with the exceptions of kaempferol, which binds to PVPP somewhat more effectively than morin, which possesses an additional 2′-hydroxyl group, and chrysin, which binds somewhat better than 5,6,7-trihydroxyflavone. As expected, the derivatization of hydroxyl groups resulted in greatly diminished binding. The effect of methylation can be seen with the pairs daidzein/formononetin, genistein/biochanin A, and hesperetin/hesperidin. The effect of glycosylation is represented with quercetin/quercitrin/rutin, apigenin/apigenin 7-glucoside/rhoifolin, luteolin/luteolin 7-glucoside, and naringenin/naringin. The effect of glycosylation on binding is especially severe, since

Table I. Flavonoids and the Fraction Bound (FB) to Polyvinylpolypyrrolidone under Standard Assay Conditions, along with the Substitution Pattern of Hydroxyl, Methoxyl, and Glycosyl Groups

flavonoid	fraction bound[a]	C-3	C-5	C-6	C-7	C-8	C-9	C-2′	C-3′	C-4′	C-5′
flavones											
5-hydroxyflavone	0.138[b]		–OH								
6-hydroxyflavone	0.071[b]			–OH							
7-hydroxyflavone	0.364[b]				–OH						
2′-hydroxyflavone	0.125[b]							–OH			
4′-hydroxyflavone	0.815[b]									–OH	
chrysin	0.327		–OH		–OH						
5,4′-dihydroxyflavone	0.135		–OH							–OH	
7,3′-dihydroxyflavone	0.281				–OH				–OH		
7,4′-dihydroxyflavone	0.307				–OH					–OH	
3′,4′-dihydroxyflavone	0.165								–OH	–OH	
apigenin	0.590		–OH		–OH					–OH	
apigenin 7-glucoside	0.160		–OH		–OGl					–OH	
rhoifolin	0.107		–OH		–OGl–ORh					–OH	
5,6,7-trihydroxyflavone	0.311		–OH	–OH	–OH						
7,3′,4′-trihydroxyflavone	0.520				–OH				–OH	–OH	
7,8,4′-trihydroxyflavone	0.338				–OH	–OH				–OH	
luteolin	0.775		–OH		–OH				–OH	–OH	
luteolin 7-glucoside	0.169		–OH		–OGl				–OH	–OH	
flavonols											
3-hydroxyflavone	0.242[b]	–OH									
3,7-dihydroxyflavonol	0.228	–OH			–OH						
3,2′-dihydroxyflavonol	0.093	–OH						–OH			
galangin	0.516	–OH	–OH		–OH						
3,7,4′-trihydroxyflavone	0.641	–OH			–OH					–OH	
kaempferol	0.814	–OH	–OH		–OH					–OH	
fisetin	0.789	–OH			–OH				–OH	–OH	
morin	0.753	–OH	–OH		–OH			–OH		–OH	
quercetin	0.860	–OH	–OH		–OH				–OH	–OH	
quercitrin	0.341	–ORh	–OH		–OH				–OH	–OH	
rutin	0.107	–OGl–ORh	–OH		–OH				–OH	–OH	
robinetin	0.844	–OH			–OH				–OH	–OH	–OH
myricetin	0.919	–OH	–OH		–OH				–OH	–OH	–OH
isoflavones											
daidzein	0.285				–OH					–OH	
formononetin	0.079				–OH					–OCH_3	
genistein	0.381		–OH		–OH					–OH	
biochanin A	0.265		–OH		–OH					–OCH_3	
flavonones											
naringenin	0.288	–OH			–OH					–OH	
naringin	0.066	–OH			–OGl–ORh					–OH	
hesperetin	0.271	–OH			–OH					–OCH_3	
hesperidin	0.065	–OH			–OGl–ORh					–OCH_3	
dihydroflavonol											
taxifolin	0.321	–OH	–OH		–OH				–OH	–OH	

[a] Mean value of data collected in triplicate. Coefficients of variation for the measurement were typically just 0.90%. [b] Fifty percent methanol in 10 mM citrate buffer (pH 6.0) was used in assay of monohydroxyflavone binding to PVPP, rather than 25% methanol, the proportion used in the standard assay for the remaining flavonoids.

not only is hydrogen bonding to PVPP by a potential hydroxyl functionality blocked but access to PVPP by other hydroxyls in the molecule is hindered. For example, quercitrin and rutin are formed by adding mono- and disaccharide units, respectively, onto quercetin, and FB values decrease from 0.860 to 0.341 to 0.107.

From the FB values for monohydroxyflavones in Table I, it can be seen that the presence of a hydroxyl group at the 4′-position (FB = 0.815) and 7-position (FB = 0.364) results in highest binding. These positions are at the extremities of the flavone nucleus and are perhaps more accessible to the PVPP surface. The importance of the 4′-OH and 7-OH groups for binding is further exemplified when FB values of more highly substituted flavonoids are compared. 7,3′,4′-Trihydroxyflavone and 3,7,4′-trihydroxyflavone bind nearly 3 times better than do 3′,4′-dihydroxyflavone and 3,7-dihydroxyflavone, respectively.

In comparing flavonoids with identical hydroxyl substitution, such as apigenin, genistein, and naringenin (all 5,7,4′-trihydroxy compounds), it is clear that at least for these compounds the order of binding among classes is flavones > isoflavones > flavanones. The relatively poor binding of flavanones (and dihydroflavonols) is possibly due to the lack of planarity of the C ring. In comparing quercetin (FB = 0.860) and taxifolin (FB = 0.321), further evidence is provided to illustrate the poor binding of dihydroflavonols relative to flavonols with the same OH-substitution pattern. The fraction bound (FB) of the chalcone isoliquiritigenin was measured to be 0.850, which was higher than that of most other trihydroxyflavonoids.

The compounds morin and quercitrin were sufficiently soluble in citrate buffer (10 mM, pH 6.0) that their binding to PVPP could be measured in the absence of the methanol component of the standard assay solution. The FB for quercitrin increased from 0.341 (standard assay) to 0.442, and that of morin increased from 0.753 (standard assay) to 0.838. When in the aqueous system the molar ratio of PVPP to each compound was increased from 100:1 to 1000:1, quercitrin and morin possessed FBs of 0.760 and 0.922, respectively.

From the data presented in Figure 3, where FB values were determined as a function of quantity of PVPP in the assay mixture, we have determined FB values at infinite [PVPP]:[guest] ratios and this ratio required for half-maximal binding. These results are summarized in Table II, where the [PVPP]:[guest] ratio at half-maximal binding

Table II. For Representative Flavonoids, Fractions Bound (FB) at Infinite [PVPP]:[Guest] Ratios and [PVPP]:[Guest] Ratio at Half-Maximal Binding

guest	maximum FB[a] ± standard error	[PVPP]:[guest] at half-maximal binding
quercitrin	0.779 ± 0.013	118.06 ± 7.89
quercetin	0.947 ± 0.022	18.59 ± 2.65
apigenin	0.849 ± 0.016	45.66 ± 4.21
kaempferol	0.922 ± 0.007	16.75 ± 0.78
naringenin	0.684 ± 0.016	134.77 ± 12.50
taxifolin	0.715 ± 0.011	115.34 ± 7.52
genistein	0.585 ± 0.010	43.48 ± 3.86

[a] Determined from data presented in Figure 3; ± values represent standard error term.

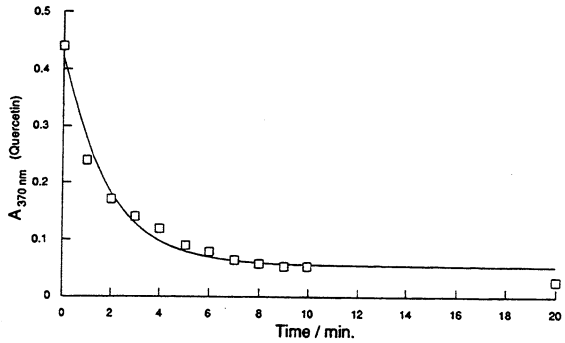


Figure 4. Determination of half-life to equilibrium of quercetin. See data analysis under Materials and Methods. For quercetin, $A = 0.37 \pm 0.02$, $k = 0.53 \pm 0.04\ \text{min}^{-1}$, and $A_\infty = 0.054 \pm 0.003$. ± Values represent the standard area term.

is a measure of the affinity of PVPP for each compound. The relatively high affinity of heavily hydroxylated and nonmethylated and nonglycosylated flavonoids is apparent. The half-lives to equilibrium for quercetin, quercitrin, and apigenin were calculated after the change in absorbance was determined over frequent time intervals, under standard assay conditions. These results are represented graphically in Figure 4 for quercetin, which has a half-life in solution of 1.31 min. Values for quercitrin and apigenin were 1.39 and 0.60 min, respectively.

CONCLUSIONS

A procedure was developed by which to monitor the binding to PVPP of representatives of each class of flavonoids. Methanol was included in the assay medium to provide solubility to all of the compounds tested. PVPP is a very effective binder of flavonoids, even in the presence of methanol and, considering the heterogeneous reaction system, at relatively low PVPP/flavonoid ratios. Binding, with a few exceptions, increases with the number of hydroxyl groups on the flavonoid nucleus. Compounds that contain 7- and 4′-hydroxyl groups bind most effectively. Our results suggest that PVPP incorporation into growth media will facilitate the trapping of soluble flavonoids (and other phenolics) for subsequent characterization and will also be useful in intercepting key signal compounds from plants to microbes. Binding will be even more effective than under the conditions described here, since the media are aqueous and the flavonoids are exuded at such low concentrations. Morin and quercitrin, for example, were shown to bind to a greater extent in an aqueous buffer medium than they did in the methanol-containing standard assay medium.

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