Quantification of Tannins in Tree Foliage

A laboratory manual for the FAO/IAEA Co-ordinated Research Project on 'Use of Nuclear and Related Techniques to Develop Simple Tannin Assays for Predicting and Improving the Safety and Efficiency of Feeding Ruminants on Tanniniferous Tree Foliage'

JOINT FAO/IAEA DIVISION
OF NUCLEAR TECHNIQUES IN FOOD AND AGRICULTURE
Animal Production and Health Sub-Programme

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Foreword

Tanniniferous trees and shrubs are of importance in animal production because they can provide significant protein supplements, but unfortunately the amounts of tannins that they contain vary widely and largely unpredictably, and their effects on animals range from beneficial to toxicity and death. The toxic or antinutritional effects tend to occur in times of stress when a very large proportion of the diet is tanniniferous. With a better understanding of tannin properties and proper management, they could become an invaluable source of protein for strategic supplementation. As the demand for food rises, tanniniferous plants must play an increasingly important part in the diet of animals, in particular for ruminants in small-holder subsistence farming in developing countries. It is therefore critical that techniques be developed to measure and manage the anti-nutritional components that they contain.

Keeping the above in mind, a Joint FAO/IAEA Co-ordinated Research Project (CRP) on "Use of Nuclear and Related Techniques to Develop Simple Tannin Assays for Predicting and Improving the Safety and Efficiency of Feeding Ruminants on Tanniniferous Tree Foliage" has been initiated. In order to provide sound basis for this CRP, an FAO/IAEA Consultants Meeting was held in August 1997 in Vienna, at which the tanniniferous plants to be studied, the analytical methods, the test animals and the animal response evaluation techniques were defined.

This publication contains methodologies for the analysis of tannins using chemical-, protein precipitation/binding- and bio-assays recommended by the consultants. Dr. Ann Hagerman has contributed the procedure for radio-labelled assay and Dr. Irene Mueller-Harvey for characterization of phenolic compounds using Thin Layer Chromatography. Harinder P.S. Makkar was responsible for compiling the detailed experimental protocols presented in this manual, and for publication of this document.

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1. INTRODUCTION

Tannins are generally defined as naturally occurring polyphenolic compounds of high enough molecular weight to form complexes with proteins. These are classified into two groups based on their structural types a) hydrolysable tannins and b) condensed tannins.

Methods for quantification of tannins may be based on the chemical properties of tannins or their capability to bind substrates, particularly proteins. The methods described here can be categorized as follows:

A. Chemical methods

(a) *Determination of total phenolics*. The method is based on the fact that phenolics are reducing agents. It may be noted that all tannins are phenolics but not all phenolics are tannins.

(b) *Determination of total tannins*. It is partly chemical, based on reducing property of tannins, and partly physical because tannins are measured as the reduction in phenolics that occur when a binding agent (polyvinyl polypyrrolidone, PVPP) is added to the extract.

(c) *Determination of condensed tannins (proanthocyanidins)*. It is based on oxidative depolymerization of condensed tannins in butanol-HCl reagent. The presence of iron is considered to increase the reproducibility and sensitivity of the assay.

(d) *Determination of gallotannins*. It is based on hydrolysis of gallotannins to gallic acid under acidic conditions and measurement of the released gallic acid by reaction with rhodanine or by HPLC.

B. Protein precipitation/binding methods

(a) *Determination of protein precipitable phenolics*. It is based on the formation of tannin-protein complexes (tannins in the plant extract and the protein, bovine serum albumin). Tannins present in the complex are determined using ferric chloride assay for total phenolics. Iron forms a complex with phenols to give a pink chromatophore which is measured spectrophotometrically.

(b) *Filter paper-protein Ponceau S dye assay*. The protein-tannin complexes are formed on a sheet of filter paper, and the protein bound to the complex is measured by dyeing it with Ponceau S dye. This dye is specific for proteins and does not bind tannins. The colour of the dye bound to proteins is eluted and measured spectrophotometrically.

(c) *Radial diffusion assay*. In this method, tannin molecules migrate through agarose gel which is impregnated with the protein, bovine serum albumin (BSA). The tannin-protein complex is formed in the gel which appears as a ring. The diameter of the ring is a measure of protein precipitation/binding capacity of tannins.

C. Tannin bioassay

The feeds under investigation (or tannins) are incubated in absence and presence of a tannin-complexing agent, polyethylene glycol (PEG) in the *in vitro* rumen fermentation system containing rumen microbes. The affinity of PEG for binding to tannins is very high; it even breaks the already formed tannin-protein complexes and releases proteins from the complex. In the *in vitro* rumen fermentation system, release of gas and production of microbial mass are

measured. The difference between these parameters observed in absence and presence of PEG is a measure of tannin activity in relation to their effects on rumen fermentation.

2. PREPARATION OF PLANT EXTRACT

The general problem in the study of natural plant products is that their nature and amount are dependent on various factors, which must be controlled as far as possible. Some of these factors are: i) stress; the metabolic state of the plant may change when it is stressed in any manner. This can be a problem before as well as after harvesting a plant part for analysis. As cells die (the senescent process), the cellular integrity is lost and as a result the enzymes come in contact with substrates to which they are not normally exposed in living cells. In addition, it also increases the oxidation process, which is a problem with phenolics since these are prone to oxidation. On oxidation, phenolics oxidise to quinones and then polymerization reaction could follow. If a plant is cut and dried under 'near ambient' conditions, which generally requires a large time to dry, the nature and content of phenolic compounds can change. In order to avoid these changes, the metabolic activities of the cells need to be curbed immediately. The next important step is to bring the chemical constituents into solutions for their measurement.

2.1. Collection, drying and storage of plant material

Leaf age and stage of development affect levels and nature of phenolics. Therefore, it is important to define the stage of maturity of plant and leaf as close as possible before collecting leaf material for analysis. When the collection site is close to the laboratory, the material can be transported to the laboratory in fresh state. The fresh material should be kept on ice and transported under dark conditions. Transportation of large amount of leaves in plastic bags should be avoided, since temperature in the bag could rise leading to sweating and wilting which can change the nature and level of phenolics. If liquid nitrogen is available, the better option is to freeze the sample and then freeze-dry the material without thawing it. Thawing can rupture cell membranes leading to changes in phenolics. If the material is frozen using a freezer, make sure that the material is not thawed during transport. Solid carbon dioxide should be used to transport such material. Once the material is dried, it should be kept in a dry place (preferably in a desiccator) in the dark. The freeze-dried material generally is hygroscopic. Light is also known to change the nature of phenolics. After freeze-drying, the cell structure is broken and the enzymes are in the native state. With the absorption of water, enzymes and phenolics can react, which can produce drastic changes in phenolics. The freeze-drying, though considered to be one of the safest method for preservation of phenolics, can lead to drastic changes if the storage conditions are not appropriate.

If a lyophilizer is not available, the plant material has to be dried under far from ideal conditions. The sample can be dried at about 50–52°C using a forced air oven. This will hasten the process of drying, and the enzymes present in the plant sample will not have much time to react with phenolics. Drying at temperatures higher than 55°C should be avoided, since it can lead to inactivation of phenolics or could decrease their extractability in solvents and affect the quantification.

Since the amount of plant material required for *in vivo* studies is large, the material should be dried under shade. In places of high humidity, use of fans is advised. The sample should be stored in a cool, dark and dry place only after it has dry matter content of > 90 %. A representative sample should be kept for tannin analysis. Representative samples should be taken just before storage and then again before feeding to livestock, and both sets of samples

should be analysed. If possible, a representative sample of the fresh leaf material should also be frozen as soon as collected and freeze-dried.

2.2. Grinding of sample

Fresh or frozen materials are difficult to work with. Grinding could be a problem using these materials. Fresh material, when frozen using liquid nitrogen, can be ground using 'Polytron' homogenizers. One has to be cautious that the temperature does not rise during homogenization; increase in temperature can lead to enzymatic changes in phenolics. Phenolics are generally extracted in aqueous organic solvents. The moisture present in the fresh material needs to be taken into account while preparing organic solvents for extraction.

It is suggested to grind the sample after drying the sample. About 500 g of the plant material should be ground first to pass a 2 mm screen. All the ground material including those parts remaining inside the mill should be taken, mixed well and approximately 100 g of this sample is again ground to pass through a 0.5 mm screen. This sample should be taken for tannin analysis. If the ground samples in a desiccator are kept in a refrigerator, the desiccator must be opened after the contents has reached the ambient temperature, otherwise moisture will condense on the sample which will lead to changes in the state of phenolics during storage.

For fibre analysis, and *in situ* nylon bag and *in-vitro* gas techniques, the sample passed through 2 mm screen should be taken.

Note:.

Take care that at any stage of the grinding, the sample temperature does not rise above 40 C.

2.3. Extraction of tannins

The aim is to quantitatively diffuse phenolics present in the plant material to liquid phase. For the extraction process, a suitable solvent is required. Generally, aqueous methanol (50%) and aqueous acetone (70%) are popular choices. The latter has been reported by various workers to be better in extracting phenolics from tree leaves. One can try both these solvents for extraction, and then based on the efficiency of extraction of phenolics (using Folin-Ciocalteu method) and/or condensed tannins (using butanol-HCl method), one can decide the solvent to be used for a particular plant material.

Dried (finely ground) plant material (200 mg) is taken in a glass beaker of approximately 25 ml capacity. Ten ml of aqueous acetone (70%) is added and the beaker is suspended in an ultrasonic water bath (Branson 3210) and subjected to ultrasonic treatment for 20 min at room temperature. The contents of the beaker is then transferred to centrifuge tubes and subjected to centrifugation for 10 min at approximately 3000g at 4°C (if refrigerated centrifuge is not available, cool the contents by keeping the centrifuge tube on ice and then centrifuge at 3000g using an ordinary clinical centrifuge). Collect the supernatant and keep it on ice. Transfer the pellet left in the centrifuge tube to the beaker using two portion of 5 ml each of 70% aqueous acetone and again subject the contents to ultrasonic treatment for 20 min. Centrifuge and collect supernatant as described above. Folin-Ciocalteu method is used for determination of total phenols in the two supernatants. Under our conditions (when the sample was ground using a rotating-ball mill) the particle size of the ground plant material was very fine and the recovery of total phenols in the second supernatant was < 5% of that in the first supernatant. Therefore, the second extraction step was omitted. One can follow this approach and decide to extract the sample once or twice.

Notes:

Very long extraction at too high a temperature may lead to degradation and loss of phenolics.

Freshly prepared extract should be used for tannin analysis.

Tubes/container containing the extract should be kept on ice till the analysis is complete.

Pigments and fat can be removed from the dried leaf sample by extracting with diethyl ether containing 1% acetic acid before extracting tannins.

3. MEASUREMENT OF TOTAL PHENOLICS AND TANNINS USING FOLIN-CIOCALTEU METHOD

(According to Makkar *et al.* [1])

The method for total phenol is useful in order to know the efficiency of extraction of phenolics in solvents. This method can be coupled with the use of insoluble matrix, polyvinyl polypyrrolidone (PVPP; binds tannin-phenolics) for measurement of tannins (see below). The results can be expressed as tannic acid equivalent. The nature of tannic acid varies from one commercial source to the other [2]. Tannic acid from Merck was found to be the best; highest efficiency of binding to BSA, the least threshold at which it starts precipitating BSA and also the highest change in absorption per unit change in tannic acid level. Furthermore, there does not seem to be batch-to-batch variation in tannic acid from Merck. It is therefore suggested to use tannic acid from Merck.

3.1. Reagents

(1) *Folin Ciocalteu reagent (1 N):* Dilute commercially available Folin-Ciocalteu reagent (2 N) with an equal volume of distilled water. Transfer it in a brown bottle and store in a refrigerator (4°C). It should be golden in colour. Do not use it if it turns olive green.

(2) *Sodium carbonate (20%):* Weigh 40 g sodium carbonate (x10 H_2O), dissolve it in about 150 ml distilled water and make up to 200 ml with distilled water.

(3) *Insoluble polyvinyl pyrrolidone (polyvinyl polypyrrolidone, PVPP):* This is commercially available from Sigma (P 6755).

(4) *Standard tannic acid solution (0.1 mg/ml):* Dissolve 25 mg tannic acid (TA) obtained from Merck in 25 ml distilled water and then dilute 1:10 in distilled water (always use a freshly prepared solution).

3.2. Preparation of calibration curve

TABLE I. PREPARATION OF CALIBRATION CURVE

Tube	Tannic acid solution (0.1 mg/ml) (ml)	Distilled water (ml)	Folin reagent (ml)	Sodium carbonate solution (ml)	Absorbance at 725nm	Tannic acid (µg)
Blank	0.00	0.50	0.25	1.25	0.000	0
T1	0.02	0.48	0.25	1.25	0.112	2
T2	0.04	0.46	0.25	1.25	0.218	4
T3	0.06	0.44	0.25	1.25	0.327	6

T4	0.08	0.42	0.25	1.25	0.432	8
T5	0.10	0.40	0.25	1.25	0.538	10

3.3. Analysis of total phenols

Take suitable aliquots of the tannin-containing extract (initially try 0.02, 0.05 and 0.1 ml) in test tubes, make up the volume to 0.5 ml with distilled water, add 0.25 ml of the Folin-Ciocalteu reagent and then 1.25 ml of the sodium carbonate solution. Vortex the tubes and record absorbance at 725 nm after 40 min. Calculate the amount of total phenols as tannic acid equivalent from the above calibration curve. Express total phenolic content on a dry matter basis (x%).

Example:

50 µl tannin-containing extract in the assay mixture gives 0.531 absorption
= 9.896 µg tannic acid (TA) equivalent (from the standard curve).
Therefore, 1 ml extract has 9.896 / 0.050 = 197.9 µg TA = 0.198 mg TA.

200 mg leaf sample was extracted in 10 ml solvent.
Therefore, 100 mg leaf has 0.198 x 5 = 0.99 mg TA or 100 g leaf has 0.99 g TA.

If leaf contains 95% dry matter (DM),
TA in DM = 0.99 / 0.95 = 1.04%.

Call this x in subsequent calculations.

3.4. Removal of tannin from the tannin-containing extract

PVPP binds tannins. Weigh 100 mg PVPP in a 100 x 12 mm test tube. Add to it 1.0 ml distilled water and then 1.0 ml of the tannin-containing extract (100 mg PVPP is sufficient to bind 2 mg of total phenols; if total phenolic content of feed is more than 10% on a dry matter basis, dilute the extract appropriately). Vortex it. Keep the tube at 4°C for 15 min, vortex it again, then centrifuge (3000g for 10 min) and collect the supernatant. This supernatant has only simple phenolics other than tannins (the tannins would have been precipitated along with the PVPP; the procedure for binding of tannins to PVPP is presently being modified, and the modification is to bind tannins to PVPP at pH 3 since PVPP binds maximally to tannins at this pH [3]). Measure the phenolic content of the supernatant as mentioned above (take at least double the volume (preferably three times) you used for total phenol estimation, because you have already diluted the extract two-fold and expect to lose tannin-phenols though binding with PVPP). Express the content of non-tannin phenols on a dry matter basis (y%).

Example, continued:

100 µl of the supernatant after PVPP treatment in the assay mixture gives 0.312 absorption
= 5.75 µg tannic acid (TA) equivalent (from the standard curve).

Therefore, 1 ml supernatant = 5.75 / 0.1 = 57.54 µg TA = 0.058 mg TA.

10 mg leaf has 0.058 mg TA (since the extract is diluted 2-fold during the test)

Therefore, 100 mg leaf sample has 0.058 x 10 = 0.58 mg TA

y = 0.58 / 0.95 (since dry matter of feed or seed = 95%)
y = 0.611%

But total phenolics in dry matter, x = 1.04%

(x–y) is the percentage of tannins as tannic acid equivalent on a dry matter basis.

Tannins (as tannic acid equivalent) = 1.04 - 0.611 = 0.43% in the dry matter.

Note: Ascorbic acid (generally added to prevent oxidation of phenols) interferes in this assay, and therefore should not be added to the solvent used for extraction of phenols/tannins.

4. DETERMINATION OF CONDENSED TANNINS (PROANTHOCYANIDINS)

(According to the method of Porter *et al.* [4])

4.1. Reagents

(1) *Butanol-HCl reagent (butanol-HCI 95:5 v/v):* Mix 950 ml n-butanol with 50 ml concentrated HCl (37%).

(2) *Ferric reagent (2% ferric ammonium sulfate in 2N HCl):* Make 16.6 ml of concentrated HCl up to 100 ml with distilled water to make 2N HCl. Dissolve 2.0 g ferric ammonium sulfate in this volume of 2N HCl. This reagent should be stored in a dark bottle.

4.2. Analysis

In a 100 mm x 12 mm glass test tube, pipette 0.50 ml of the tannin extract diluted with 70% acetone. The quantity of acetone should be large enough to prevent the absorbance (550 nm) in the assay from exceeding 0.6. It will depend on the quantity of condensed tannin expected in the sample, and occasionally will need to be determined by trial and error. To the tubes add 3.0 ml of the butanol-HCl reagent and 0.1 ml of the ferric reagent. Vortex the tubes. Cover the mouth of each tube with a glass marble and put the tubes in a heating block adjusted at 97 to 100°C (or in a boiling water bath) for 60 min. Cool the tubes and record absorbance at 550 nm. Subtract a suitable blank, which is usually the absorbance of the unheated mixture. If the extract has flavan-4-ols, a pink colour develops without heating. If this happens, use one heated blank for each sample, comprising 0.5 ml of the extract, 3 ml of butanol and 0.1 ml of the ferric reagent. Condensed tannins (% in dry matter) as leucocyanidin equivalent is calculated by the formula:

(A 550 nm x 78.26 x Dilution factor*) / (% dry matter)

This formula assumes that the effective $E^{1\%,\ 1\ cm,\ 550\ nm}$ of leucocyanidin is 460 [4].

(2) The dilution factor is equal to 1 if no 70% acetone was added and the extract was made from 200 mg sample in 10 ml solvent. Where 70% acetone is added (for example to prevent the absorbance from exceeding 0.6) the dilution factor is:

0.5 ml / (volume of extract taken)

Notes:

The presence of pigments may interfere in this method. The pigments can be removed by extracting the dried leaves with petroleum ether containing 1% acetic acid.

Vanillin-HCI is also used for determination of condensed tannins but this method is not specific. It measures condensed tannins as well as simple flavonoids. This method also has several other disadvantages. Readers are referred to Refs. [5, 6].

Ascorbic acid (generally added to prevent oxidation of phenols) does not interfere in this assay.

5. GALLOTANNIN DETERMINATION BY RHODANINE ASSAY

(According to Inoue and Hagerman [7])

The plant material was extracted as described above (Section 2). The supernatant is designated as **supernatant A**.

5.1. Reagents

(1) *Rhodanine solution (0.667% w/ v in methanol):* Weigh 667 mg rhodanine (Sigma, R 4375) and dissolve it in 100 ml methanol. Stable for at least 2 weeks when stored in a refrigerator.

(2) *KOH (0.5 N):* Dissolve 2.8 g potassium hydroxide in 100 ml distilled water.

(3) *Sulphuric acid solutions:* Prepare 0.3, 0.4, 0.6, 22 and 26 N sulphuric acid solutions by appropriately diluting 98% commercially available sulphuric acid (36 N).

5.2. Determination of free gallic acid

Pipette 200 µl **supernatant A** in a 160 mm x 12 mm culture test tube (4 tubes per sample). Remove acetone from the sample using a vacuum drying oven adjusted at 40°C (pressure 300 mbar) for about 2 h or by flushing with nitrogen gas (bring nitrogen gas beam as close as possible to liquid surface). Using nitrogen gas, it takes about 8–10 min to dry the sample. One can construct a multi-tube (8–10 outlets) assembly for flushing nitrogen gas, which allows simultaneous drying of 8–10 samples.

Add to 200 µl of 0.2 N sulphuric acid to the tubes containing dried **supernatant A**. To three tubes add 300 µl of the rhodanine solution and to the fourth tube 300 µl methanol. This fourth tube acts as a proper blank. After 5 min add 200 µl of 0.5 N potassium hydroxide solution to all the tubes. Wait for 2.5 min and then add 4.3 ml of distilled water. After 15 min measure absorbance at 520 nm against proper blank.

5.3. Determination of gallic acid present in free and in gallotannin forms

Pipette 3.34 ml of **supernatant A**, in duplicate, in the above mentioned culture test tube. Remove acetone by flushing the tubes with nitrogen gas. After removal of acetone, 1 ml of the supernatant remains (or make to 1 ml with distilled water). To it add 0.1 ml of 22 N sulphuric acid so that the final sulphuric acid concentration is 2 N. Freeze the contents and remove air from these culture tubes by using a vacuum pump. A special assembly can be constructed for removal of air. This assembly allows simultaneous removal of air and tightening of the cap of the culture tube. Keep these tubes at 100°C for 26 h to hydrolyse gallotannins to gallic acid. After hydrolysis, make up the volume to 11 ml by adding 9.9 ml distilled water. Sulphuric acid concentration in this solution is 0.2 N. This solution is addressed as '**hydrolysed supernatant A**'.

Pipette 200 µl of the **hydrolysed supernatant A** (4 tubes per sample; 1 blank and the rest 'test' samples). Now add 300 µl of the rhodanine solution to the 'test' tubes and 300 µl methanol to the blank tube. Wait for 5 min, and then add 200 µl of 0.5 N KOH solution. Again wait for 2.5 min and add 4.3 ml distilled water. After 10 min, measure absorbance at 520 nm.

5.4. Calibration curve

Stock gallic acid solution (1 mg/ml in 0.2 N sulphuric acid): Weigh 100 mg gallic acid and dissolve in approximately 80 ml of 0.2 N sulphuric acid and then make up the volume to 100 ml with 0.2 N sulphuric acid. It can be kept frozen for at least one month.

Working gallic acid solution (0.1 mg/ml): Dilute the gallic acid stock solution 1:10 with 0.2 N sulphuric acid (1 ml of the stock solution plus 9 ml distilled water). It can be stored in a refrigerator for at least two weeks.

Note: Vortex stir these solutions well.

TABLE II. CALIBRATION CURVE

Gallic acid (µg)	Working solution (µl)	0.2 N Sulphuric acid (µl)	Rhodanine (µl)	Wait for at least 5 min	0.5 N KOH (µl)	Wait for at least 2.5 min	Distilled water (ml)
4	40	160	300		200		4.3
8	80	120	300		200		4.3
12	120	80	300		200		4.3
16	160	40	300		200		4.3
20	200	0	300		200		4.3
Blank	0	200	300		200		4.3

Wait for approximately 10 min, then measure absorbance at 520 nm.

Total gallic acid minus free gallic acid = gallotannins as gallic acid equivalent.

Note: Ascorbic acid (generally added to prevent oxidation of phenols) interferes in this assay, and therefore should not be added to the solvent used for extraction of phenols/tannins.

6. GALLOTANNIN DETERMINATION USING HPLC

(According to Makkar H.P.S. and Becker, K. 1999, unpublished)

6.1. Preparation of sample for determination of free gallic acid

Pipette 1 ml of **supernatant A** (see Section 5) into the above mentioned culture tubes, remove acetone in a vacuum oven adjusted at 40°C (pressure 300 mbar). It takes about 3 h to remove acetone. Acetone can also be removed by flushing with nitrogen gas. Dry the contents completely in a heating block at 40°C by flushing with nitrogen. It takes about 25 min to dry the contents completely. It can be stored under nitrogen in a freezer.

Just before the analysis, add 750 µl distilled water to the residue, and dissolve the contents using an ultrasonic water bath (keep the tubes for about 5 min). Filter the contents through a 0.45 µm membrane filter (cellulose acetate or cellulose nitrate) before loading to the HPLC.

6.2. Preparation of sample for determination of gallic acid present in free and in gallotannin forms

Pipette 2 ml of the **hydrolysed supernatant A or B** (see above) into a 25 to 50 ml capacity tube/beaker and then add 2 ml buffer (2.304 g $NH_4H_2PO_4$ dissolved in 1000 ml distilled water) to it. Adjust the pH between 6.3 and 6.8 using 8 M KOH solution (approximately 120 µl will be required), and record the exact amount required for the pH adjustment. Do not exceed pH7.0 because the sample will immediately get oxidized. In case the pH rises beyond 7, discard it, and start afresh with 2 ml of the hydrolysed supernatant. Freeze overnight, thaw it and then filter through 0.45 µm membrane filter before loading to the HPLC.

HPLC conditions and analysis:

Column: 250 mm x 4.6 mm filled with Nucleosil 120-5 C18 (Macherey Nagel GmbH & Co.KG, Postfach 10 13 52, D- 52313 Duren).

Flow rate: 1.2 ml/min.

Column temperature: Ambient (ca 22°C).

Injection volume: 20 µl.

Gallic acid stock solution: Dissolve 400 mg gallic acid in 1000 ml distilled water.

Gallic acid working solution (6 mg/1000 ml): Pipette 150 µl of the stock solution and make the volume to 10 ml with distilled water. Linearity between area and concentration was observed in the range of 2 to 10 mg gallic acid/ml on injection of 20 µl of gallic acid solution).

Elution time of gallic acid: Between 14 and 15.5 min.

Solvents: Buffer A; H_2O - methanol - H_3PO_4 (975.5:19.5:1; v/v/v), and Buffer B; methanol - H_2O (700:300; v/v)

TABLE III: GRADIENT USED

Time (min)	Solvent A (%)	Solvent B (%)
0.0	100	0
15.0	100	0
22.0	0	100
25.0	0	100
30.0	100	0
33.0	100	0

Detection wavelength: 280 nm.

Note: Ascorbic acid does not interfere in this assay, and therefore can be added to the solvent used for extraction of phenols/tannins.

7. DETERMINATION OF PROTEIN-PRECIPITABLE PHENOLICS

(According to Makkar *et al.* [8]).

7.1. Reagents

(1) *Acetate buffer (pH4.8 to 4.9, 0.2 M):* Pipette 11.40 ml glacial acetic acid to about 800 ml distilled water. Adjust pH of this solution to 4.8 to 4.9 with 4 N sodium hydroxide solution, and bring the final volume to 1 litre. To it add 9.86 g NaCl to make its concentration 0.17 M.

(2) *Sodium dodecyl sulfate solution (SDS) (1% w/v):* Dissolve 1 g SDS in 100 ml of distilled water.

(3) *SDS-triethanolamine (TEA) (1% SDS (w/v) and 7% (v/v) triethanolamine in distilled water) solution:* To 7 ml of triethanolamine add 93 ml distilled water and dissolve 1 g SDS in this solution.

(4) *Ferric chloride reagent (0.01 M ferric chloride in 0.1 M HCl:*. For making 0.1 M HCI, dilute 4.2 ml concentrated HCl (37%) to 500 ml with distilled water. Dissolve 0.81 g ferric chloride in 500 ml of 0.1 M HCI. Filter and store the contents in a brown bottle.

(5) *Glacial acetic acid*.

(6) *BSA solution:* Dissolve 100 mg BSA (fraction V) in 100 ml of the acetate buffer.

7.2. Formation of the tannin-protein complex

To 2 ml of the BSA solution (containing 1 mg BSA/ml acetate buffer), add 50% methanol and increasing levels of the tannin-containing extract to make 3 ml. For example, use 0.95, 0.90, 0.85, 0.80, 0.75, 0.70 ml of 50% methanol with 0.05, 0.10, 0.15, 0.20, 0.25, 0.30 ml of the extract; this may vary depending on the amount of tannins in the sample. Vortex the contents. Allow the mixture to stand in a refrigerator (4°C) overnight. Centrifuge at about 3000g for about 10 min. Remove the supernatant carefully without disturbing the precipitate. Add 1.5 ml of 1% SDS solution to the precipitate and vortex it to dissolve the precipitate.

Note: It is essential to use 50% aqueous methanol for extraction of tannins from the plant material since acetone interferes in the protein-precipitation assay.

7.3. Determination of tannins (phenolics) in tannin-protein complex

Take an aliquot (1 ml) of the above dissolved complex. Add 3 ml of SDS-triethanolamine solution. Then add a 1-ml portion of the ferric chloride reagent. Record absorbance at 510 nm after 15–30 min. Convert the absorbance to tannic acid equivalent, using a standard curve (see below). Multiply the values obtained by 1.5 to obtain tannins in the complex. Draw a linear regression between tannins precipitated as tannic acid equivalent and mg leaf (in aliquot taken for the assay). The slope of the curve (mg tannic acid precipitated/mg leaf; let it be x) represents the protein-precipitable phenolics in the sample.

TABLE IV. EXAMPLE (Acacia cyanophylla)

Tube	Extract (µl)	Leaf (mg)*	Absorbance at 510nm	TA (mg)**	TA in complex (mg)***
1	100	2	0.121	0.054	0.081
2	150	3	0.167	0.077	0.116
3	200	4	0.234	0.109	0.164
4	250	5	0.292	0.136	0.204
5	300	6	0.341	0.160	0.240
6	350	7	0.422	0.199	0.299
7	400	8	0.472	0.222	0.333
8	500	10	0.591	0.280	0.420

TA, tannic acid (Merck)

* 200 mg leaf is extracted in 10 ml 50% aqueous methanol.

**Conversion of absorbance at 510 nm to mg tannic acid by the standard curve (see below).

***Obtained by multiplying values in the previous column (which correspond to 1 ml of the soluble tannin-protein complex) by 1.5, because the tannin-protein complex is dissolved in 1.5 ml of 1% SDS.

Protein precipitable phenolics (x; mg tannic acid equivalents precipitated/mg leaf dry matter) for the above example = 0.043/0.953 = 0.045, since dry matter of the leaves was 95.3%.

7.4. Protein precipitable phenolics as percentage of total phenolics

7.4.1. Determination of total phenolics in the original extract

Take different aliquots (generally 0.05, 0.10, 0.15, 0.2, 0.25, 0.30 ml, but this may vary depending on the amount of phenolics in the sample) of the extract (200 mg in 10 ml of 50% methanol), make up to 1 ml with 1% of SDS, and add 3 ml of the SDS-triethanolamine solution and 1 ml of the ferric chloride reagent. Record absorbance at 510 nm as described

above. Convert the absorbance to tannic acid equivalent using the standard curve. Draw a linear regression between tannic acid equivalent and mg leaf (in the aliquot taken). The slope of the curve (mg tannic acid equivalent/mg leaf; let it be y) represents total phenolics.

Protein-precipitable phenolics have already been measured as x (Section 7.3).

The percentage of total phenolics which precipitate protein = (x / y) x 100.

TABLE V. EXAMPLE *(Acacia cyanophylla)*

Tube	Extract (µl)	Leaf* (mg)	Absorbance at 510nm	TA**
1	50	1	0.145	0.066
2	100	2	0.280	0.131
3	150	3	0.404	0.190
4	200	4	0.532	0.251
5	250	5	0.674	0.319
6	300	6	0.824	0.391

TA, tannic acid (Merck)
* 200 mg leaf is extracted in 10 ml 50% aqueous methanol.
** Calculated from the calibration curve below.

Total phenolics (y; mg tannic acid equivalent/mg leaf dry matter)
= 0.064/0.9535 = 0.067, since dry matter of the leaves was 95.35%.

Therefore, protein precipitable phenolics as percentage of total phenolics
= (x / y) x 100 = (0.045 / 0.067) x 100 = 67.2.

7.4.2. Calibration curve for the above example

TABLE VI. CALIBRATION CURVE

Tube	TA solution * (ml)	SDS, 1% (ml)	SDS-TEA (ml)	Ferric chloride (ml)	Absorbance at 510 nm	TA (mg)
Blank	0	1.0	3.0	1.0	0.000	0.00
T1	0.1	0.9	3.0	1.0	0.107	0.05
T2	0.2	0.8	3.0	1.0	0.225	0.10
T3	0.3	0.7	3.0	1.0	0.319	0.15
T4	0.4	0.6	3.0	1.0	0.426	0.20
T5	0.5	0.5	3.0	1.0	0.527	0.25

TA, tannic acid (Merck).

* TA solution: 0.5 mg/ml in 1% SDS.

8. PROTEIN-BINDING CAPACITY BY FILTER PAPER ASSAY

(According to Dawra *et al.* [9]

8.1. Reagents

(1) *Tannic acid solution:* Dissolve 100 mg tannic acid (Merck, Germany) in 100 ml of 50% aqueous methanol. Add 100 mg ascorbic acid to it to minimize oxidation of tannic acid during handling.

(2) *Acetate buffer (pH5, 0.05 M):* Pipette 2.85 ml glacial acetic acid to about 800 ml distilled water. Adjust pH of this solution to 5.0 with 4 N sodium hydroxide solution, and bring the final volume to 1 litre.

(3) *Bovine serum albumin (BSA) solution:* Dissolve 200 mg of bovine serum albumin (fraction V) in 100 ml of 0.05 M of the acetate buffer (pH5, 0.05 M).

(4) *Dye solution:* Prepare 3% (w/v) trichloroacetic acid (TCA) solution in distilled water. Dissolve 0.2 g of Ponceau S dye in 100 ml of the TCA solution.

(5) *Acetic acid solution (0.2%, v/v):* Add 2 ml of glacial acetic acid to 998 ml distilled water.

(6) *Sodium hydroxide solution (0.1 N):* Weight 4 g sodium hydroxide and dissolve it in approximately 500 ml distilled water and then make up the volume to 1 litre with distilled water.

(7) *Acetic acid solution (10%, v/v):* Add 10 ml of glacial acetic acid to 990 ml distilled water.

8.2. Preparation of plant extract

As described above in 70% acetone (see Section 2). Acetone does not interfere in this method.

8.3. Assay procedure

Take 1 MM Whatman paper chromatography sheet and cut it into an appropriate size (depends on the number of samples to be analysed). Draw squares of approximately 1.5–2 cm using a light lead pencil on the chromatography sheet. Apply different aliquots (5–25 µl containing 5–25 µg tannic acid) on the sheet; each aliquot, at least in triplicate and on three different squares. Similarly, apply appropriate aliquots (10–50 µl; depending on the amount of tannins present) of the plant extract on the middle of squares on the chromatography sheet. Allow the spots to dry and spray immediately with the BSA solution until the paper is wet. After 30 min, wash the paper with the acetate buffer (pH5, 0.05 M) with three 10-min changes with slight shaking to remove the unbound BSA. The paper was stained with 0.2% Ponceau S dye solution by keeping the strips dipped for 10 min in the stain solution. The staining solution should not be used in successive experiments. Wash the stained strips in 0.2% acetic acid solution until no more colour is eluted from the strips. Normally, this requires three washings to make the background clear. Air-dry the strips and cut the stained areas. To prepare a corresponding blank, stain simultaneously a chromatography sheet and wash in a manner similar to other samples. Cut the stained area into small pieces and elute the colour by adding 3 ml of 0.1 N sodium hydroxide solution and vortexing, followed by addition of 0.3 ml of 10% acetic acid and centrifugation at approximately 2500g. Measure the absorbance of the colour at 525 nm against corresponding blank. Convert these absorbance to protein content by using a standard curve. For preparing a standard curve, apply different concentrations of BSA (5–50 µg; 5–50 µl of 1 mg/ml BSA solution in the acetate buffer) as separate spots (at least in triplicate for each concentration) on a chromatography sheet and cut into strips. Stain these strips with the dye solution, wash and read the colour as described above for the samples.

Note: Ascorbic acid does not interfere in this assay, and therefore can be added to the solvent used for extraction of phenols/tannins.

9. RADIAL DIFFUSION ASSAY

(According to Hagerman [10])

Note: Acetone does not interfere in this method. The plant can be extracted in 70% aqueous acetone.

9.1. Chemicals

Agarose: Type I, gel point 36°C (Sigma A-6013), bovine serum albumin (fraction V) (Sigma A-3350), glacial acetic acid, ascorbic acid and sodium hydroxide.

9.2. Reagents

(1) *Sodium hydroxide solution (4 N):* Weigh 8 g of sodium hydroxide and dissolve it in approximately 40 ml distilled water, make up the volume to 50 ml with distilled water.

(2) *Acetate buffer (pH5.0, 0.05 M) containing 60 µM ascorbic acid:* Pipette 2.85 ml glacial acetic acid to about 800 ml distilled water and add to it 10.6 mg ascorbic acid. Adjust pH of this solution to 5.0 with 4 N sodium hydroxide solution, and bring the final volume to 1 litre.

9.3. Preparation of plates

Weigh 2.5 g agarose into 250 ml of the acetate buffer. Heat (boil for approx. 15 min) with continuous stirring on a magnetic stirrer until the agarose dissolves. Bring this solution to 45°C (takes approx. 5 min) by keeping the vessel containing this agarose solution into a water bath set at approximately 45°C. Add 250 mg BSA and dissolve it in the agarose solution without allowing the solution to cool. This solution will be sufficient for preparation of approximately 20 Petri dishes (internal diameter 8.5 cm). Use a 10 ml of glass pipette with a large tip opening or 10 ml of disposable syringes and dispense approximately 10 ml of the solution into each Petri plates kept on a flat surface. Take care not to introduce air bubble while transferring the agarose solution containing BSA. Make sure that the solution covers all the surface of Petri plates. Allow the solution to harden. Cover the Petri dishes and seal them with a strip of Parafilm in order to prevent drying and cracking of the agarose layer. These plates can be stored for two to three weeks in a refrigerator without losing sensitivity of the assay.

9.4. Assay procedure

On the day of performing the assay, take out the Petri dishes from the refrigerator, bring them to room temperature and then open them. Using a well puncher (4 mm) punch three to four wells, far apart, in the solidified agarose in Petri dishes. Remove the plugs of agarose from the Petri dishes gently so that it comes along the puncher or remove the plug using a gentle suction.

Pipette 10 µl (using a micropipette) of the extract in each well. If the preliminary experiment showed that tannin content is low, add 10 µl portion two or three times. The wells should not be allowed to completely dry between successive addition of aliquots. Cover the Petri dishes and again seal using Parafilm. Place the plates on a horizontal platform in an incubator adjusted at 30°C. After 96 h, remove the Petri dishes, uncover and measure the diameter of the ring formed. The amount of tannins is proportional to the square of the diameter. The square of the diameter of the ring formed can be calibrated by taking different concentration of tannic acid or any other standard tannin, and the results can be expressed equivalent to a standard.

10. TANNIN BIOASSAY

(According to Makkar H.P.S., Bluemmel M., Becker K., [3])

10.1. Sample preparation

Dried leaves should be passed through a 1 mm sieve.

10.2. Reagents

(1) *Bicarbonate buffer solution:* Dissolve 35 g sodium bicarbonate ($NaHCO_3$) and 4 g ammonium carbonate (NH_4HCO_3) in approximately 500 ml distilled water and then make up the volume to 1 litre with distilled water.

(2) *Macromineral solution:* Dissolve 6.2 g potassium dihydrogen phosphate (KH_2PO_4), 5.7 g disodium hydrogen phosphate (Na_2HPO_4), and 0.6 g magnesium sulphate ($MgSO_4.7H_2O$) in approximately 500 ml distilled water and then make up the volume to 1 litre with distilled water.

(3) *Micromineral solution:* Dissolve 10 g manganese chloride ($MnCl_2.4H_2O$), 13.2 g calcium chloride ($CaCl_2.2H_2O$), 1 g cobalt chloride ($CoCl_2.6H_2O$), 8 g ferric chloride ($FeCl_3.6H_2O$) in approximately 50 ml distilled water and then make up the volume to 100 ml with distilled water.

(4) *Resazurine:* Dissolve 0.1 g resazurine in 100 ml distilled water.

(5) *Reducing solution:* Dissolve 996 mg sodium sulphide ($Na_2S.9H_2O$) in 94 ml distilled water and then add 6 ml of 1 N sodium hydroxide solution (dissolve 4 g sodium hydroxide in 100 ml distilled water for 1 N sodium hydroxide).

10.3. Weighing of samples and preparation of syringes

Tear a specially made scoop (approximately 4 cm in length and 1 cm in depth/radius; standard sodium hydroxide-containing plastic container can be cut horizontally to half to form the scoop) on an analytical balance. Weigh 500 mg feed sample (passes through 2 mm sieve) in the scoop and then insert a 5 ml capacity pipette or a glass rod into the narrow end of the scoop and transfer the sample from the scoop into 100 ml calibrated glass syringes. Weigh 1 g tannin-complexing agent, polyethylene glycol, PEG (MW. 4000 or 6000) on the scoop and transfer it also into syringes similar to those for the feed samples. The feedstuffs with and without the tannin-complexing agent are incubated at least in triplicate.

10.4. Preparation of *in vitro* rumen fermentation buffer solution

Collect the rumen fluid and particulate matter before the morning feed from two cattle fed on roughage diet, homogenize, strain and filter through four layers of cheese cloth. Keep all glassware at approximately 39°C and flush these with carbon dioxide before use. Carbon dioxide is heavier than air and hence it remains in the glassware for a reasonable period provided the container is not inverted up-side down. The strained rumen fluid is kept at 39°C under carbon dioxide and should be prepared just before start of the incubation. As the amount of feed taken is 500 mg, composition of the medium is according to Tilley and Terry [11]. Menke *et al.* [12] reduced the rumen buffer volume per syringe by half as they used 200 mg of the substrate because of the limited volume of the syringes and the inconvenience of emptying the syringes. Here, besides recording the gas volume, we are interested to use the fermented material for various analyses; therefore, the amount of substrate taken is 500 mg. There is an inherent error associated with gravimetric determination of the fermented residue which (error) is large if 200 mg feed is taken in place of 500 mg.

10.4.1. Medium composition and incubation procedure

Rumen buffer solution	630.00 ml
Macromineral solution	315.00 ml
Micromineral solution	0.16 ml
Resazurine	1.60 ml

Distilled water	975.00 ml
Freshly prepared reducing solution	60.00 ml
The rumen fluid (see above for collection and preparation)	660.00 ml

The above volume is sufficient for 60 syringes (40 ml/syringe) plus 10% extra.

Mix, in the order mentioned above, all the above-mentioned solutions, except rumen fluid and the reducing solution, in a 3 or 5 litre capacity glass container. The container is kept in a water bath adjusted at 39°C. This water bath is a plastic rectangular container (400 cm x 300 cm x 200 cm) filled with water, the temperature of which is adjusted at 39°C using a portable thermostat suspended from the top of the plastic container in water. This plastic water bath is kept on a magnetic stirrer. The contents are flushed with carbon dioxide and kept stirred using a magnetic stirrer. After about 5 min, add the reducing solution and keep the mixture stirring and flushing with carbon dioxide at 39°C. When the mixture has been reduced (blue colour of the dye changes to pink and then to colourless; it takes about 15–20 min for the reduction process to complete and during this time we generally homogenized and strained the rumen liquor and the particulate material collected from cattle), add 660 ml of the rumen fluid. Keep this mixture stirring and flushing with carbon dioxide for another 10 min. Transfer a portion (40 ml) of the rumen-fluid medium into each syringe using a dispenser, and incubate in a water bath at 39°C. For filling 60 syringes, after some practice should take 30–35 min. After completion of the filling-up process, shake the syringes well and then transfer them to the water bath. Shake all the syringes every hour for the first four hours and then every two hours. Generally, the incubation is started at about 7.30 a.m. and after 12 h of the incubation, the syringes are not shaken till the termination of the incubation (16 h and/or 24 h). The gas volume is recorded after 2, 4, 6, 8, 10, 12, and 16 h or 24 h. The net gas production is calculated by subtracting values for the blank. The blanks (at least three in number) contain only the rumen-fluid medium and no feed material. Addition of PEG to blanks does not affect the gas production from blanks, suggesting that it is inert.

The difference between the net volume of gas produced from syringes with and without PEG is a measure of tannin effect. PEG binds tannins and inactivates them. From the gas volume, production of short-chain fatty acids (SCFA) can be calculated from the following equation (Girma G., Makkar H.P.S. and Becker, K. Under preparation).

Relationship between SCFA (mmol/syringe) and atmospheric pressure-corrected gas volume (the experiment was conducted in Hohenheim, Stuttgart which is at a height of 500 m from the mean see level). The equations are valid at 1 atmospheric pressure.

SCFA (mmol/40 ml) = -0.0601 + (0.0239 x Gas (ml)); n = 39 (without PEG), R^2 = 0.953; Standard error of estimate = 0.109; P<0.001.

SCFA (mmol/40 ml) = 0.0521 + (0.0207 x Gas (ml)); n = 37 (with PEG); missing observations = 2, R^2 = 0.925; Standard error of estimate = 0.108; P<0.001

SCFA (mmol/40 ml) = -0.00425 + (0.0222 x Gas (ml)); n = 76 (with and without PEG), R^2 = 0.935; Standard error of estimate = 0.115; P<0.001

These equations have been generated on incubation of leaves from trees and browses (39 in number).

One of the many factors which determines gas volume is the molar proportions of SCFA. 'Near-one' R^2 observed (see above) suggest that the total SCFA production can be calculated with reasonable accuracy from the volume of gas produced.

10.5. Measurement of microbial protein in the residue (apparent undigested residue) left after fermentation

(According to Makkar and Becker [13])

10.5.1. Preparation of apparent undigested residue

After 16 or 24 h of fermentation, centrifuge (20,000g, 30 min, 4°C) the contents and discard the supernatant. Wash the pellet with distilled water followed by centrifugation (20,000g, 30 min, 4°C). Lyophilize the pellet consisting of undigested substrate and microbial mass. The lyophilized residue has been termed as apparent undigested residue. Use a representative sub-sample of this preparation for analyses. In order to get representative sub-sample, it is advised to grind the entire residue in a pestle and mortar or in a small rotating-ball grinding mill.

10.5.2. Preparation of lyophilized rumen microbial fraction

Two hours after the morning feed, collect about 1 L rumen liquor from a cow fed a roughage-based diet. Pass the liquor through two layers of muslin cloth and then keep at 4°C for 30 min in a carbon dioxide-flushed cylinder of 1 litre capacity. Separate the rumen fluid devoid of heavy and light particles by pipetting the liquor from the cylinder between the heavy particles which settle down and the light particles which float on the top. This method for collection of rumen fluid is essentially adapted from Yang and Russell [14]. Centrifuge (20,000*g*, 20 min, 4°C) several portions (each 30–35 ml) of this liquor. Wash the pellets with distilled water followed by centrifugation (20,000g, 20 min, 4°C). Repeat this washing step two more times, lyophilize the pellets and pool them. Use a sub-sample of one preparation. The nitrogen content of this fraction should be approximately 7.7% [15]. In the lyophilized rumen microbial fraction prepared in our laboratory in the manner as described above, the nitrogen content was 7.7%.

10.5.3. *Spectrophotometric method for determination of purines (marker for microbial mass/protein)*

(According to Zinn and Owens [16], with some modification as described in Makkar and Becker [13]).

10.5.3.1. Reagents

(1) *Ammonium dihydrogen phosphate (0.2 M):* Dissolve 23 g ammonium dihydrogen phosphate in about 700 ml distilled water, and then make up the volume to 1 litre with distilled water.

(2) *Sodium hydroxide (10 M):* Dissolve 40 g NaOH in approximately 70 ml distilled water and then make up the volume to 100 ml with distilled water.

(3) *$AgNO_3$ (0.4 M):* Dissolve 1.6987 g $AgNO_3$ in approximately 15 ml distilled water and then make up the volume to 25 ml with distilled water. Protect the solution from light. Store in a brown bottle and surround the bottle with black paper.

(4) *HCl (0.5 M):* Dilute 10 ml HCl (37%) to 240 ml with distilled water.

(5) *Ammonium dihydrogen phosphate (28.5 mM):* Dilute 100 ml of 0.2 M solution of ammonium dihydrogen phosphate to 700 ml with distilled water.

(6) o-*Phosphoric acid (85%).*

(7) *Perchloric acid (0.6 M):* Dilute 10 ml perchloric acid (70%; 12 M) to 200 ml with distilled water.

10.5.3.2. Procedure

Weigh the lyophilized microbial fraction or the apparent undigested residue (25–75 mg) in 25 ml screw-cap tubes and add 2.5 ml of 0.6 M perchloric acid. Incubate the mixture in a water bath at 90–95°C for 1 h. After cooling, add 7.5 ml of 28.5 mM ammonium dihydrogen phosphate and return the tubes to a water bath (90-95°C) for 15 min. After cooling, centrifuge (3,000g, 10 min) the contents and collect the supernatant. Add an aliquot (0.25 ml) of the supernatant to 4.5 ml of 0.2 M ammonium dihydrogen phosphate and adjust the pH between 2 and 3 (generally to 2.5) using o-phosphoric acid. After the pH adjustment (usually 5 µl o-phosphoric acid gives pH2.3), add 0.25 ml of $AgNO_3$ (0.4 M) and keep the mixture overnight at 5°C in the dark. Centrifuge (3,000g, 10 min) the contents and discard the supernatant. Take care not to disturb the pellet. Wash the pellet with 4.5 ml distilled water adjusted to pH 2 (with sulphuric acid) followed by centrifugation. Suspend the pellet in 5 ml of 0.5M HCl, vortex thoroughly and transfer to the 90–95°C water bath for 30 min after covering the tubes with marbles. Centrifuge (3,000g, 10 min) the tubes and record absorbance of the supernatant at 260 nm against 0.5 M HCl. For studies with RNA in the range of 25–75 mg (instead of lyophilized microbial preparation or the apparent undegraded residue), read the absorbance at 260 nm after 1:10 dilution of the supernatant.

Without adjustment of the pH (which is generally 3.4) to between 2 and 3 before addition of the $AgNO_3$ solution (see below), the recovery of purine basis from yeast RNA (Sigma) is generally lower (80–90% vs. 94–99%), suggesting the importance of the pH-adjustment step in obtaining satisfactory recoveries. Addition of the $AgNO_3$ solution does not change the pH of the solution. Use o-phosphoric acid for adjustment of pH to 2.7.

Express results either based on RNA or lyophilized microbial preparation.

10.5.4. HPLC method for determination of purine bases (adenine and guanine)

(According to Balcells *et al.* [17], with some modifications as described in Makkar and Becker [13]).

10.5.4.1. Equipment, reagents, HPLC conditions and analysis

The HPLC equipment, which we used, consisted of a Merck Hitachi L-7100 HPLC pump, an L-7450 photo diode array detector, an L-7200 autosampler, a D-700 interphase module and an LC organiser.

Analytical column: Reverse phase C18 (LiChrospher 100, endcapped 5 µm) 250 mm x 4 mm I.D. (Lichrocart; Merck, Darmstadt, Germany) protected by a guard column containing the material as in the main column.

HPLC solvent A: 10 mM $NH_4H_2PO_4$ and adjust pH to 6 with 10% NH_4OH.

(Dissolve 11.503 g $NH_4H_2PO_4$ in about 500 ml distilled water and then make the volume to 1 litre with distilled water. It is 100 mM solution. Pipette 100 ml of this solution and dilute to 1 litre to obtain 10 mM solution)

HPLC solvent B: Add 150 ml of acetonitrile to 600 ml of 12.5 mM $NH_4H_2PO_4$ (75 ml of 100 mM solution plus 525 ml of distilled water) and adjust pH to 6 with 10% NH_4OH.

(Filter solvents A and B through a 0.45 µm filter and degas by ultrasonication and by application of vacuum).

Purine bases and internal standard solution for converting integration units to the concentration: Prepare 1 mM stock solution. Put a measuring flask (250 ml capacity)

containing approximately 50 ml distilled water on a magnetic stirrer fixed with a hot plate. Add to the flask 100 µl of 10 M sodium hydroxide solution. Heat at about 90°C and then transfer 33.77, 37.77 and 34.025 mg of adenine and guanine and allopurinol respectively to the flask. Wait (generally 30 min) till the contents dissolve. Cool the contents and make up the volume to 250 ml with distilled water. This solution can be stored at 4°C for 10 days. Dilute this stock solution 12.5 times; pipette 2 ml of the stock solution into 25 ml measuring flask and make up the volume to 25 ml with buffer A of the HPLC. Inject 40 µl of this solution into the HPLC.

Preparation of 3 mM allopurinol solution: Take 100 ml measuring flask and weigh-in 40.83 mg allopurinol. Add approximately 50 ml of distilled water and 20 µl of 12 N (70%) perchloric acid. Heat the contents to approximately 90°C with stirring on a magnetic stirrer. Cool the contents to room temperature and make up the volume to 100 ml with distilled water.

Preparation of 8 mM caffeine solution: Dissolve 155.36 mg caffeine in 80 ml of distilled water and then make up the volume to 100 ml with distilled water.

Gradient: A 30-min linear gradient from 0 to 100% solvent B. After 40 min, increase solvent A to 100% in the following 5 min and equilibrate the column to the starting condition (100% A) in the next 15 min before injecting the next sample.

Time (min)	Solvent A (%)	Solvent B (%)
0.0	100	0
30	0	100
40	0	100
45	100	0
60	100	0

Detection wavelength: 254 nm with a full scale deflection set at 0.2 absorbance

Column temperature: Ambient (approximately 22°C)

Guanine and adenine appear at about 11 and 15.5 min respectively. Allopurinol or caffeine can be used as internal standards. These appear at about 13.5 and 29.5 min respectively. For tannin-containing feeds, do not use caffeine since it binds with tannins, which lowers the recovery of caffeine [13].

10.5.4.2. Sample hydrolysis

Weigh 25–100 mg sample in 25 ml screw-cap tubes and add 2.5 ml of perchloric acid (0.6 M) and 0.5 ml of an internal standard (3 mM allopurinol or 8 mM caffeine). Incubate the mixture in a water bath at 90–95°C for 1 h. After cooling, add 7.0 ml of Buffer A of the HPLC system, adjust the pH between 6.6 and 6.9 using concentrated KOH (approximately 8 M) and freeze overnight. Next day thaw the sample and then centrifuge (approx. 10,000g) to remove the precipitate formed (freezing overnight before the centrifugation step improves the removal of suspended material). Filter through 0.45 µm filter and inject appropriate volume (15–50 µl) into the HPLC.

Express results based on adenine plus guanine.

10.5.5. Some useful data on purines

(According to Makkar and Becker [13])

TABLE VII. PURINE DETERMINATION (AS A260 NM) USING SPECTROPHOTOMETRIC METHOD

	Absorbance at 260 nm	
	Mean	S.D.
25 mg LRM	0.215	0.006
50 mg LRM	0.456	0.005
75 mg LRM	0.675	0.005
25 mg RNA*	0.210	--
50 mg RNA*	0.414	--

LRM, lyophilized rumen microbes
A260nm = (0.009207).mg LRM - 0.01178 (r^2 = 0.99; n = 3)
* Average of two values and after 1:10 dilution

TABLE VIII. PURINE BASE DETERMINATION USING HPLC METHOD

	Adenine (µmol)		Guanine (µmol)	
	Mean	SD (n=3)	Mean	SD (n=3)
50 mg LRM	2.35	0.03	2.95	0.02

LRM, lyophilized rumen microbes

Note: It may be noted that workers can optimize their own system for a particular material being studied once equipped with a sound grasp of the rationale behind these procedures.

11. RADIOLABELLED BSA PRECIPITATION METHOD

This method provides the most sensitive method for determining amounts of protein precipitated by a tannin. It is also useful for running competitive binding assay to characterize tannin-protein interactions. The method described here [18] has been scaled down from the original method [19].

Caution: Proper handling of isotopes is required for this method. you must be approved for isotope use by the appropriate committee of your institution.

11.1. Reagents

(1) *pH 4.9 acetate buffer (Buffer A):* Acetic acid (0.20 M), NaCl (0.17 M), pH adjusted to 4.9 with NaOH (11.4 ml glacial acetic acid, 9.86 g NaCl dissolved in about 800 ml water, then adjust to pH 4.9 with a solution of NaOH, then bring to a final volume of 1 liter).

To make large volumes of Buffer A conveniently, prepare the following

2 M acetic acid, 1.7 M NaCl. Add 114 ml glacial acetic acid to about 800 ml distilled water, add 99.4 g NaCl, and bring to 1 L with distilled water. Store in refrigerator.

2 M sodium acetate, 1.7 M NaCl. Add 164.1 g sodium acetate to about 800 ml distilled water. Add 99.4 g NaCl and bring to 1 L with distilled water. Store in refrigerator.

Buffer A: Mix 40 ml of the acetic acid solution with 60 ml of the sodium acetate solution and bring to 1 L. Check the pH; it should be 4.9.
(stored Buffer A in refrigerator)

(2) *Radioiodinated bovine serum albumin (provided by IAEA in Buffer A for groups participating in the FAO/IAEA Co-ordinated Research project):* IAEA will obtain radioiodinated BSA from ICN, catalogue number 68031 or equivalent. This is

radioiodinated by the chloramine T method, to a specific radioactivity of about 0.85 mCi/mg, and is provided as a 2 mg/ml solution of BSA in phosphate buffer.

IAEA will dilute the initial batch of radioiodinated protein 100-fold with unlabelled 2 mg/ml BSA in buffer A. Thus the 150 µl provided by ICN will be diluted to a total volume of 15.0 ml by IAEA, and will then be dispensed into 200 µl aliquots and frozen for shipment to laboratories.

The labelled protein will be shipped and stored frozen, and will not be thawed until just before use.

The 200 µl aliquots to be used on a given day should be thawed, diluted 10x with unlabelled 2 mg/ml BSA in buffer A and immediately dialyzed for 1–2 h at 4°C against 500 ml buffer A. Thus for a typical experiment 2 vials of labelled protein might be used; the total 400 µl of labelled protein would be diluted to 4.0 ml with unlabelled BSA and then dialyzed. After dilution, you should get around 30,000 cpm in a 50 µl aliquot of protein (although this of course depends somewhat on time since labeling and decay).

(3) *Unlabelled ("carrier") BSA*: 2.0 mg/ml in Buffer A. Prepared weekly and stored in the refrigerator. Used both to dilute the labelled protein to a suitable activity and as carrier in the TCA-precipitability controls.

(4) *Plant Extracts and Tannic acid Standard:* Prepare the tannic acid (Merck) standard at 0.1 mg/ml. It should be initially dissolved at 1.0 mg/ml in 50% methanol, and then diluted 10x with distilled water.

Plant extracts are prepared in 50% methanol as for protein-precipitable phenolics, and are then diluted 5 or 10 times with distilled water as needed to obtain linear response.

(5) *Trichloroacetic acid (100%):* Commercially purchased 100% (~6.1 N) TCA. This solution is stable indefinitely. More dilute solutions of TCA cannot be used reliably after prolonged storage.

11.2. Supplies

(1) 0.5–0.65 ml microfuge tubes (less expensive tubes which can withstand 13K rpm centrifugation but can easily be cut). For example, Life Science Products 8507-GMT 0.65 ml BesTubes, US $26/1000 tubes.

(2) Tube opener (for example, Life Science Products TT-44 Tube opener, 10 pack for US $24).

(3) Tube cutter (for example, Life Science Products TC018-A cutter @ US $13.20 and TC018-B replacement blade @ US $5.90)
(Life Science Products, http://www.lifesciprod.com, FAX 1-303-452-7689. Similar products are available from many other suppliers e.g. Fisher)

(4) Also needed are gloves and other isotope work area supplies; microfuge; gamma counter.

11.3. Method for Precipitation Assays (amount of BSA precipitated by tannic acid or plant extracts)

The assay is run in 0.5 ml microfuge tubes. Buffer A, labelled protein, and water (as needed to attain a final total volume of 400 µl) are added to the tubes, which are then capped and vortexed. The tannin or plant extract is then added with immediate vortexing. The samples are incubated overnight at 4°C and are then centrifuged for 5 min in a microfuge (13,000*g*) at

room temperature. The supernatants are carefully aspirated without disrupting the pellets. The surface of the pellet is gently washed with 100 µl buffer, the samples centrifuged again for 1 min, and the solution aspirated. The tubes are trimmed to fit into the gamma counter vials, and the pellets are then counted in a gamma counter directly, without transferring them out of the microfuge tubes.

Assay tubes contain:

(2) 50 µl labelled protein
(3) 250 µl buffer A
(4) 100-0 µl water
(5) 0-100 µl tannic acid solution or plant extract

For each assay, the following controls are run:

(2) Total counts used per aliquot labelled protein.
(3) Background: 100 µl water, no tannin. This value is subtracted from all values for counts precipitated. We usually find that the background is less than 10% of the counts added.
(4) TCA precipitable counts: 50 µl labelled protein plus 190 µl "carrier" unlabelled BSA (2 mg/ml) mixed with 60 µl of 100% TCA and incubated with the samples at 4 ^{o}C (15 min is sufficient, but overnight is OK). It is desirable to use labelled protein in which >90% of the counts are TCA-precipitable.

11.4. Calculations

The amount of protein precipitated is calculated directly from the counts in the precipitate after correcting for background (non-specific) precipitation. The initial concentration of the labelled BSA (50 µl @ 2.0 mg/ml = 100 µg) is used for the calculation. The amount of protein precipitated is plotted as a function of the dry matter equivalent of plant extract, and each plant extract is tested at $\geq$ 3 concentrations. The slope of the linear portion of the curve gives the protein precipitation capacity of the plant, as µg protein precipitated per mg forage. The capacity of the tannic acid standard is determined each time the assay is run for inter- and intra-lab quality control.

12. CHARACTERIZATION OF PHENOLIC COMPOUNDS BY THIN LAYER CHROMATOGRAPHY (TLC)

12.1. Characterization of condensed tannins

12.1.1. Samples

About 500 g of the shade dried plant material should be ground first to pass a 2 mm screen. All the ground material including those parts remaining inside the mill should be taken, mixed well and approximately 100 g of this sample is again ground to pass through a 0.5 mm screen. Take care that at any stage of the grinding, the sample temperature does not rise above 40°C.

12.1.2. Reagents

(1) *70% acetone*: Mix 70 ml acetone (Analar grade) and 30 ml distilled water.

(2) *Butanol/HCl reagent*: Thoroughly mix 95 ml butan-1-ol (Analar grade) and 5 ml HCl (12 M).

(3) *Anthocyanidin standards*: Chlorides of cyanidin, delphinidin, pelargonidin, etc (only the first two are very common, but others may also be present and can be seen on the plates) can be purchased from Apin Chemicals, Abingdon, UK, Fax: +44-1235 83200, Tel: +44-

1235 832515 or Extrasynthese, 69730 Genay, France, Fax: +33-478 98 19 45, Tel: +33-478 98 20 34 or Fluorochem, Old Glossop, UK., Fax: +44- 01457 869360, Tel: +44-01457 868921.

12.1.3. Material for TLC

(1) *Cellulose MN300 plates:* Polygram CEL 300, cat. no. 801 013, Macherey and Nagel, Dueren, Germany; Tel (024 21) 969-0, Fax (02421) 969 199; order the 20 x 20 cm plates (these have plastic at the back) and cut them in a guillotine, soft side down, to 10 x 10 cm or even 5 x 5 cm if you want to shorten the time.

(2) *Disposable micro-pipettes*: Camlab, UK or any other TLC supplier; recommended sizes 1, 2 and 5 µl.

(3) *TLC sprayers which produce a fine mist (need a fine nozzle) and are resistant to 12 M HCl*: e.g. Merck TLC sprayer cat. no 370/2000/10, £180 + VAT. This is a good sprayer but is very expensive. Glass sprayers can also be used if the nozzle is fine (Merck ca. no. 370/0642/00 or 306/6261/00 (I strongly recommend the pressure relief vent of the latter assembly, as the simple bulb of the first assembly is more likely to produce an uneven spray. However, the reagent spray atomizer is likely to give the finer mist of the two). It is vital that all glass sprayers are cleaned with distilled water immediately after use, as the nozzles become easily blocked.

(4) *TLC solvents*:

Solvent 1 (for first direction): Mix 100 ml concentrated formic (85%), 10 ml 12 M HCl and 30 ml water.

Solvent 2 (for second direction): Mix 20 ml pentan-1-ol, 10 ml glacial acetic acid and 10 ml water.

12.1.4. Extraction

Leaf samples (200 mg of dried plant material passed through a 0.5 mm screen) are taken in a glass beaker of approximately 25 ml capacity. To it is added 10 ml of aqueous acetone (70%) and the beaker is suspended in an ultrasonic water bath (Branson 3210) and subjected to ultrasonic treatment for 20 min at room temperature. The contents of the beaker are then transferred to centrifuge tubes and subjected to centrifugation for 10 min at approximately 3000g at 4°C (if refrigerated centrifuge is not available, cool the contents by keeping the centrifuge tube on ice and then centrifuge at 3000 **g** using an ordinary clinical centrifuge). Collect the supernatant and keep it on ice.

Take 1 ml of the aqueous acetone extract and evaporate the solvent to less than 200 µl in a stream of nitrogen (not oxygen, as this would oxidise the tannins!). Dilute the concentrate to 400 µl with water and mix well. Take an aliquot of 80 µl from this aqueous solution and add 1 ml of the HCl/butanol reagent, cover the tubes (marbles or loose Teflon lined Pyrex tube screw tops) heat at 100°C for 60 min. Cool the solution and spot aliquots onto the TLC plates.

12.1.5. Procedure

In a fume cupboard, fill two small TLC tanks with solvents 1 or 2 to a height of about 3 mm, ensure that the atmosphere in the tanks is well saturated with the solvent (a sheet of filter paper can be dipped in the solvent and surround around the tank walls inside to speed up the saturation process, 'seal' the lids with grease to prevent evaporation of the solvent; the TLC plate should not be run before 30 min after filling the tanks with the solvents).

Spot the sample carefully at the bottom left corner (7 mm from the edges). The diameter of the spot should not exceed 5–7 mm. The volume of sample to be spotted depends on the concentration of the anthocyanidins. It is best to try out a range of volumes (e.g. 5, 10, 20 µl) by repeatedly applying 5 µl onto the same spot and letting the spot dry between applications. Do not use hot air blower/hair drier to dry the spots.

Gently lower the plate into the TLC tank. Switch off the fume cupboard to prevent drafts crossing the tank and causing temperature gradients. Remove the plate, when the solvent front has just reached the top of the TLC plate (approx. 3 mm below the top); but switch the fume cupboard on again for this. Dry the plate in the draft of the fume cupboard (do not use hot air). When all solvent has evaporated, turn the plate by 90 degrees and repeat the separation with solvent 2. The colours of the anthocyanidins should be clearly visible. They are identified from their position (Rf-values) on the plate [see below] and by their characteristic colours.

12.2. Survey of Condensed and Hydrolysable Tannins by TLC

12.2.1. Solvents

Solvent 1: Mix 2 ml glacial acetic acid and 98 ml water.

Solvent 2: Mix 60 ml butan-1-ol, 15 ml glacial acetic acid and 25 ml water.

12.2.2. Spray reagents to detect different classes of tannins

Some of these sprays require some skill in order to extract good and reproducible information from them. The best thing, therefore, is to keep trying until one is familiar with the colour reaction conditions. A lot of information can be obtained by the combination of different sprays, the positions of the spots on the TLC plates. Note that some spots will react with several of these sprays. For example, condensed tannins will react positively with spray 1 and 2. Hydrolysable tannins (i.e. gallotannins) will react with sprays 1 and 3. Ellagitannins with sprays 1 and 4. Catechin gallate type tannins will react with sprays 1, 2 and 3 (e.g. present in Acacia nilotica). The position on the TLC plate also provides information, some tannins will hardly move away from the origin where the plant extract was applied. These tannins are likely to be of higher molecular weight and this information alone might be useful in interpreting some animal responses.

(1) *Vanillin/HCl reagent:* Every day, prepare freshly a solution containing 1 g vanillin in 10 ml 12M HCl. Be careful when spraying this reagent, it is very corrosive! Cut one side out of a large cardboard box, place it in a fumecupboard. Activate the spray, test that the droplets are finely distributed on a piece of paper, then pass this mist over the TLC plates. You will need to gauge yourself how much spray to apply per plate, initially just apply the minimum amount until red spots start to appear.

There is no problem at all with this reagent. It works very well and detects flavan-3-ols, e.g. catechin and epi-catechin, plus condensed tannins. When these are present, pink spots are obtained. You will need to keep a record of these red spots. This can be done by

(2) using a sharp tool (edge of thin spatula, scalpel or razor blade) to surround each spot with a series of small holes (recommended option);
(3) photocopying the TLC plate (but careful, the acid might damage the photocopier, consider covering it with a transparent film first); or
(4) placing some tracing paper over the TLC plate and copying the spots (but TLC surface is quite fragile).

(5) *Ferric ion reagent:* Prepare daily a fresh solution containing 1 g of $K_3Fe(CN)_6$ and 1 g $FeCl_3$ in 50 ml water. Then add 5 tiny crystals of $KMnO_4$. Lightly spray the TLC plate with a fine mist of this reagent (avoid spluttering large drops on the plate).

(6) The background of the plate tends to turn dark blue more or less quickly. This can be reduced by laying the TLC plate (Cellulose surface point up) into a glass basin containing 2 to 3M HCl soon after applying the reagent {Safety note: HCN is released, therefore, use a fumecupboard}. Once the plates are thoroughly rinsed in the HCl bath {careful not to damage the cellulose surface}**,** they are transferred to a second basin filled with water and left to soak for several hours. Ideally, blue spots are obtained on a relatively white background - this requires a bit of practice.

This reagent works well once you have sorted out the conditions, i.e. how much spray to apply. It is a general reagent for all phenolic compounds (tannins and others). If the sample has large quantities of hydrolysable tannins, these often appear as large spots. It is a useful spray, as it provides guidance for the next two sprays which can be more difficult.

Potassium iodate reagent: Prepare a saturate solution KIO_3 (potassium iodate) in distilled water (i.e. add enough KIO_3 crystals until some of them no longer dissolve in the water).

This reagent detects gallic acid and its esters (i.e. gallotannins, a subgroup of the hydrolysable tannins). It is suggest that this reagent is tried initially using gallic acid as a standard. The coloration is orange pink to brown and does not always appear clearly. Therefore, several attempts will be needed. Sometimes it is helpful to hold the TLC plates towards the window up to eye-level and to look across the white surface. This has the effect of 'concentrating' the colour and makes it easier to see the spots.

(7) *Sodium nitrite reagent:* Cool 10 ml of water to near 0^oC, add 20 mg of $NaNO_2$ (sodium nitrite) plus 1–2 drops of glacial acetic acid.

This reagent detects ellagic acid and its esters. Orange-brown spots are obtained. This is the most tricky reagent and it will require several attempts to become confident with it. Again, try it out first using ellagic acid as the standard.

12.3. TLC procedure

Apply the aqueous acetone extracts (5 to 30 µl) to several cellulose MN300 TLC plates (10 x 10 cm) and try to keep the spot at the origin as small as possible without damaging the TLC surface (approximately 7 mm diameter). Place the TLC plate in ‘solvent 1’ first and remove it when the solvent front has just reached the top of the plate. Dry in a cool stream of air, turn the plate by 90 degrees and place the plate into a tank containing ‘solvent 2’.

Then subject each TLC plate to one of the 4 spray reagents separately.

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