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Tannins

Key Words: Hydrolyzable tannins; condensed tannins; proanthocyanidins; polyvinylpolypyrrolidone; Folin-Ciocalteu reagent; butanol-HCl reagent; gallic acid; tannic acid; polyethylene glycol; ^{14}C-labeled polyethylene glycol; ferric reagent; protein precipitation assay; biological activity of tannins; in vitro rumen fermentation buffer.

1. Introduction

1.1. Nature, Mechanism of Action, and Biological Effects

Tannins are polyphenolic compounds that are broadly categorized into two major groups: (1) hydrolyzable tannins, consisting of a central core of carbohydrate to which phenolic carboxylic acids are bound by ester linkage **(Fig. 1)**; and (2) condensed tannins, or proanthocyanidins, consisting of oligomers of two or more flavan-3-ols, such as catechin, epicatechin, or the corresponding gallocatechin **(Fig. 2)**. Tannins have a very high affinity for proteins and form protein-tannin complexes. The ingestion of a plant containing condensed tannins decreases nutrient utilization, protein being affected to a great extent, and decreases feed intake. On the other hand, hydrolyzable tannins are potentially toxic to animals. Consumption of feeds containing high levels of hydrolyzable tannins cause liver and kidney toxicity and lead to death of animals. Oak and yellow wood poisonings are attributed to hydrolyzable tannins ***(1)***.

The tannin assays are generally categorized into two groups: chemical methods and protein precipitation methods. Other methods that do not fall under these categories are the gravimetric assays, a tannin bioassay, and ^{14}C-labeled polyethylene glycol binding assay ***(2)***.

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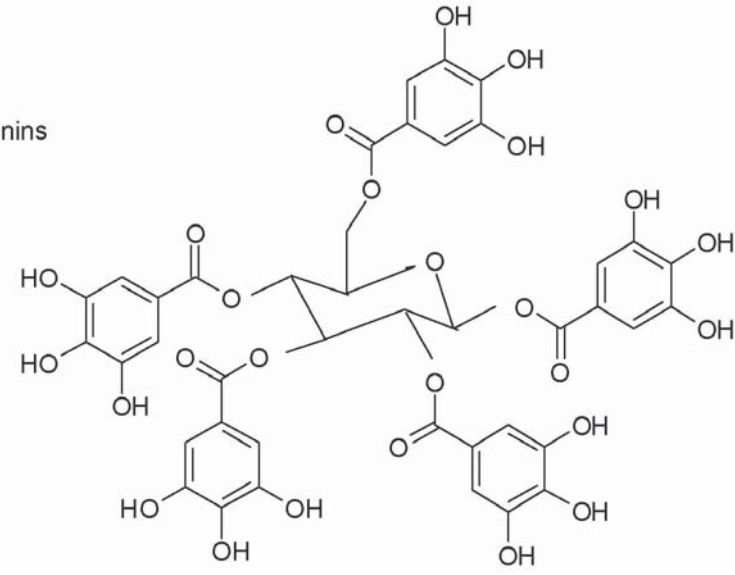
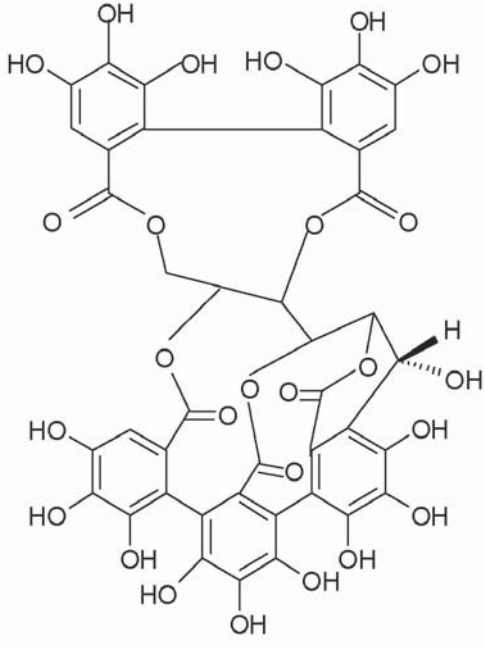
1. Gallotannins



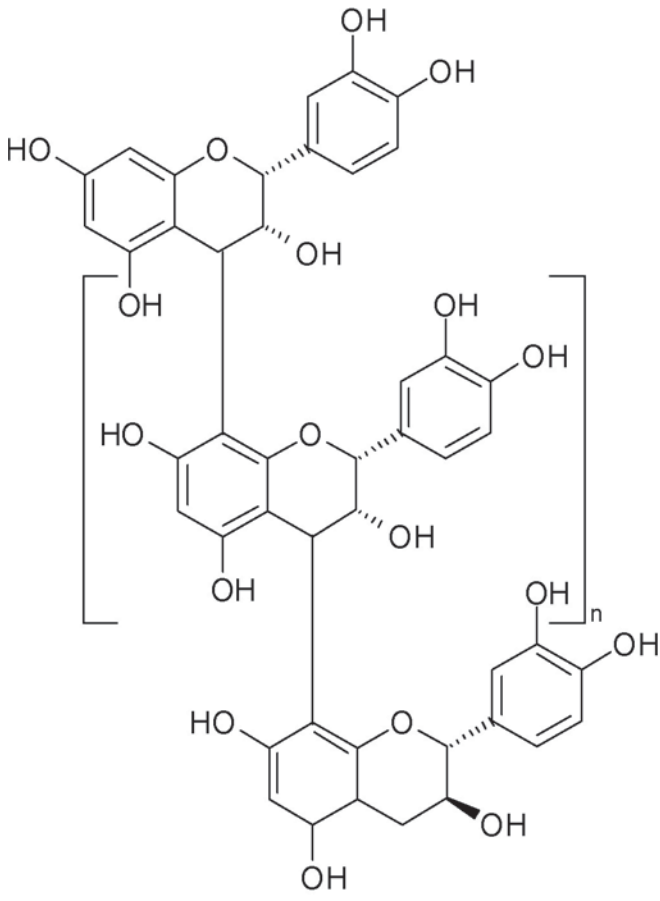
Pentagalloyl glucose (β-1,2,3,4,6-pentagallyol-O-D-glucopranose)

2. Ellagitannins



Castalagin

Fig. 1. Structure of hydrolyzable tannins.



Sorghum procyanidin

Epicatechin-[(4β ⟶ 8)-epicatechin]$_{15}$ - (4β ⟶ 8)-catechin

Fig. 2. Structure of hydrolyzable tannins.

1.2. Chemical Assays

The most commonly used procedures in the category of chemical assays are described in the following subsections.

1.2.1. Total Phenol and Total Tannin Assays

These assays are based on the oxidation-reduction principle and use the Folin-Ciocalteu or Folin-Denis reagents. Another method using the ferric chloride reagent is based on the metal-complexing property of phenolics ***(3)***. The Folin-Ciocalteu method is used widely for total phenols because of its high sensitivity and reproducibility. However, the presence of reducing agents interferes in the assay. Nontannin phenolics cannot be distinguished from tannins with both the oxidation-reduction and metal-complexing methods. Tannins are distinguished from nontannins by using a solid matrix, polyvinylpolypyrrolidone (PVPP). A portion of the plant extract is treated with PVPP. This method assumes that the phenolics that bind to proteins are the same as those that bind to PVPP. Total phenols are measured in a plant extract using the Folin-Ciocalteu method before and after treatment with PVPP. Polyvinylpolypyrrolidone has a high affinity for tannins and its removal using centrifugation, following the PVPP treatment, removes tannins from the extract. The difference between total phenol values before and after the PVPP treatment is a measure of tannins ***(4)***.

1.2.2. Vanillin Assay and Butanol-HCl Methods for Condensed Tannins

Vanillin assay is based on the metal-complexing properties of tannins ***(5,6)***, and the butanol-HCl assay with and without addition of iron on the oxidative depolymerization of tannins ***(7)***. The vanillin assay has been widely used for measuring condensed tannins in sorghum and other beans. This assay is not very specific since it measures flavan-3-ols and dihydrochalcones, which are nontannin, in addition to condensed tannins. In addition, in this method monomeric flavans give higher color yield as compared to condensed tannins, and proanthocyanidins based on 5-deoxyflavanols, such as profistinidin in quebracho tannin, do not react; the presence of acetone interferes in this assay, and reproducibility of this method is not good ***(8)***. The acidic butanol method is simple and more specific compared to the vanillin assay. This method yields pink-colored anthocyanidins on oxidative cleavage of the interflavan bonds of condensed tannins in the presence of mineral acids in alcoholic solutions at about 95°C. This method was later modified by inclusion of iron in the butanol-HCl reagent. The addition of iron was considered to enhance the sensitivity and reproducibility of the assay ***(7)***.

1.2.3. Hydrolyzable Tannin Assays

The approach used for the determination of hydrolyzable tannins is based on their conversion to a common unit and the determination of the common units by spectrophotometric or high-performance liquid chromatography (HPLC) methods. These methods are useful for simple hydrolyzable tannins but may provide limited information for complex oligomeric hydrolyzable tannins. The rhodanine method ***(9)*** determines gallotannins as gallic acid equivalent. In this method, gallotannins are hydrolyzed under anaerobic and acidic conditions to gallic acid, which is reacted with rhodanine to give pink chromophore (measured at 520 nm). The specificity and sensitivity of the assays could be increased by measuring gallic acid by the HPLC method ***(2)***. Recently, Hagerman's group ***(10)*** modified the potassium iodate method to include a first step in which all of the hydrolyzable tannins are converted to a single chemical species, methyl gallate. The advantage of this method is that the effect due to the difference in reactivity of the parent compounds is thus eliminated, and results from various laboratories can be compared directly since they are measured in terms of methyl gallate, which is commercially available.

1.3. Protein Precipitation Assays

The methods for quantification of tannins based on their property of complexing with proteins (protein precipitation assays) are considered to provide better information on the biological value of feeds and fodders containing tannins. In these methods, protein-tannin complexes are prepared. The protein in these complexes is measured using the ninhydrin assay of amino acids released by alkaline hydrolysis of the complex ***(11,12)***, which represents protein precipitation capacity; and when phenolics in the complex are determined by the ferric chloride method ***(3)*** the protein precipitable phenolics are measured. The method based on binding of ^{125}I-labeled bovine serum albumin ***(13)*** for determination of the protein precipitation capacity of tannins is accurate and sensitive. Recently, this method has been simplified ***(14)***. However, it requires special equipment and some degree of expertise.

In another method ***(15)***, the tannin-protein complexes are formed on a chromatography paper and reacted with the protein bovine serum albumin (BSA). Unbound BSA is washed off, and the protein in the tannin-protein complex is stained with Ponceau S, a dye specific for proteins. Protein-bound dye is eluted and the absorbance of the eluate is measured at 525 nm. The absorbance is converted to protein by using a calibration curve. This method is about 20 times more sensitive than the other methods ***(11,12)***. The protein bound to tannins can also be measured using amido black dye, which can be eluted and measured or can be measured using an image analyser ***(16)***. Another

advantage of these methods ***(14–16)*** is that acetone containing plant extracts can be used, whereas the presence of acetone interferes in other protein precipitation assays ***(3,11–13)***. The preparation of plant extract in aqueous acetone (generally 70%) is desirable because the solubility of tannins and phenolics is higher in aqueous acetone solution, and acetone prevents oxidation of phenols.

Another protein precipitation method that is insensitive to acetone is the radial diffusion assay ***(17)***. The tannin-protein complex is formed in the gel containing BSA, which appears as a ring. The diameter of the ring is a measure of protein precipitation/binding capacity of tannins.

1.4. Gravimetric Methods

The spectrophotometric methods determine tannins relative to one or another standard, namely tannic acid, gallic acid, catechin, quebracho tannins, leukoanthocyanins, etc. The gravimetric method ***(18)*** based on the precipitation of phenolics by ytterbium acetate measures total phenolics and not tannins. This method is not specific for phenolics. It precipitates other moieties in addition to phenolics. In addition, the precipitation is not complete at low phenolic concentration, and some phenolics, for example, rutin, is not precipitated ***(19)***, leading to underestimation of phenolics. Another gravimetric method ***(4)*** is based on weighing the tannin extract before and after removal of the tannin by treatment with PVPP to bind tannin and removal of the PVPP-tannin complexes by centrifugation. Gravimetric methods have generally lower sensitivities than colorimetric methods and are time-consuming. The gravimetric method ***(4)*** also suffers from these disadvantages. So it should be used when the dry matter digestibility of tannin-rich feeds needs to be corrected for the presence of tannins. For other routine applications the principle of the gravimetric method is used in conjunction with a spectrophotometric method. Total phenols are determined spectrophotometrically using the Folin-Ciocalteu reagent in the extract before and after the PVPP treatment. The difference in these phenolic values is a measure of tannins. This difference (tannins) when expressed as tannic acid equivalent is quite close to the tannin levels determined gravimetrically in leaves from various trees and browses; each gram of tannins (by mass) had a reducing power equivalent to 0.76 to 1.25 g tannic acid ***(4)***.

1.5. Tannin Bioassay

All available protein precipitation assays measure tannins under conditions different from those of the rumen, and therefore the results obtained have limited applicability for predicting the nutritive value of tannin-containing feedstuffs. Polyethylene glycol and polyvinylpyrrolidone bind to tannins and make them inert ***(20)***. This property has been exploited together with the in

vitro gas method to quantify the effects of tannins on rumen fermentation. Incubation of polyethylene glycol 6000 along with a tannin-containing feedstuff in the in vitro system increases gas production. The percentage increase in gas production, for example at 24 h of incubation, represents the effects of tannins. The higher the percentage increase in gas production, the greater is the effect ***(20)***.

1.6. ^{14}C-Labeled Polyethylene Glycol Binding Assay

Polyethylene glycols (PEGs) of molecular weights 4000 or 6000 have a very high affinity for both hydrolyzable and condensed tannins over a wide range of pH ***(20)***. In this method the feed samples are kept in contact with PEG spiked with ^{14}C-labeled PEG, and the radioactivity bound to feed sample is a measure of tannins; the higher the activity, the higher the tannins. The method is reportedly useful since there is no need to extract tannins, and it is considered to be a measure of both bound and extractable tannins ***(21,22)***. The formation of soluble tannin-PEG complexes, which is not recovered in the feed sample, can underestimate the tannin values. There is a need to study the extent of formation of soluble versus precipitable PEG-tannin complexes under the conditions of the assay (and to study the nutritional significance of these soluble complexes); to standardize the assay for parameters such as optimum particle size of the sample, temperature, and treatment time; and to investigate the extent of nonspecific binding of ^{14}C-PEG. Additionally, correlations should be established between the PEG-binding assay and protein precipitation assays or the tannin bioassay using the in vitro gas method or the increase in the nitrogen degradability of feeds on addition of PEG. Inclusion of in vivo parameters such as intake, nitrogen balance, and degradability, production parameters (growth, wool production, etc.) for these feeds in the correlation studies, would reveal the usefulness of the in vitro methods in predicting nutritional and physiological effects of feeding tannin-containing diets to ruminants.

An increase in nitrogen degradability of a feed when incubated in an in vitro rumen fermentation system in the presence of PEG is also a measure of tannin activity, and this increase in nitrogen degradability has also been found to predict the effects of tannins in ruminants ***(23,24)***.

1.7. Near-Infrared–Based Method

Tannins in legume forages were quantified using near-infrared reflectance spectroscopy. The wavelength, 2.150 μm, was identified for prediction of condensed tannins ***(25)***. This method may be applicable to the determination of tannins in large sample sets of homogeneous feeds such as forage legumes.

Among the above methods, only three methods are presented here:

1. Total phenolic and tannin assays
2. Condensed tannin assay
3. Biological activity of tannins

For other methods, see *A Laboratory Manual on Tannin Assays* (Makkar, 2003).

2. Materials

2.1. Measurement of Total Phenolics and Tannins Using the Folin-Ciocalteu Method, Based on Makkar et al. (4)

1. *Folin-Ciocalteu reagent (1 N).* Dilute commercially available Folin-Ciocalteu reagent (2 N) with an equal volume of distilled water. Transfer it into a brown bottle and store in a refrigerator (4°C). It must be golden-yellow. Do not use it if it turns olive green or green.
2. *Sodium carbonate (20%).* Weigh 40 g sodium carbonate.10 H_2O, dissolve it in about 150 mL distilled water, and make the volume up to 200 mL with distilled water.
3. *Insoluble polyvinylpyrrolidone (polyvinylpolypyrrolidone, PVPP).* This is commercially available from Sigma, St. Louis, MO (P 6755).
4. *Standard tannic acid solution (0.1 mg/mL).* Dissolve 25 mg tannic acid obtained from Merck in 25 mL distilled water and then dilute 1 : 10 in distilled water (always use a freshly prepared solution).

2.2. Measurement of Condensed Tannins (Proanthocyanidins, Based on Porter et al. (7)

1. *Butanol-HCl reagent (butanol-HCl 95 : 5 v/v).* Mix 950 mL *n*-butanol with 50 mL concentrated HCl (37% w/v).
2. *Ferric reagent (2% ferric ammonium sulfate in 2 N HCl).* To prepare 2 N HCl, take 16.6 mL of concentrated HCl and make the volume up to 100 mL with distilled water. Dissolve 2.0 g ferric ammonium sulfate in 100 mL of 2 N HCl. This reagent should be stored in a dark-brown bottle.

2.3. Determination of Biological Activity of Tannins, Based on Makkar et al. (20)

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 the volume up to 1 L 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 sulfate ($MgSO_4 \cdot 7H_2O$) in approximately 500 mL distilled water and then make the volume up to 1 L with distilled water.
3. *Micromineral solution:* Dissolve 10 g manganese chloride ($MnCl_2 \cdot 4H_2O$), 13.2 g calcium chloride ($CaCl_2 \cdot 2H_2O$), 1 g cobalt chloride ($CoCl_2 \cdot 6H_2O$), 8 g ferric

chloride ($FeCl_3 \cdot 6H_2O$) in approximately 50 mL distilled water and then make the volume up to 100 mL with distilled water.
4. *Resazurin:* Dissolve 0.1 g resazurin in 100 mL distilled water.
5. *Reducing solution:* Dissolve 996 mg sodium sulfide ($Na_2S \cdot 9H_2O$) 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).

3. Methods

3.1. Measurement of Total Phenolics and Tannins Using Folin-Ciocalteu Method, Based on Makkar et al. (4)

3.1.1. Extraction of Phenolics (Simple Phenolics and Tannins)

Dried and finely ground (ground preferably using a ball mill) plant sample (leaves, seeds, root, stem, etc.) is placed in a glass beaker of approximately 25-mL capacity (*see* **Note 1**); 10 mL of aqueous acetone (70%) is added and the beaker is suspended in an ultrasonic water bath and subjected to ultrasonic treatment at 300 W for 20 min at room temperature (*see* **Notes 2** and **3**). The content of the beaker is then transferred to centrifuge tubes and centrifuged for 10 min at approximately 3000 *g* 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 (*see* **Notes 4** and **5**).

3.1.2. Preparation of Calibration Curve

Take 0.0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 µL of standard tannic acid solution in 5-mL glass test tubes separately. Then add an adequate quantity of distilled water for respective test tubes to bring the volume to 500 µL. To this solution, add 250 µL of the Folin-Ciocalteu reagent (1 N) and 1.25 mL of the sodium carbonate solution (final volume will be 2.0 mL; tannic acid concentration will be 0.0 to 10 µg). Vortex the contents and incubate at room temperature under dark condition. After 40 min read absorbance at 725 nm against the reagent blank.

3.1.3. Analysis of Total Phenols

Take suitable aliquots of the above sample extract in test tubes and make the volume to 500 µL with distilled water. Add to it 250 µL of the Folin-Ciocalteu reagent (1 N) and then 1.25 mL of the sodium carbonate solution. Vortex the tubes and record absorbance at 725 nm after incubation for 40 min under dark conditions. Calculate the amount of total phenols as tannic acid equivalent from the above calibration curve. Express the results as total phenolics as grams per 100 g on a dry matter basis.

3.1.4. Determination of Total Tannins

Weigh 100 mg PVPP in a 100 × 12 mm test tube. Add 1.0 mL distilled water and then 1.0 mL of the sample 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). Vortex and keep the tube at 4°C for 15 min, vortex it again, and centrifuge at 3000 *g* for 10 min. Collect the supernatant. This supernatant has only simple phenolics other than tannins (the tannins get bound to PVPP). Measure the phenolic content of the supernatant as mentioned above and express the content of nontannin phenolics on a dry matter basis. From the above results, the tannin content of the sample can be calculated as follows:

$$\text{Total phenolics (\%)} - \text{Nontannin phenolics (\%)} = \text{Tannin (\%)}$$

The results can be expressed as tannic acid equivalent on a dry matter basis.

3.2. Measurement of Condensed Tannins (Proanthocyanidins), Based on Porter et al. (7)

3.2.1. Determination of Condensed Tannins

In a 100 mm × 12 mm glass test tube, take a suitable aliquot (0.1 to 0.5 mL) of the sample extract (for preparation of the extract see **Section 3.1.1**; also *see* **Note 6**) and dilute it with 70% aqueous acetone to 0.5 mL (the aliquot volume depends on the amount of condensed tannins in the extract). To the tubes add 3.0 mL of the butanol-HCl reagent and 0.1 mL of the ferric reagent. Vortex the tubes and cover the mouth of each tube with a glass marble and transfer the tubes in a heating block adjusted at 97° to 100°C or in a boiling water bath for 60 min. After cooling the tubes, record absorbance at 550 nm. Subtract absorbance of a suitable blank, which is usually the unheated mixture. If the extract has flavan-3,4-diols or flavan 4-ol, a pink color develops without heating. If this happens, use one heated blank for each sample, composed of 0.5 mL extract, 3 mL *n*-butanol (no HCl), and 0.1 mL ferric reagent. Condensed tannins (% in dry matter) as leukocyanidin equivalent is calculated by the following formula:

$$(\text{Absorbance at 550 nm} \times 78.26 \times \text{Dilution factor})/(\%\ \text{Dry matter})$$

This formula takes the effective $E^{1\%,1cm,550nm}$ of leukocyanidin to be 460. The dilution factor is equal to 1 if 0.5 mL of the sample extract is taken before addition of 3 mL of the butanol-HCl reagent and the extract was made from a 200-mg sample in 10 mL 70% aqueous acetone (the dilution factor would be 5 if, for example, 0.1 mL of the sample extract is taken and to it is added 0.4 mL of 70% aqueous acetone before the addition of 3 mL of the butanol-HCl

reagent; the amount of sample extract depends on the amount of condensed tannins in the extract; if by taking 0.5 mL of the sample extract the absorbance is greater than 0.6, take the lower amount of the sample extract and dilute to 0.5 mL with 70% aqueous acetone). *See* **Note 7**.

3.3. Determination of Biological Activity of Tannins, Based on Makkar et al. (20)

3.3.1. Sample Preparation

Dried leaves should be passed through a 1-mm sieve.

3.3.2. Weighing of Samples and Preparation of Syringes

Tare a specially made scoop (approximately 4 cm in length and 1 cm in depth/radius; a standard sodium hydroxide–containing plastic container can be cut horizontally in half to form the scoop) on an analytical balance. Weigh a 500-mg feed sample (passed through a 1-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 (molecular weight 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.

3.3.3. Preparation of In Vitro Rumen Fermentation Buffer Solution

Collect the rumen fluid and particulate matter before the morning feed from two cattle fed on a 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 upside down. The strained rumen fluid is kept at 39°C under carbon dioxide and should be prepared just before the start of the incubation. As the amount of feed taken is 500 mg, the composition of the medium is based on Tilley and Terry ***(26)***. Menke et al. ***(27)*** 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, the fermented material is taken for various analyses and hence the amount of substrate taken is 500 mg. There is an inherent error associated with gravimetric determination of the fermented residue, which is large if 200 mg feed is taken in place of 500 mg.

Medium composition and incubation procedure (following volume in milliliters)	
Rumen buffer solution	630.00
Macromineral solution	315.00
Micromineral solution	0.16
Resazurin	1.60
Distilled water	975.00
Freshly prepared reducing solution	60.00
The rumen fluid (see above for collection and preparation)	660.00

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

Except for the rumen fluid and the reducing solution, mix all the above-mentioned solutions in the order listed above in a 3- or 5-L capacity glass container.

3.3.4. Incubation and Determination of Gas Released

The container holding the above solutions is kept in a water bath adjusted at 39°C. This water bath is a plastic rectangular container (400 cm × 300 cm × 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 color of the dye changes to pink and then to colorless; it takes about 15 to 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. Filling 60 syringes, after some practice, should take 30 to 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 4 hours and then every 2 hours. Generally, the incubation is started at about 7.30 a.m. and after 12 h of the incubation, the syringes are not shaken until 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 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. The addition of PEG to blanks does not affect the gas production from blanks, suggesting that it is inert.

3.3.5. Determination of Biological Activity of Tannins

The difference between the net volume of gas produced from syringes with and without PEG, generally at 16 h and 24 h, is measured, which is a measure of tannin effect. The PEG binds tannins and inactivates them. The higher the percent increase in gas (without PEG being 100%) on addition of PEG, the higher the biological activity of tannins with regard to rumen microbes ***(20)***.

4. Notes

1. Pigments and fat can be removed from the dried leaf sample by extracting with diethyl ether containing 1% acetic acid before extracting phenolics.
2. A first extraction with 50% aqueous methanol followed by a second extraction with 70% aqueous acetone can also be used for extraction of phenolics.
3. A very long period of phenolic extraction and extraction at high temperature may lead to inactivation of phenolics.
4. Freshly prepared extract should be used for phenolic and tannin analysis.
5. Tubes/containers containing the extract should be kept under cold conditions until the analysis is complete.
6. The presence of pigments may interfere with this method. The pigments can be removed by extracting the dried leaves with petroleum ether containing 1% acetic acid. Ascorbic acid (generally added to prevent oxidation of phenolics) does not interfere in the condensed tannin assay and can be added while preparation of the plant extract (ascorbic acid interferes in the phenolic assay by Folin-Ciocalteu method).
7. Vanillin-HCl ***(5,6)*** is also used for determination of condensed tannins but this method is not specific ***(8,28)***. It measures condensed tannins as well as simple flavonoids. This method also has several other disadvantages.

References

1. McSweeney, C.S., Makkar, H.P.S., and Reed, J.D. (2003) Modification of rumen fermentation to reduce adverse effects of phytochemicals. In: *Proceedings of the Sixth International Symposium on the Nutrition of Herbivores,* Mannetje, L.'t, Ramirez-Aviles, L., Sandoval-Castro, C., and Ku-Vera, J.C., (eds.), Merida, Mexico, 19–24 October, pp. 239–270.
2. Makkar, H.P.S. (2003) *Quantification of Tannins in Tree and Shrub Foliage,* Makkar, H.P.S., (ed.), Kluwer Academic Press, New York, p. 102.
3. Hagerman, A.E., and Butler, L.G. (1978) Protein precipitation method for the quantitative determination of tannins. *J. Agric. Food Chem.* **26**, 809–812.
4. Makkar, H.P.S., Blummel, M., Borowy, N.K., and Becker, K. (1993) Gravimetric determination of tannins and their correlations with chemical and protein precipitation methods. *J. Sci. Food Agric.* **61**, 161–165.

5. Broadhurst, R.B., and Jones, W.T. (1978) Analysis of condensed tannins using acidified vanillin. *J. Sci. Food Agric.* **29**, 788–794.
6. Price, M.L., Van Scoyoc, S., and Butler, L.G. (1978) A critical evaluation of the vanillin reaction as an assay for tannin in sorghum grain. *J. Agric. Food Chem.* **26**, 1214–1218.
7. Porter, L.J., Hrstich, L.N., and Chan, B.G. (1986) The conversion of procyanidin and prodelphinidins to cyaniding and delphinidin. *Phytochemistry* **25**, 223–230.
8. Makkar, H.P.S., and Becker, K. (1993) Vanillin-HCl method for condensed tannins: effect of organic solvents used for extraction of tannins. *J. Chem. Ecol.* **19**, 613–621.
9. Inoue, K.H., and Hagerman, A.E. (1988) Determination of gallotannins with rhodanine. *Anal. Biochem.* **169**, 363–369.
10. Hartzfeld, P.W., Forkner, R., Hunter, M.D., and Hagerman, A.E. (2002) Determination of hydrolyzable tannins (gallotannins and ellagitannins) after reaction with potassium iodate. *J. Agric. Food Chem.* **50**, 1785–1790.
11. Makkar, H.P.S., Dawra, R.K., and Singh, B. (1987) Protein precipitation assay for quantitation of tannins: Determination of protein in tannin-protein complex. *Anal. Biochem.* **166**, 435–439.
12. Makkar, H.P.S., Dawra, R.K., and Singh, B. (1988) Determination of both tannin and protein in a tannin-protein complex. *J. Agric. Food Chem.* **36**, 523–525.
13. Hagerman, A.E., and Butler, L.G. (1980) Determination of protein in tannin-protein precipitates. *J. Agric. Food Chem.* **28**, 944–947.
14. Henson, G.L., Niemeyer, L., Ansong, G., Forkner, R., Makkar, H.P.S., and Hagerman, A.E. (2004) Modified method for determining protein binding capacity of plant polyphenolics using radiolabeled protein. *Phytochemical Anal.* **15**, 159–163.
15. Dawra, R.K., Makkar, H.P.S., and Singh, B. (1988) Protein binding capacity of microquantities of tannins. *Anal. Biochem.* **170**, 50–53.
16. Hoffmann, E.M., Muetzel S., and Becker, K. (2002) A modified dot-blot method of protein determination applied in the tannin-protein precipitation assay to facilitate the evaluation of tannin activity in animal feeds. *Br. J. Nutr.* **87**, 421–426.
17. Hagerman, A.E. (1987) Radial diffusion method for determining tannin in plant extracts. *J. Chem. Ecol.* **13**, 437–449.
18. Reed, J.D., Horvath, P.J., Allen, M.S., and Van Soest, P.J. (1985) Gravimetric determination of soluble phenolics including tannins from leaves by precipitation with trivalent ytterbium. *J. Sci. Food Agric.* **36**, 255–261.
19. Lowry, J.B., and Sumpter, E.A. (1990). Problems with ytterbium precipitation as a method for determination of plant phenolics. *J. Sci. Food Agric.* **52**, 287–288.
20. Makkar, H.P.S., Blümmel, M., and Becker, K. (1995) Formation of complexes between polyvinyl pyrrolidone and polythylene glycol with tannins and their implications in gas production and true digestibility in *in vitro* techniques. *Br. J. Nutr.* **73**, 897–913.

21. Silanikove, N., Perevolotsky, A., and Provenza, F.D. (2001) Use of tannin-binding chemicals to assay for tannins and their negative postingestive effects in ruminants. *Anim. Feed Sci. Technol.* **91**, 69–81.
22. Mlambo, V., and Makkar, H.P.S. (2005) Calibration and validation of the 14C-labelled polyethylene glycol-binding assay for tannins in tropical browse. *Anim. Feed Sci. Technol.* **122**, 29–40.
23. Getachew, G., Makkar, H.P.S., and Becker, K. (2000) Effect of polyethylene glycol on *in vitro* degradability of nitrogen and microbial protein synthesis from tannin-rich browse and herbaceous legumes. *Br. J. Nutr.* **84**, 73–83.
24. Jones, R.J., Meyer, J.H.F., Bechaz, M., and Stoltz, M.A. (2000) An approach to screening potential pasture species for condensed tannin activity. *Anim. Feed Sci. Technol.* **85**, 269–277.
25. Goodchild, A.V., El Haramein, F.J., Makkar, H.P.S., El Monein, A.A., and Williams, P.C. (1997) *18th International Conference on Near-Infrared Spectroscopy*, September 15–19, Haus der Technik, Essen, Germany.
26. Tilley, J.M., and Terry, R.A. (1963) A two-stage technique for the in vitro digestion of forage crops. *J. Br. Grasslands Soc.* **18**, 104–111.
27. Menke, K.H., Raab, L., Salewski, A., Steingass, H., Fritz, D., and Schneider, W. (1979) The *estimation* of the digestibility and metabolizable content of animal feedstuffs from the gas production when they are incubated with rumen liquor *in vitro. J. Agric. Sci. (Cambridge)* **93**, 217–222.
28. Makkar, H.P.S., and Becker, K. (1994) Some problems in the determination of tannins and possible solutions. *Acta Horticultura* **381**, 782–788.