Gravimetric Determination of Tannins and their Correlations with Chemical and Protein Precipitation Methods

Harinder P S Makkar, Michael Blümmer, Norbert K Borowy and Klaus Becker*

Institute for Animal Production in the Tropics and Subtropics, University of Hohenheim, Box 700562, 7000 Stuttgart 70, Germany

(Received 3 September 1992; revised version received 13 October 1992; accepted 30 October 1992)

Abstract. A method for gravimetric determination of tannins based on binding with insoluble polyvinylpyrrolidone (PVP) is presented. The gravimetric method gives the absolute amount of tannins and avoids problems of standards associated with spectrophotometric methods. The method was applied to nine browse and tree leaves. The values obtained correlate significantly with tannins determined spectrophotometrically, protein precipitation capacities and protein precipitable phenolics. This method together with other tannin assays will be useful in nutritional studies. The present study also demonstrates the different behaviour of tannic acids from different commercial sources towards PVP suggesting the presence of different moieties in tannic acids from different commercial sources and even among batches from the same source thereby affecting the results obtained using the spectrophotometric methods. Use of well-defined tannic acid as a standard in spectrophotometric methods is suggested which will allow meaningful comparison of values obtained from different laboratories.

Key words: tannins, polyvinylpyrrolidone, gravimetric method, chemical methods, protein precipitation methods.

INTRODUCTION

Tannins are complex polyphenolic compounds with great structural diversity and wide phylogenetic distribution. Several methods are available for quantitation of tannins (see the reviews of Tempel 1982; Deshpande et al 1986; Makkar 1989). None of the available methods determines tannins in absolute terms but measures relative to one or the other standard, namely, tannic or gallic acids in methods based on the oxidation-reduction principle, catechin or quebracho tannins in methods of condensed tannin determination, and bovine serum albumin (BSA) as a standard protein in precipitation methods. Gravimetric methods do not suffer from disadvantages associated with colorimetric methods (Reed et al 1985). Gravimetric methods are not necessarily perfect—these have generally lower sensitivity than colorimetric methods.

The present method to quantify tannins gravimetrically originated in order to overcome the possible overestimation of dry matter digestibilities in tannin-rich feeds when determined gravimetrically as in the methods of Ørskov et al (1980), Tilley and Terry (1963) or in a modified Hohenheim gas test (Blümmer et al 1990). Under such experimental conditions tannins are solubilised but might be indigestible (Chesson 1981; Ørskov 1991) or partially digested (Makkar, H P S, unpublished). The present method is based on weighing the tannin extract before and after treatment with insoluble polyvinylpyrrolidone (PVP) and removal of the PVP by centrifugation. The PVP binds to tannins (Loomis and Battaile 1966) and therefore the difference in weight is due to the tannins. The colorimetric methods for tannin determination based on binding with the PVP are available (Laurent 1975; Watterson and Butler, 1983).

* To whom correspondence should be addressed.
EXPERIMENTAL

Materials

Air-dried samples of various leaves were used in the study (Table 1). PVP, BSA and catechin were obtained from Sigma (Deisenhofen, Germany). Tannic acids (TA) were from Merck (Darmstadt, Germany), lots from Sigma (Deisenhofen, Germany). Tannic acids (TA) respectively; and Sigma lot 120H3387, termed as II.

All other chemicals were of analytical grade. The disposable aluminium dishes (10 cm in diameter, weighing 2.5 g) were from Sartorius (Goettingen, Germany).

Methods

Preparation of tannin extract

Dried leaf (200 mg) ground to pass through an 80 mesh screen was suspended in 10 ml aqueous acetone (acetone:water, 7:3 v) in a centrifuge tube. Ice-cold suspensions were subjected to Ultra-turrax (Janke and Kunkel, IKA-Labortechnik, Heitersheim, Germany) treatment at 20000 rpm at 4°C (tubes kept in ice) for a total period of 6 min (content of total phenol (TP) in the supernatant as determined by Folin Ciocalteu (FC) reagent (see below) reached plateau after 5 min of the treatment) with cooling after every 2 min of the treatment. The tubes were centrifuged at about 2000 x g for 20 min and the supernatant collected was kept on ice until analysed (generally within 4 h).

Gravimetric determination of tannins

About 110 ml of the extract prepared as described above was divided into two portions of 65 ml and 45 ml. The PVP (6.5 g, 50 mg ml-1) was added to the former after diluting 1:1 with distilled water, the mixture stirred for 15 min at about 4°C, centrifuged and supernatant collected. Two disposable aluminium weighing dishes, one for the PVP treated and other for the untreated extracts were weighed. Aliquots (10 ml of untreated and 20 ml of the PVP treated extracts) were pipetted into the respective dishes and dried to a constant weight (about 30 min for the untreated and 60 min for the treated extracts) on a hot plate or in an oven at 100°C. The dishes were transferred to a desiccator and weighed when room temperature was achieved. This procedure of drying the aliquots and weighing was repeated three times to obtain four values for each treated and untreated extract. The values observed did not deviate by more than 5% from their mean. The average weight corresponded to 10 ml of the undiluted extracts. Thus, the difference in weight for the treated and untreated extracts gave the weight of tannins in 10 ml of the extract or 200 mg of the leaves. The results were expressed as percent tannins on dry matter basis.

Other analytical procedures

The FC method (Julkunen-Tiitto 1985) for TP was used for optimisation of the conditions for the gravimetric method. The difference in the TP values before and after the PVP treatment (unbound) represent the tannin levels gravimetrically.

The vanillin-HCl method of Broadhurst and Jones (1978) was used for determination of condensed tannins (CT) with some modifications which allow determination of CT in presence of acetone (Makkar and Becker 1993). The results are expressed as catechin equivalent. The CT were also determined by the proanthocyanidin assay using butanol-HCl-Fe3+ reagent (Porter et al 1986). Protein precipitation capacity (PPC), specific activity, protein precipitable phenolics (PPP) and percentage PPP were determined by the method of Makkar et al (1988).

### Table 1

<table>
<thead>
<tr>
<th>Name</th>
<th>Tannins (g kg⁻¹, gravimetrically)</th>
<th>TP (g kg⁻¹)</th>
<th>Tannins (g kg⁻¹)</th>
<th>CT (g kg⁻¹)</th>
<th>Proanthocyanidins (A₅₂₀ nm g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Panicum maximum</em></td>
<td>0</td>
<td>11.1 ± 0.4</td>
<td>2.2 ± 0.3</td>
<td>1.0 ± 0.04</td>
<td>13.04 ± 1.43</td>
</tr>
<tr>
<td><em>Dialium guineense</em></td>
<td>0.4</td>
<td>53.4 ± 2.5</td>
<td>39.1 ± 2.4</td>
<td>26.5 ± 0.2</td>
<td>1346 ± 70</td>
</tr>
<tr>
<td><em>Acioa barterii</em></td>
<td>85.0</td>
<td>96.6 ± 1.1</td>
<td>82.0 ± 1.1</td>
<td>72.7 ± 3.1</td>
<td>2686 ± 110</td>
</tr>
<tr>
<td><em>Quercus incana</em></td>
<td>63.5</td>
<td>71.1 ± 3.6</td>
<td>56.4 ± 3.6</td>
<td>12.3 ± 1.1</td>
<td>291 ± 5.4</td>
</tr>
<tr>
<td><em>Cassia sieberiana</em></td>
<td>21.0</td>
<td>40.5 ± 3.6</td>
<td>26.5 ± 3.0</td>
<td>6.6 ± 0.6</td>
<td>142.6 ± 2.9</td>
</tr>
<tr>
<td><em>Piliostigma reticulatum</em></td>
<td>60.7</td>
<td>50.2 ± 2.3</td>
<td>40.5 ± 1.9</td>
<td>20.7 ± 1.4</td>
<td>778 ± 20</td>
</tr>
<tr>
<td><em>Guiera senegalensis</em></td>
<td>60.6</td>
<td>60.1 ± 1.6</td>
<td>40.5 ± 2.3</td>
<td>9.9 ± 0.6</td>
<td>136.9 ± 4.7</td>
</tr>
<tr>
<td><em>Cajanus cajan</em></td>
<td>48.2</td>
<td>50.4 ± 2.2</td>
<td>34.4 ± 1.2</td>
<td>22.0 ± 0.5</td>
<td>394 ± 5.0</td>
</tr>
<tr>
<td><em>Dichrostachys cinerea</em></td>
<td>58.7</td>
<td>89.8 ± 1.6</td>
<td>65.5 ± 2.6</td>
<td>38.5 ± 0.5</td>
<td>672.7 ± 7.66</td>
</tr>
</tbody>
</table>

* The data are expressed as mean ± SD (n = 3 or 4) and are on dry weight basis.

# Difference of TP before and after PVP treatment using Folin Ciocalteu method.

" Tannic acid equivalent.

" Catechin equivalent.
The PPC was also determined as described by Asquith and Butler (1985) except that the reaction volume was 3 ml. To the BSA-dye (2 ml) containing 2 mg ml\(^{-1}\) BSA was added 50% methanol and then increasing amount of tannin-containing extract to make 3 ml. The slope of the BSA precipitation curves represents mg BSA precipitated per mg leaf. The extracts for the determination of PPC and PPP were in 50% aqueous methanol and not in aqueous acetone, as acetone interferes with the formation of the protein–tannin complex.

The PVP was purified using 10% v/HCl as described by Loomis (1974).

**RESULTS AND DISCUSSION**

**Effect of increasing amount of PVP**

Different quantities of the PVP were added to the solutions of TA I and III (2 mg ml\(^{-1}\)) prepared in water, 50% v/aqueous methanol and 70% v/aqueous acetone and stirred for 15 min at about 4°C. The TA as TP was determined using the FC method in the supernatant obtained after centrifuging the contents. In case of TA I, the PVP bound TA to a greater extent in aqueous methanol or aqueous acetone than in water, whereas on using TA III the binding was higher in water followed by aqueous methanol and aqueous acetone (Fig 1). PVP is known to bind tannins mainly through hydrogen bonds. Organic solvents, such as alcohols or acetone decreased the affinity of PVP towards tannins (Loomis and Battaile 1966). Higher binding of TA I to the PVP in aqueous acetone or aqueous methanol is therefore surprising. The TA I was at least 2 years old, but recently obtained tannic acid (TA II) also gave similar results except that the extent of binding to PVP was 1–10% higher for the new batch. Another point worth noting is the higher degree of binding of TA III to the PVP (Fig 1). These results suggest the presence of some moieties in TA I of non-tannin nature but with positive reaction in the FC reagent, and these moieties could differ from batch to batch. Hagerman et al (1992) also noticed different components in different preparations of TA. In 70% aqueous acetone, addition of the PVP beyond 50 mg ml\(^{-1}\) did not increase the binding of TA substantially (Fig 1). Therefore, 50 mg PVP ml\(^{-1}\) was added initially for binding the tannins of the leaf extracts with TP content < 2 mg TA equivalent ml\(^{-1}\). As binding efficiency of leaf tannins to the PVP could differ from that of the TA, the PVP at a level of 50 mg ml\(^{-1}\) was also added to 1:1 diluted (with 70% aqueous acetone) extracts, which is equivalent to 100 mg ml\(^{-1}\) of the undiluted extract. The levels of TP in the supernatant after the PVP treatment were lower in the diluted extracts of all the leaves studied, which gave higher values of tannin by 190% and 84% than that observed using undiluted extract for Acioa barterii and Dichostachys cinerea, respectively, and 1–6% for others. Further increase in the PVP did not increase the apparent tannin level substantially. In colorimetric methods, some workers (Loomis and Battaile 1966; Watterson and Butler 1983) have used the PVP after treatment with 10% HCl. After the HCl treatment and drying, clumps of PVP were formed. Loomis and Battaile (1966) have suggested not breaking the clumps, as it could lead to the formation of soluble PVP. The efficiency of HCl-treated PVP when used without breaking the clumps was poorer compared to untreated PVP (Fig 1). However, when the clumps were broken by grinding, there was no difference in the binding efficiencies of the treated and untreated PVP, suggesting that poorer binding of HCl-treated PVP in the clump form was due to lesser surface area available for binding to TA.

**Tannin levels by gravimetric, chemical and protein precipitation methods**

It is interesting to compare the tannin values obtained by the present method with the FC method (Table 1). For *Cassia sieberiana* and *D cinerea*, the values obtained by the gravimetric method were lower than those by the colorimetric method. Tannins in *Panicum maximum* were too low to be measured by the gravimetric method (Table 1). Phenolic compounds of different structures give different response to the FC reagent. The difference in values obtained by these two methods may be due to the different nature of tannin of the leaves and the TA. Each g of tannins from different sources has different reducing powers varying from 0.75 to 1.26 g TA equivalent (ratio of colorimetric to gravimetric values; Table 1), and also different protein binding/precipitating capacities (Table 2), suggesting different biological
TABLE 2
Tannin levels by protein precipitation methods

<table>
<thead>
<tr>
<th>Name</th>
<th>PPC (mg BSA precipitated per mg leaf)</th>
<th>PPP (μg TA equivalent per mg leaf)</th>
<th>Specific activityb</th>
<th>PPP (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dye-binding method</td>
<td>Makkar et al (1988) x</td>
<td>y</td>
<td></td>
</tr>
<tr>
<td>Panicum maximum</td>
<td>ndc</td>
<td>0.106 ± 0.002</td>
<td>8.40 ± 0.55</td>
<td>13.25</td>
</tr>
<tr>
<td>Dialium guineense</td>
<td>0.161 ± 0.002</td>
<td>0.124 ± 0.006</td>
<td>19.47 ± 1.49</td>
<td>6.37</td>
</tr>
<tr>
<td>Acioa barterii</td>
<td>0.604 ± 0.007</td>
<td>0.634 ± 0.008</td>
<td>34.44 ± 0.66</td>
<td>18.41</td>
</tr>
<tr>
<td>Quercus incana</td>
<td>0.338 ± 0.007</td>
<td>0.124 ± 0.006</td>
<td>19.47 ± 1.49</td>
<td>6.37</td>
</tr>
<tr>
<td>Cassia sieberiana</td>
<td>0.067 ± 0.006</td>
<td>0.043 ± 0.003</td>
<td>6.10 ± 0.59</td>
<td>6.97</td>
</tr>
<tr>
<td>Ptilistigma reticulatum</td>
<td>0.185 ± 0.004</td>
<td>0.086 ± 0.002</td>
<td>12.24 ± 0.33</td>
<td>7.03</td>
</tr>
<tr>
<td>Guiera senegalensis</td>
<td>0.161 ± 0.008</td>
<td>0.078 ± 0.002</td>
<td>8.91 ± 1.05</td>
<td>8.75</td>
</tr>
<tr>
<td>Cajanus cajan</td>
<td>0.170 ± 0.012</td>
<td>0.060 ± 0.004</td>
<td>9.05 ± 0.27</td>
<td>6.63</td>
</tr>
<tr>
<td>Dichostachys cinerea</td>
<td>0.507 ± 0.006</td>
<td>0.287 ± 0.006</td>
<td>32.10 ± 0.53</td>
<td>8.94</td>
</tr>
</tbody>
</table>

* The data are expressed as mean ± SD (n = 3 or 4) and are on dry weight basis.

b mg BSA pptd/mg TA equivalent (x/y, y in mg TA equivalent/mg leaf).

c nd—not detected.

PPC, protein precipitation capacity; PPP, protein precipitable phenolics.

TABLE 3
Correlations between tannin levels obtained by different methods

<table>
<thead>
<tr>
<th></th>
<th>TP</th>
<th>Tannins-C</th>
<th>CT</th>
<th>PAs</th>
<th>PPC-dye binding</th>
<th>PPC-Makkar et al (1988)</th>
<th>PPP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tannins-G</td>
<td>0.88**</td>
<td>0.91***</td>
<td>0.69*</td>
<td>0.63</td>
<td>0.79*</td>
<td>0.67*</td>
<td>0.75*</td>
</tr>
<tr>
<td>TP</td>
<td>0.99***</td>
<td>0.80**</td>
<td>0.63</td>
<td>0.95***</td>
<td>0.95***</td>
<td>0.94***</td>
<td></td>
</tr>
<tr>
<td>Tannins-C</td>
<td>0.84**</td>
<td>0.70*</td>
<td>0.96***</td>
<td>0.87**</td>
<td>0.96***</td>
<td>0.84**</td>
<td></td>
</tr>
<tr>
<td>CT</td>
<td>0.93***</td>
<td>0.70*</td>
<td>0.96***</td>
<td>0.87**</td>
<td>0.96***</td>
<td>0.84**</td>
<td></td>
</tr>
<tr>
<td>PAs</td>
<td>0.70*</td>
<td>0.88**</td>
<td>0.90***</td>
<td>0.99***</td>
<td>0.99***</td>
<td>0.88**</td>
<td></td>
</tr>
<tr>
<td>PPC-dye binding</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* P < 0.05; ** P < 0.01; *** P < 0.001.

Tannins-G, Tannins-C, tannins measured gravimetrically and colorimetrically respectively; TP, total phenols; CT, condensed tannins; PAs, proanthocyanidins.

responses of different tannins even at the same level in animal diets. This emphasizes the need to study structure–activity relationship of polyphenols. Insoluble PVP also binds to simple phenols (Loomis 1974). However, contribution of simple phenols towards observed tannin levels would be negligible, as strength of binding of phenols to the PVP is in proportion to the number of phenolic hydroxyl groups (Clifford 1974) and presence of organic solvents decrease the extent of binding (Anderson and Sowers 1968). The values in Table 1 for the tannin levels (as tannic acid equivalent) are obtained from the calibration curve using TA I. The ratio of slopes of the curves (amount of tannic acid, x-axis versus absorbance at 725 nm, y-axis) for the TA I:TA III was 1:07 (moisture content of both TA I and III was similar), which would give higher values for tannins by 7% on using TA III as a standard. The PPC of TA III was also substantially lower than of TA I (Makkar, H P S, unpublished). These observations together with different affinities of different TAs towards the PVP (see above) suggests the need to use well-defined standards. Recently, Hagerman et al (1992) have fractionated TA into different fractions using HPLC. It is suggested that the chemical companies should produce well-characterised tannic acid (analogous to fraction V of BSA used as a standard in protein assays). Use of such a standard would ensure reproducibility between and within laboratories, and would allow meaningful comparison of values obtained from different laboratories.

There was wide variation in the tannin levels in the leaves studied (Tables 1 and 2). Tannin levels were negligible in P maximum and high in A barterii. About 89% of the TP bind protein, and the protein-binding affinity of phenols as evident from the specific activity was also highest in A barterii (Table 2). The PPC by the dye-binding method (Asquith and Butler 1985) were
Correlations between gravimetric and other methods

The best correlation of values obtained gravimetrically was with tannins ($r = 0.91$, $P < 0.001$), followed by TP ($r = 0.88$, $P < 0.01$), PPC-dye binding ($r = 0.79$, $P < 0.05$), CT ($r = 0.69$, $P < 0.05$) and PPC-Makkar et al (1988) ($r = 0.67$, $P < 0.05$). The PPC values are the slopes of the curves which do not represent the true values as these do not account for the intercept. Poorer correlation with PPC methods could be due to the inherent disadvantage of the BSA precipitation curves generated by increasing the tannin-containing extract in an assay containing fixed amount of protein.

The gravimetric method in combination with other methods may be useful in providing better insight into the effect of tannins on biological systems. It would also help in the proper interpretation of dry matter digestibilities for tannin-rich feeds obtained gravimetrically by in-sacco and in-vitro methods.

ACKNOWLEDGEMENT

One of the authors (HPSM) is thankful to the Alexander von Humboldt Foundation, Bonn for awarding the AvH-doctoral Research Fellowship under which this work was undertaken. The authors are thankful to Dr H. Steingass, Institute for Animal Nutrition and Dr M. Yuonan for supplying the leaves.

REFERENCES


Clifford MN 1974 The use of poly-n-vinylpyrrolidone as the adsorbent for the chromatographic separation of chlorogenic acids and other phenolic compounds. *J Chromatogr* 94 261–266.


Laurent S 1975 Étude comparative de différentes méthodes d’extraction et de dosage des tannins chez quelques pteridophytes. *Arch Int Physiol Biochem* 83 735–752.


