Analysis of Condensed Tannins Using Acidified Vanillin

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(Manuscript received 23 January 1978)

The acidified vanillin method for determination of plant tannins has been reassessed. Excluding light and controlling the temperature of the reaction mixture increased the colour stability of the vanillin tannin adduct. Selection of optimum concentrations of reactants and solvents enabled the method to be used in the range 5–500 μg of condensed tannin with a precision and accuracy greater than 1 μg.

1. Introduction

The occurrence of condensed tannins (polymeric flavanols) in forage legumes1,2 is of interest since these substances may affect ruminant performance. The presence of tannins has been associated with control of bloat,3 improved protein utilisation4–6 and reduced digestibility and palatability of the forage.7,8

Investigations of these effects rely in part on estimates of tannins in the forage and on their astringency or reactivity with proteins.6,9 A convenient method for determining astringency has been developed.10 A number of colorimetric methods which vary in the degree of discrimination against non-tannin phenolics, also present in the plant material, have been employed to estimate tannin.11–13 Flavanols (condensed tannins, monomers, dimers, etc)14,15 unlike the majority of natural phenolic compounds16,17 react with vanillin in acidic medium to yield a coloured product18 with an absorbance maximum at 500 nm. The vanillin method for estimating tannin is most attractive in that it is specific but suffers from reported lack of reproducibility between samples, days and laboratories.11–13

A method for tannin estimation has been developed with improved reproducibility and sensitivity.

2. Experimental

2.1. Materials

Leaves and roots were collected from individual plants of Lotus pedunculatus, Lotus tenuis, Coronilla varia and Onobrychus vicifolia grown in a glasshouse. Condensed tannins isolated from these plants15 were used as standards. The physical and chemical properties and purity of these condensed tannins have been reported.15 The tannins are stable when stored as freeze-dried solids under dessicants in the dark at 5°C. All other chemicals and solvents were AR grade. Reactions were carried out in glass tubes (14 × 120 mm) which had been thoroughly cleaned by soaking in detergent for 24 h.

2.2. Tannin extraction

Plant tissue (0.2–1 g fresh weight) was macerated in glass–glass homogenisers (Davil type, Kontes Glass Company, Vineland, New Jersey) with 70% v/v acetone: water (tissue weight: vol., 1:3), containing 0.1 % w/v ascorbic acid. The macerate and washings were centrifuged (2000 g, 5 min) and the supernatant liquid transferred to a separating funnel. The residue was re-extracted (5 ×) with 70 % acetone. The combined extracts, on saturation with NaCl, separated into a lower aqueous and upper acetone phase.19 The aqueous phase was re-extracted with the upper (acetone) phase of a NaCl saturated solution of 70 % acetone. The combined acetone phases were evaporated to remove
acetone. Water (2 ml) was added to the solution and extracted (3×) with diethyl ether and then (3×) with ethyl acetate. Traces of solvent were removed from the aqueous phase by rotary evaporation. The aqueous solution (containing condensed tannins) was made up to a standard volume (5 or 10 ml).

2.3. Preparation of standards
Purified tannin,15 isolated from the species under investigation, was dissolved in distilled water to give a stock solution of 1 mg ml⁻¹. Dilutions of the stock solution were made to give final concentrations of 10 to 1000 μg tannin ml⁻¹. The standards were made up fresh each day.

2.4. Instrumentation
Absorbance (500 nm) was determined either on a Unicam SP800 or a Hilger Watts ‘Uvichem’ spectrophotometer.

2.5. Environmental variables
In preliminary studies it was found that light and temperature conditions were important variables and they were therefore examined in detail.

The effect of light on complex formation and stability was studied on reaction mixtures (a) exposed to direct sunlight, (b) in diffuse sunlight and (c) in tubes covered with aluminium foil to exclude light. In all later experiments, tubes were covered with aluminium foil.

Complex formation and stability was measured at 15, 20 and 25°C to cover the range of temperature normally encountered in laboratories.

2.6. Reagent variables
Sample volume was 0.5 ml (100 μg of tannin in water) and the total reaction volume was 5 ml. The concentrations of vanillin, hydrochloric acid and methanol were varied.

Studies on the effect of hydrochloric acid concentration were performed using (a) 0.5 ml sample or water, (b) 1.5 ml of 8% w/v vanillin in methanol and (c) 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 ml of concentrated hydrochloric acid diluted with water to a final volume of 3.0 ml. The effect of methanol concentration was determined in a reaction mixture containing 0.5 ml of sample or water, a final concentration of 2.4% w/v vanillin and hydrochloric acid (up to 3.0 ml) diluted with methanol instead of water as in the previous experiment.

Vanillin concentration was varied from 0.3 to 7.2% w/v final concentration. The reaction mixture contained 0.5 ml of sample or water, 3.0 ml of vanillin in methanol and 1.5 ml of concentrated hydrochloric acid.

2.7. Interferences
Interference from anthocyanins in tannin estimation were determined by measuring absorbance at 500 nm of solutions containing 0–500 μg anthocyanin (0.5 ml sample volume) after the addition of the following reagents: (i) 3.0 ml 4% w/v vanillin in methanol and 1.5 ml concentrated hydrochloric acid or (ii) 3.0 ml methanol and 1.5 ml concentrated hydrochloric acid. The experiment was repeated on solutions of anthocyanins also containing 50 μg tannin and the interference from anthocyanin in the tannin estimation was determined.

Since ascorbate and sodium chloride concentration could vary in the final extract their effect on complex formation was measured.

2.8. Tannin determination using other methods
Tannins were determined using the vanillin/HCl method of Burns11 and the vanillin/H2SO4 method of Swain and Hillis.13

3. Results and discussion
3.1. Effect of light and temperature on complex formation
The stability of the tannin/vanillin complex was greatly affected by the light conditions (Table 1) and to a lesser extent, the temperature at which the experiment was performed. Exposure of the
Table 1. Effect of light on stability of the tannin–vanillin complex

<table>
<thead>
<tr>
<th>Light condition</th>
<th>Maximum absorbance 500 nm (at 15 min)</th>
<th>Decrease in absorbance maximum (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>At 25 min</td>
</tr>
<tr>
<td>Tubes in dark</td>
<td>1.285</td>
<td>&gt;0.4</td>
</tr>
<tr>
<td>Diffuse light</td>
<td>1.265</td>
<td>2.5</td>
</tr>
<tr>
<td>Sunlight</td>
<td>1.260</td>
<td>4.5</td>
</tr>
</tbody>
</table>

250 μg of *Lotus pedunculatus* tannin estimated at 500 nm.

![Absorbance spectra](image)

**Figure 1.** Visible spectra for vanillin–tannin adducts. A, Spectra after 5 min for both B and C; B reaction carried out in darkened tubes (after 60 min); C, reaction mix exposed to light (after 60 min).

reaction mixture to light caused a change in absorbance spectra (Figure 1), accompanied by a decrease in absorbance at 500 nm. This decline in absorbance could be prevented by covering the reaction tubes with aluminium foil to exclude light. This enabled samples to be measured 15 to 60 min after mixing with errors of ~ 1 μg tannin.

Temperature (15–25°C) affected the rate of reaction (Table 2) rather than the stability of the complex. Twenty minutes\(^1\)\(^,\)\(^2\) after mixing a 6% difference in absorbance was observed for reactions carried out at 20°C compared with 15°C.

Errors which resulted from light and temperature conditions could account for the variation previously reported for the method.\(^1\)\(^,\)\(^2\) By enclosing the reaction tubes in aluminium foil and

Table 2. Effect of temperature on complex formation

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Time to reach maximum absorbance 500 nm (min)</th>
<th>Error of readings taken at 20 min(^a) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>35</td>
<td>6</td>
</tr>
<tr>
<td>20</td>
<td>15</td>
<td>&lt;1</td>
</tr>
<tr>
<td>25</td>
<td>12.5</td>
<td>&lt;1</td>
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\(^a\)Previous methods\(^1\)\(^–\)\(^3\) have measured absorbance (500 nm) 20 min after mixing reagents.
carrying out the experiments in a laboratory at a controlled temperature, samples could be measured between 15 and 60 min with a precision $> \pm 1 \mu g$ tannin.

3.2. Effect of reagent concentration on complex formation

Sensitivity of the method was increased when hydrochloric acid and methanol concentrations were increased (Figure 2).

In 60% v/v hydrochloric acid the sensitivity was twice that at 30% v/v acid. However at concentrations $> 30$% v/v hydrochloric acid the absorbance at 500 nm increased with time. The stability of the tannin complex and the corresponding blanks (no tannin) are shown in Figure 3. The change in absorbance was due to the slow self-reaction of vanillin in strong acids and not to the vanillin–tannin complex which was stable ($< 1$% variation in absorbance of sample minus blank). To eliminate errors arising from variable blank corrections and to achieve maximum sensitivity, the hydrochloric acid and methanol concentrations were maintained at 30% v/v and 60% v/v respectively.

![Figure 2](image2.png)

Figure 2. Effect of hydrochloric acid and methanol concentrations on absorbance (500 nm) of the tannin–vanillin complex. •, HCl diluted with water; ○, HCl diluted with methanol.

![Figure 3](image3.png)

Figure 3. Change in absorbance (500 nm) with time at different hydrochloric acid concentrations. (a) 30% acid blank; (b) 30% acid sample; (c) 60% acid blank; (d) 60% acid sample; (d–c) 60% sample minus 60% blank.
The sensitivity of the tannin–vanillin reaction increased sharply with increase in vanillin concentration up to 2.4% final concentration but was relatively insensitive to vanillin above this concentration (Figure 4). The reagent concentrations chosen for optimum sensitivity and reproducibility of the reaction were 2.4% w/v vanillin, 30% v/v concentrated hydrochloric acid and 60% v/v methanol.

Figure 4. Effect of vanillin concentration on complex formation (100 μg tannin).

3.3. Sources of possible interferences in method

Ascorbate and NaCl used in the extraction of tannins had no effect on the sensitivity or stability of the reaction. This is in contrast to the vanillin/H₃SO₄ method¹ in which ascorbate affected the sensitivity (Broadhurst, R. B, unpublished data) of the reaction.

Sakar and Howarth¹⁶ reported that dihydrochalcones and anthocyanins react with acidified vanillin to give a similar product to tannins. Dihydrochalcones are removed in the ether fraction during extraction of impurities (section 2.2). Anthocyanins (which are observed as red–blue pigments) are usually at low concentration in leaf and root tissue but can be high in flowers and fruit. Therefore they constitute the only possible interference with this method. At concentration ≤1 mg

<table>
<thead>
<tr>
<th>Anthocyanin in 0.5 ml sample volume (μg)</th>
<th>Absorbance (500 nm)</th>
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<tbody>
<tr>
<td>0</td>
<td>0.002  0.002   0.204  0.202</td>
</tr>
<tr>
<td>20</td>
<td>0.010  0.008   0.211  0.203</td>
</tr>
<tr>
<td>50</td>
<td>0.024  0.023   0.228  0.205</td>
</tr>
<tr>
<td>100</td>
<td>0.049  0.045   0.252  0.207</td>
</tr>
<tr>
<td>300</td>
<td>0.137  0.131   0.333  0.202</td>
</tr>
<tr>
<td>500</td>
<td>0.237ₐ 0.217   0.437  0.220</td>
</tr>
</tbody>
</table>

1. Anthocyanin + 3 ml of 4% w/v vanillin in methanol + 1.5 ml concentrated hydrochloric acid.
2. Anthocyanin + 3.0 ml methanol + 1.5 ml hydrochloric acid.
3. Same as (1) but 50 μg sainfoin tannin added to sample.
4. Absorbance due to tannin (3 minus 2).
ₐ Anthocyanin contains some measurable vanillin reactive material, possibly grape tannin.
ml⁻¹, interference by anthocyanins can be eliminated by carrying out the reaction as for tannin estimation but in the absence of vanillin and subtracting the absorbance (at 500 nm) from that obtained with the vanillin present (Table 3).

Higher concentrations of anthocyanins can be reduced to workable levels by back extraction of the initial acetone phase with NaCl saturated aqueous phase (ex. 70% v/v acetone/H₂O:2.2). However this has only been necessary when extracting skins of black grapes (e.g. Pinot Noir). The method has been used to determine the tannin content of leaf, root, flower and fruits of several plant species as well as the determination of free tannin in animal digesta.

3.4. Procedure for tannin estimation

3.4.1. Stock solutions
1. Concentrated hydrochloric acid.
2. Freshly prepared 4% w/v vanillin in methanol.
3. Methanol.

3.4.2. Procedure
1. Wrap thoroughly cleaned tubes in aluminium foil.
2. Pipette 0.5 ml of sample in water into tube.
3. Add 3.0 ml of vanillin reagent to sample and mix thoroughly.
4. Add 1.5 ml of concentrated hydrochloric acid and mix thoroughly.
5. Allow reaction mixture to stand for 15 min at 20 ± 2°C.
6. Read samples and blank against water at 500 nm.
7. Prepare standard curves as above using tannin standards (section 2.3).
8. Correct for presence of anthocyanins (section 3.3).

3.5. Comparison with other methods
A comparison of previously reported methods¹¹⁻¹³ with the method reported in this paper is shown in Table 4 and the standard curves in Figure 5.

<table>
<thead>
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<th>Table 4. Comparison of reagent concentration for three methods</th>
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<tbody>
<tr>
<td>Vanillin conc. (%)</td>
</tr>
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<td>-------------------</td>
</tr>
<tr>
<td>New method</td>
</tr>
<tr>
<td>Burns¹¹ and Maxson¹²</td>
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<tr>
<td>Swain¹³</td>
</tr>
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</table>

₄ Hydrochloric acid.
⁵ Sulphuric acid.

Our method is ≅ 3.3 times as sensitive as other vanillin/HCl¹¹,¹² methods and equivalent in sensitivity to the vanillin/H₂SO₄¹³ method. The lower sensitivity of the Burns¹¹ and Maxson¹² method is a result of the lower HCl concentration (3.3% v/v) used by these workers.

Of the previously reported methods, the vanillin/H₂SO₄¹³ reagent gave the greatest colour stability. If light is excluded in the vanillin/H₂SO₄ method, it becomes equivalent in reproducibility and sensitivity to the vanillin/HCl method developed in this paper. The vanillin/H₂SO₄¹³ method does however suffer from interference by ascorbate (Broadhurst unpublished) due presumably to the oxidative nature of sulphuric acid.
4. Conclusion

The vanillin/hydrochloric acid method reported in this paper is more sensitive and not subject to the variability previously reported in acidified vanillin methods.\textsuperscript{11-13} This was achieved by optimising the reagent concentrations and controlling external variables (light and temperature). Interferences from other phenols reported to react with the vanillin reagent\textsuperscript{16} have been eliminated by the extraction procedure and suitable blank corrections.

Acknowledgments

The authors wish to thank Dr J. Wilson and Dr E. Wong, Applied Biochemistry Division, DSIR, Palmerston North, New Zealand, for helpful advice during the course of this work.

References