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Evaluation of assays for phenolic compounds on the basis of in vitro gas production by rumen micro-organisms

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Abstract

12 Bolivian fodder tree samples were selected in order to achieve a range of phenolic compositions as gauged by analyses for total phenols (TP) by the Prussian Blue method, protein precipitation activity (PPA) by radial diffusion and condensed tannin (CT) by an acid butanol method. Aqueous acetone extracts of phenols from these samples were prepared and added, with and without the phenol binding agent polyvinylpyrrolidone (PVP), to an in vitro gas production system which used rumen micro-organisms to ferment glucose. Differences in the cumulative volume of gas produced with and without PVP were taken as measures of the effects of phenols on fermentation. Two measures of inhibition were used, the maximum differences achieved (generally after 9 or 12 h. incubation) termed Difference T max and the difference when gas production had virtually ceased (after 166 h incubation) termed Difference T166. Linear regression analysis was used to test the hypothesis that the assays correlated with the inhibition of fermentation. Significant ($P < 0.01$) correlations were found between Difference T max, Difference T166 and TP ($R^2 = 0.82$ and 0.83 respectively) and PPA ($R^2 = 0.63$ and 0.74 respectively). There was no significant ($P > 0.5$) correlation between CT and either measure of inhibition.

It was concluded that TP and, less accurately, PPA were useful indicators of the degree of inhibition of rumen micro-organisms by phenolic compounds in tree leaves. The acid butanol assay for condensed tannins was not an indicator of rumen micro-organism inhibition when used for a range of tree species.

Keywords: Phenolic compounds; Gas production

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1. Introduction

Tree leaves are used as sources of fodder in many tropical countries, particularly in the dry season when other feeds can be in short supply. The assessment of the nutritive value of such fodders is difficult due to the presence of anti-nutritive factors in many tree leaves, phenolic compounds such as tannins being the most widespread of these factors (reviewed by Mangan, 1988). Phenolic compounds can be broadly classified into three types: hydrolysable tannins, condensed tannins and non-tannin phenols (such as low molecular weight phenols). A wide range of assays for measuring phenols has been proposed (reviewed by Hagerman and Butler, 1989). For this study three assays, recommended by Hagerman and Butler (1989), which appeared suitable for routine use were selected. Total phenols (TP) is a chemical measure of all the phenolic material. It was preferred to the similar Folin assay as it is less susceptible to interference from proteins. Protein-binding assays give measures of protein precipitation activity (PPA).

PPA is a biochemical measure of the activity of all the tannins. The radial diffusion assay for PPA was selected for its simplicity. The acid butanol assay for condensed tannins (CT) is a chemical measure of the condensed tannin (proanthocyanidin) content. Hagerman and Butler (1989) regarded it as generally better than the vanillin assay for the selective determination of condensed tannin.

Simple correlations between nutritive value or digestibility and phenolic assays are unlikely to be reliable across a range of species. Other components, such as fibre and protein content, are also of major importance. To investigate such correlations one approach has been to use agents which strongly bind to tannins and thus inhibit their usual biological effect. Comparisons can then be made between the properties of tanniniferous feeds with and without binding agent, the difference being a measure of the effect of the tannins. Barry and Forss (1983) ground and extracted plant material in the presence and absence of polyethylene glycol (PEG) and observed higher protein solubilities in the PEG treated extracts. PEG treatment also increased the apparent digestibility of tanniniferous material eaten by sheep (Barry and Duncan, 1984). Polyvinylpyrrolidone (PVP) similarly binds tannins (Garrido et al., 1991).

Poor correlations have been obtained in this laboratory between phenol assays and the differences in in vitro gas production caused by PVP and PEG treatment of dried Colombian tree fodders (M. Rosales and C.D. Wood, unpublished data); Khazaal and Ørskov (1994) have reported similar findings using polyvinylpolypyrrolidone (PVPP) to treat various Greek browse species. The effectiveness of PEG at releasing protein from protein-tannin complexes is dependent on the age of the complex at the time of addition of PEG (Jones and Mangan, 1977). The poor correlations observed may have been due to the binding agents only being able to bind to a variable proportion of the tannins, the remaining tannins remaining bound to the substrate and inhibiting its fermentation. As only dried and ground samples were available for this study, it was considered that the treatment of the dried substrates with binding agents could not be relied upon to bind all the tannins. To overcome this the tannins were extracted from the leaves before treatment with PVP.

The objective of this study was to investigate correlations between tannin assays and their effects on an in vitro fermentation method which uses rumen micro-organisms

(Theodorou et al., 1994) to identify which assays most reliably indicated the extent of these effects. Linear regressions between two measures of these effects and the various assays were then investigated.

2. Materials and methods

2.1. Tree leaf samples

A range of tree leaf samples of species of interest to agroforestry workers and of potential use as fodders were obtained from Santa Cruz province in the tropical lowland region of Bolivia. All the samples were analysed for phenolics. From these, 12 samples were selected so as to give a range of phenolic compositions. The tree species selected, with their sample codes (in brackets), were: *Flemingia macrophylla* (= F. *congesta*) (FCb); *Tipuana tipa* (TTa); *Calliandra calothyrsus* (CCb); *Gliricidia sepium* (GSb); *Erythrina ulei* E2 (EU2b); *Inga nobidis* 14 (IN14a); *Inga nobidis* 17 (IN17a); *Inga nobidis* 20 (IN20a); *Inga nobidis* 21 (IN21b); *Inga nobidis* 31 (IN31b); *Piptadenia macrocarpa* 18 (PM18a); *Piptadenia macrocarpa* 18 (PM18b). The number refers to different accessions, code (a) signifies young leaves, code (b) mature leaves. Separate samples of young and mature leaves, pooled from three trees of each species, were collected, oven-dried at 50°C and ground to pass through a 1 mm screen.

2.2. Preparation of extracts and analysis for phenols

The 12 samples were extracted with 70% aqueous acetone (70 ml acetone per 100 ml aqueous acetone solution). 10 g sample was mixed in a Hobart blender for 1 min with 100 ml aqueous acetone and the mixtures centrifuged at 2000 G for 10 min. The supernatant was decanted from the residue. The extracts were analysed for PPA (method of Hagerman, 1987, as modified by Wood et al., 1994), acid butanol (procedure of Porter et al., 1986) for condensed tannins (CT) and total phenols (TP) by the Prussian blue assay described by Price and Butler (1977). The TP method was adapted by taking an aliquot of 10 μl of extract instead of 100 μl, as the acetone extract used was more concentrated than that proposed in the original method.

2.3. In vitro fermentation

The gas production method of Theodorou et al. (1994) as outlined by Prasad et al. (1994) was used. This involved the anaerobic fermentation of substrates in a buffered medium contained in sealed serum bottles using an inoculum prepared from fresh rumen fluid and measuring the gas produced using a pressure transducer. The following modifications to the method were made for the particular requirements of this study.

1. 0.5 g glucose was added as a substrate to each bottle. Tripticase peptone was not added to the basal medium. The substrates potentially available to the rumen micro-organisms were the glucose plus those in the added inoculum and the extract itself.

2. Polyvinlypyrrolidone (PVP; average molecular weight 40 000, Sigma Chemicals, UK) was added in aqueous solution to the basal medium prior to the addition of substrate and inoculum so as to achieve a concentration of 5 g l^{-1} in the final incubation mixture.
3. Extracts were fermented with and without PVP, water being added to mixtures without PVP to ensure that all bottles had the same volume of liquid.
4. The volume of water added to dilute the basal medium was adjusted so that after glucose and PVP/water addition the concentration of buffer and minerals was the same as the standard medium.
5. For 10 h gas production experiments readings were taken every 2 h. For full length (166 h) experiments they were taken every 3 or more h.

2.4. Development of method for preparation of phenolic extracts

Phenols were extracted in 70% aqueous acetone. To assess whether acetone would interfere with the assay, the effect of adding different volumes of 70% aqueous acetone to gas produced in 10 h experiments was investigated.

The effects of different volumes of phenolic extracts on gas production were investigated to establish a level which produced measurable effects and which lay in a range where there was a linear relationship between the volume of extract added and its effect. Horsechestnut leaves, being in plentiful supply and high in tannins, were used for convenience. Up to 15 ml (equivalent to 1.5 g dried leaf) of extract were used in full length (166 h) experiments.

2.5. Effects of extracts from Bolivian leaf samples

The effects of extracts from the Bolivian leaf samples were assessed in two separate 166 h fermentations. To correct for differences between the inocula, the volumes of gas produced in the second experiment were standardised to those of the first by multiplying the cumulative gas production (with and without PVP) by the cumulative gas production after 166 h from 0.5 g of glucose in experiment 1 divided by that attained in experiment 2 (procedure developed by A. Robinson and C.D. Wood, unpublished results).

2.6. Regression analysis

Linear regression analysis was conducted using an ordinary least squares regression procedure by means of the computer programme Statgraphics.

3. Results

3.1. Composition of the aqueous acetone extracts

TP, PPA and CT levels are shown in Table 1. The analyses estimate different chemical or biochemical properties of phenols using independent scales.

Table 1
Measurement of total phenol, PPA and condensed tannins in the acetone extract

Sample	Total phenol mg g^{-1} [a]	PPA cm^2 g^{-1} [b]	Condensed tannin Abs g^{-1} [c]	Inhibition of gas production (ml)	
				T166	Tmax
FCb	45.0	271	629		
TTa	4.8	141	0		
CCb	91.7	417	355		
GSb	2.3	100	0		
EU2b	5.0	174	0		
IN14a	74.2	539	1554		
IN17a	67.8	602	1469		
IN20a	58.6	291	917		
IN21b	39.0	306	260		
IN31b	15.3	138	174		
PM18a	96.9	563	224		
PM18b	72.5	785	160		

[a] Data expressed as mg gallic acid equivalent g^{-1} dry matter. [b] Data expressed as the diameter squared of tannin-protein precipitate ring formed during the radial diffusion assay per gram dry sample. [c] Data expressed as optical density per gram dry sample. [d] Difference Tmax poorly defined.

Regression analysis indicated that there were statistically significant linear correlations between TP and PPA ($P < 0.01$, $R^2 = 0.730$). There was also an apparent correlation (not statistically significant, $P > 0.05$, $R^2 = 0.277$) between TP and CT, with CCb, PM18a and PM18b as outliers (all 3 samples having a relatively high TP for their CT content). Similarly, linear correlation between PPA and CT was not statistically significant ($P > 0.05$, $R^2 = 0.243$), samples IN20a and PM18a having high PPAs but low CT contents.

The analytical data as a whole indicated that the samples used had a wide range of phenolic components, with GSb having very few phenols. TTa, GSb and EU2b did not apparently contain condensed tannins so it may be surmised that the tannins giving rise to the PPA were hydrolysable tannins, although these were not assayed directly.

3.2. *Effect of aqueous acetone and PVP on gas production*

Volumes up to 0.5 ml of aqueous acetone had minimal effects on gas production, but 3–4 ml doubled gas production after 10 h (data not shown). This was presumably because the micro-organisms used acetone as a substrate. 5 ml of aqueous acetone (about 3.5% acetone in the incubation mixture) gave gas production levels similar to those obtained without added acetone, presumably due to anti-microbial properties of acetone at these levels compensating for its use as a substrate. To avoid interference from acetone, extracts were placed into serum bottles and dried under CO_2 before buffer was added. Any residue from the acetone which may have remained in the bottles did not appear to affect gas production, although blanks containing such residues were prepared by drying down 10 ml aqueous acetone in the bottles for use in the experiments to assess the Bolivian tree leaf extracts.

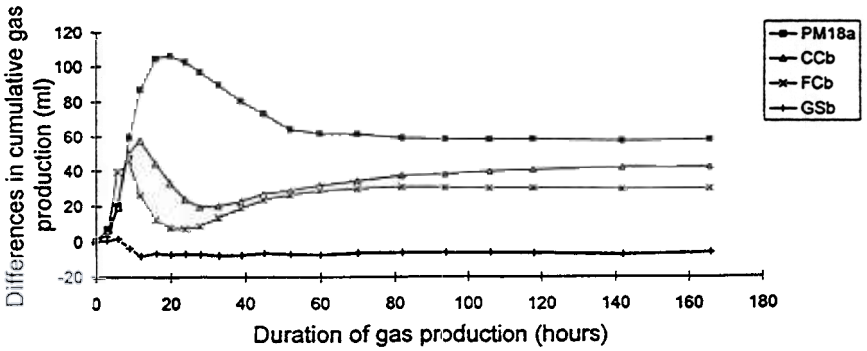

Fig. 1. Difference in cumulative gas production of extracts from Bolivian fodder trees fermented with and without PVP. 0.5 g glucose was added as substrate.

Glucose was fermented with and without PVP in two separate fermentation runs. Bottles with PVP had slightly higher gas production levels after 10 h. The difference between the controls of 10 ml dried-down aqueous acetone, with and without PVP, were subtracted from values obtained for extracts (up to 8.1 ml and 5.5 ml for experiments 1 and 2 respectively) to make allowance for this.

3.3. Effect of PVP on gas production with extract

Using horsechestnut leaf extracts in a 166 h experiment, it was found that from about 15 h fermentation, cumulative gas production from extracts plus glucose substrate (without PVP) exceeded that from control samples (no extract but with glucose substrate, data not shown). With 15 ml extract, these differences achieved statistical significance ($P < 0.05$) from 20 h of incubation.

At all incubation times horsechestnut extracts fermented with PVP had cumulative gas productions greater than the same extract without PVP. For 15 ml extract differences were statistically significant ($P < 0.05$) for all incubation times except 20, 24 and 28 h when differences were at or near a minimum (see below) and not statistically significant ($P > 0.05$). Initially, PVP caused a greatly increased gas production rate which peaked at 9 h (data not shown, but the shapes of the plots of the differences between horsechestnut extracts fermented with and without PVP were similar to that found for FCb, given in Fig. 1). After this peak, gas production was relatively high in the bottles without PVP leading to a marked closing of the differences in cumulative gas productions to a minimum after 24–28 h. The differences in cumulative gas production increased again from 28 h and stabilised after about 120 h when gas production had virtually ceased. This increase in difference in cumulative gas production was due to a marked decline in gas production in bottles without PVP from 28 h; the gas production in bottles with PVP declined less quickly.

The extract apparently contained appreciable levels of fermentable materials as well as phenols which could be bound by PVP. Different components of the extracts, therefore, tended to stimulate and inhibit gas production making interpretation difficult.

Hence, the differences in cumulative gas production between an extract fermented with and without PVP was used as a measure of the effect of phenols rather than the effects of an extract against a control fermentation without extract.

3.4. Effect of different volumes of leaf extract on gas production

There was an approximately linear relationship at all incubation times between the effect of extract and the volume added (up to 15 ml tested, data not shown). The horsechestnut leaves had similar levels of extractable tannins to the more tanniniferous of the Bolivian leaf samples, as judged by the radial diffusion assay and by their effect on gas production. In subsequent trials 10 ml of extract (originating from 1 g dried leaf) per bottle was used as a standard procedure since, as judged from the above, the PVP added appeared to be more than adequate to bind the phenols in the extracts.

3.5. Effect of extracts from Bolivian fodder trees on cumulative gas production

Fig. 1 shows the effect of PVP on cumulative gas production for selected Bolivian fodder tree leaf extracts. GSb contained very low levels of extractable phenols and decreased fermentation from 12 h indicating that there was no inhibition of fermentation by PVP-bound components in the extract. FCb and CCb had similar plots to that described earlier for horsechestnut. Samples IN14a, IN21b, IN17a and PM18a also had plots closely similar to this shape. The timing of the initial peak did vary slightly between samples, 9 h for FCb and 12 h for CCb for example. PM18a (see Fig. 1) did not show increasing differences in the later stages of the gas production run; IN20a behaved similarly. TTa showed an almost constant difference from 6 h into the run, while IN31b showed no early differences while having differences later in the run.

The variability in the shapes of the plots of the effect of PVP on cumulative gas production for the different species complicated the process of making comparisons. There were two times at which it appeared that comparisons could be made:

Difference Tmax: the time (about 9–12 h) when the differences were generally at a maximum

Difference T166: the end of the run (i.e. 166 h) when gas production had virtually ceased.

The values obtained for these two measures of the effects of the polyphenolic extracts on gas production are given in Table 1. There was a statistically significant ($P < 0.001$, $R^2 = 0.723$) correlation between the differences at Tmax and T166. IN20a was the only clearly outlying point as this extract had a large difference Tmax, but a low difference T166.

3.6. Correlations between assays for phenolic compounds and the effects of PVP bound phenols

Significant linear correlations were obtained between PPA and Difference Tmax ($P < 0.01$, $R^2 = 0.627$), and PPA and Difference T166 ($P < 0.01$, $R^2 = 0.741$), the

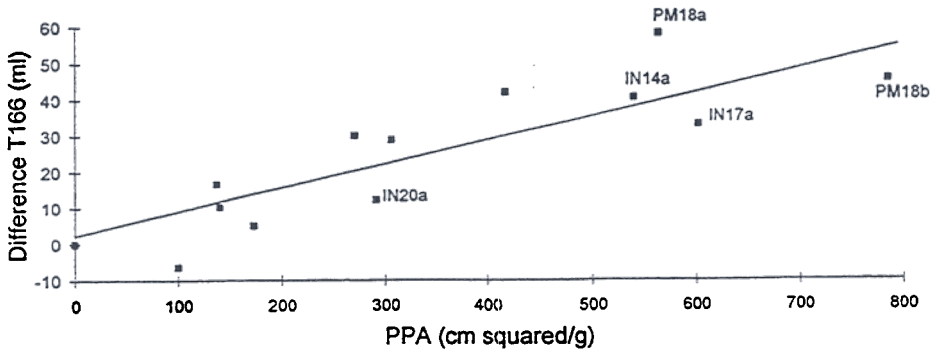

Fig. 2. Correlation between protein precipitation activity (PPA) of Bolivian tree leaf extracts and the difference at 166 h fermentation (Difference T166) between extract plus 0.5 g glucose with and without PVP.

latter being illustrated in Fig. 2. PPA appeared to give a fair indication of the inhibitory effect of polyphenols on rumen micro-organisms.

Regression analysis also gave significant correlations between TP and Difference Tmax ($P < 0.01$, $R^2 = 0.822$), TP and Difference T166 ($P < 0.01$, $R^2 = 0.832$). As noted above IN20a had a large difference Tmax, but a low difference T166. IN20a was an outlying point on the TP vs. difference T166 correlation as can be seen in Fig. 3, removal of this point giving $R^2 = 0.918$. The correlation between TP and differences at both Tmax and T166 had higher values for R^2 than that between PPA and these parameters, however for sample IN20a the PPA gave a better measure than TP of the effect of phenols on the Difference T166.

Correlation between the levels of condensed tannins and both Difference Tmax and Difference T166 was very poor, neither achieving statistical significance ($P > 0.05$).

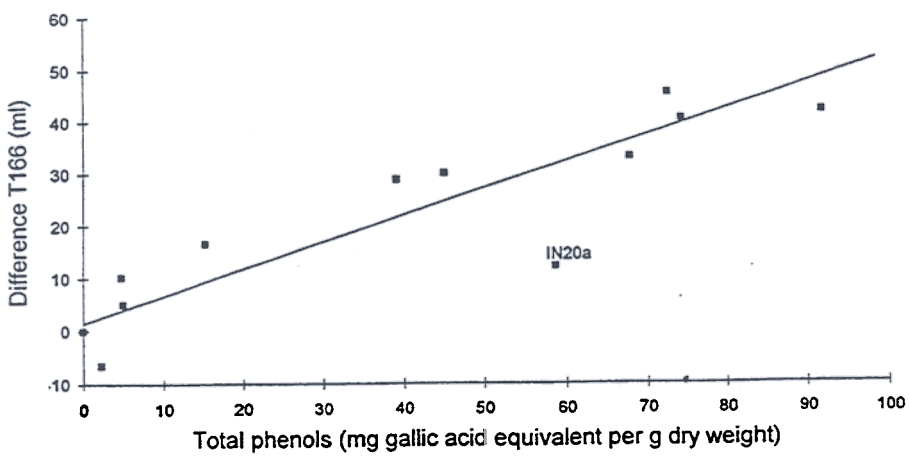

Fig. 3. Correlation between total phenols (TP) of Bolivian tree leaf extracts and the difference at 166 h (Difference T166) between extract plus 0.5 g glucose fermented with and without PVP (all data included).

4. Discussion

4.1. Effects of PVP-bound tannins on gas production

The initial inhibitory effect of the PVP-bound tannins was presumably due to an inhibition of microbial activity. The subsequent reduced difference in gas production with and without PVP may have been due to the relative exhaustion of readily fermentable substrate in bottles with PVP, adaptation of the micro-organisms to phenols, degradation of phenols reducing their inhibitory effect or a combination of all of these factors. The increase in the differences in cumulative gas production after about 28 h, seen for most extracts, may have been due to phenols permanently binding to substrate and thus preventing its degradation, the formation of toxic compounds from the degradation of phenols, or changes in the stoichiometry of gas production induced by the phenols.

4.2. Previous studies on correlations between assays for phenolic compounds and their effects on ruminants

There is only limited experimental evidence to indicate the usefulness of assays for phenols in nutritive value assessment. Robbins et al. (1987) correlated crude protein content to the proportion of digestible protein for feeds such as grasses and agriculturally-produced legumes and grains with very low tannin contents. Using the correlation equation obtained to predict protein digestibility and comparing this to the measured protein digestibility of tanniniferous feeds, an estimate of the reduction in digestible protein due to tannins was obtained. This was found to correlate with the activity of the tannins as assayed by a protein precipitation assay ($R^2 = 0.90$). Digestibility trials were conducted using mule deer fed on a range of plants. Hanley et al. (1992) used predictive equations developed by Robbins et al. (1987) to predict successfully the protein and dry matter digestibilities of tanniniferous forage leaves from seven species and one sample of twigs when fed to black-tailed deer. A protein precipitation assay gave a useful estimate of the effect of tannins in vivo.

McKey et al. (1978) have also found that TP (by the Folin method) and soluble proanthocyanidins (condensed tannins, by acid butanol) correlated negatively with the dry matter digested by rumen inoculum during 96 h incubation ($R = -0.5011$, $P < 0.001$; $R = -0.4915$, $P < 0.001$ respectively). Data were quoted for 30 species of trees from which samples of mature leaves had been taken. For 72 West African fodder trees and shrubs, Rittner and Reed (1992) found that in vitro protein degradability was negatively correlated with soluble phenols (by ytterbium precipitation; $R = -0.34$, $P < 0.01$) and soluble proanthocyanidins (by acid butanol; $R = -0.47$, $P < 0.001$). However, the behaviour of some species deviated greatly from that indicated by the correlation studies.

Other workers have had less success in finding correlations. Makkar et al. (1989) were unable to find significant correlations between TP (by the Folin method), condensed tannins (by vanillin) and protein precipitation with in sacco dry matter loss for leaves of 10 species of trees from India. Khazaal and Ørskov (1994) found that the

increase in gas production resulting from treating eleven leaf samples with PVPP was not related ($P > 0.05$) to TP (by Folin method and by the gravimetric method of Makkar et al., 1993), extractable condensed tannins (by acid butanol and vanillin methods) and total condensed tannins (by acid butanol). Mole and Waterman (1987) found little correlation between chemical assays for TP (by the Folin method), condensed tannins (by the vanillin method), hydrolysable tannins (by various methods) and biochemical activities as assayed by a protein precipitation method and cellulase inhibition.

4.3. Correlations achieved in this study

From the brief review above it can inferred that there is no broad consensus as to which tannin assays most reliably indicate their biological effects in the rumen. The Prussian Blue assay for TP has been recommended by Hagerman and Butler (1989) as it is less susceptible to interference from proteins than the alternative Folin assay. TP assays do not, however, discriminate between tannin and non-tannin phenols and are susceptible to interference to some extent (Hagerman and Butler, 1989). It was, therefore, perhaps surprising to find good correlations between biochemical activities as measured in the in vitro gas production system described here and TP by the Prussian Blue method. The radial diffusion assay also appeared to be a useful, although less accurate, indicator of the effect of tannins on gas production. Hagerman and Butler (1989) have advocated the use of protein-binding assays as indicators of biological activity; there is some support for their view from this study.

The extracts apparently contained variable mixtures of condensed and hydrolysable tannins with several extracts having a measurable PPA but no response to the CT assay. If the effects of tannins are due to the combined effects of both hydrolysable and condensed tannins it would be expected that measures of one type alone will not correlate well over a range of tree species, even if the assay does truly reflect the biological effects of that component. The data obtained strongly indicated that the inhibition of gas production by rumen micro-organisms by phenols is not due to condensed tannins alone.

It must also be noted that the in vitro system used here was not susceptible to any toxic effects that tannins may have directly on animals and effects which may be exerted by their binding to feeds (including consideration of non-extractable tannins). Other assays may well be of use in assessing these effects.

5. Conclusions

Earlier studies have given some contradictory indications as to the usefulness of various assays for phenols and tannins as measures of their likely effects on the nutritive value of feeds. This may in part have been due to the difficulty of isolating the effects of the tannins in complex mixtures. Also the assays have their limitations and may be subject to interference from non-tannin materials. This study has demonstrated that the in vitro gas production method is sensitive to inhibition by phenols. The total phenol assay by the Prussian blue method appeared to be a useful indicator of the degree of

inhibition of rumen micro-organisms by phenols. Protein precipitation activity by the radial diffusion assay also appeared to be useful, but was less accurate across the range of samples compared. The acid butanol assay for condensed tannins was not an indicator of rumen micro-organism inhibition when used for a range of tree species. It must be noted, however, that the inhibition of rumen micro-organisms is not necessarily the only effect of phenols in vivo and other assays may be useful indicators of other effects.

Acknowledgements

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