



In Vitro Gas Production Test: towards Rapid Nutritional Evaluation of Roughages for Ruminant Feeding

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ABSTRACT: The gas production *in vitro* method was used to evaluate the degradability and gas production of browse plants in the absence or presence of polyethylene glycol 8,000 (PEG). Substrates (leguminous and browse plants; 500mg) were incubated for 24h and the accumulated gas produced recorded. The incubation contents of the syringes were transferred into nylon bags and the undegraded residues weighed after washing and drying to constant weight (SNB method). Substrates were also incubated in the rumen in nylon bags for 24 h to determine *in sacco* degradability. Gas production ranged between 10.3 to 64.4ml whereas the dry matter degradability ranges between 27.3 and 70.9%. Addition of PEG, which minimised the inhibitory effects of tannin on microbial fermentation resulted in an increase in both gas production and degradability *in vitro*, which ranged from 25.7 to 64.2 ml and 34.2 to 75.0%, respectively. Correlation analysis of the DM degradability estimated by the SNB method and *in sacco* method was better in presence of PEG ($y = 0.71x + 14.9$; $r^2 = 0.92$) as compared to absence of PEG ($y = 0.59x + 15.0$; $r^2 = 0.72$). Partitioning factor (PF) of substrate to gas, which was expressed as mg DM degraded/ml gas, reflects the variation in microbial biomass yield. The PF figures varied from 4.94 -11.05 to PF+PEG values of 4.74-6.84 upon the addition of PEG. The present study showed that presence of tannin has a potentially beneficial effect to protein nutrition of the host animal by altering partitioning of nutrients towards more microbial yield rather than short chain fatty acids. PF values of browse plants determined both on the absence and presence of PEG may indicate the relative importance of tannins in different plant species on substrate degradability and partitioning of nutrients.

Keywords: Browse Plants, Tannin, Partition of Nutrient, Gas Production, Polyethylene Glycol

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INTRODUCTION

The potential degradability and degradation rate of feeds can be estimated by the gas production *in vitro* method (Menke, *et al*, 1979). It is based upon the measurement of gas produced, which is a result of short chain fatty acids (SCFA) and gases produced from substrate fermentation by the rumen microbes. This method was shown to be a reliable tool in feed evaluation because the gas production has been well correlated with microbial protein synthesis (Krishnamoorthy, *et al*, 1991), *in vivo* digestibility (Khazaal, *et al*, 1993), and intake (Blümmel and Ørskov, 1993). Microbial biomass yield from substrate fermentation is the most important protein source for the host animal. The relationship between the SCFA produced in the rumen and microbial protein available to ruminant have been demonstrated *in vivo* (Leng, 1993) and *in vitro* (Blümmel, M., Makkar, H.P.S., and Becker, K. 1997) to be negative. This implies that feed samples should not only be evaluated based on gas production data since partition of energy released from degraded substrate contribute both to SCFA production and for microbial protein synthesis (Blümmel, *et al*, 1994a). The concept of partitioning of fermentation products (partitioning factor; PF) was introduced recently to express the conversion of energy from truly degraded substrate (mg DM) required to yield 1ml of gas (Blümmel, 1997b). The former is estimated by weighing the fermentation residue remaining after refluxing the fermented substrate with neutral detergent solution (Blümmel, M. and Becker, K. 1997). PF value is unique to each substrate, and is not necessarily correlated to chemical composition. High PF values were shown to be peculiar of highly digestible roughage and they were well correlate with dry matter intake in ruminants (Blümmel, *et al*, 1997b). Thus, assessment of PF values may be an important used as an important tool for feed evaluation.

Apparently no references have been described the effect of tannins on PF values of browse plants. This is largely due to the difficulty in quantifying the amount of substrate degraded *in vitro*. It is important to determine the PF values for browse plants because most of these plants contain condensed tannins, which may cause harmful effects on rumen microbial fermentation (Jones, G. A., *et al*, 1994). When incubated in the presence of PEG the gas produced from substrate fermentation increased (Khazaal, *et al*, 1994; Makkar, *et al*. 1995a), and this has been attributed to the binding of PEG to tannins, which are released during fermentation (Khazaal, *et al*. 1993). Formation of PEG-tannin complexes has been suggested (Makkar, *et al*, 1995a) to prevent the accumulation of these compounds in the medium to a level which might be inhibitory to the microorganisms. Therefore, the degradability of browse plants in the presence of PEG is also expected to increase. However, the analysis of true *in vitro* degradability (Blümmel, M. and Becker, K. 1997) can be misleading in browse plants due to the presence of tannins (Makkar, *et al*. 1995a). Tannins form complexes with proteins (both from plants and microbial sources), which are insoluble in neutral detergent solution, thus forming precipitates, which in turn will overestimate the undegradable fraction (Makkar, *et al*, 1995a). Attempts to separate tannins from fermented substrate by centrifugation method associated with detergent digestion were also reported. But, they give

incorrect values because the centrifugal forces appeared to enhance further the formation of tannin-protein complexes in the fiber (Makkar, H.M.S. and Blümmel, M. 1997).

The aim of this study was to validate a method to estimate the effect of tannin on degradability and partitioning of nutrients of browse plant incubated *in vitro*.

THEORETICAL REVIEW

In situ degradability

In situ degradability analysis¹⁸ was carried out with the following changes; feed sample (1.5g, 1.0mm) in triplicate was transferred into pre-weighed nylon bag and the bag was tied with elastic band to allow a substrate:nylon bag surface area ratio of 5mg/cm². The bags were incubated in the rumen for 24 h and subsequently washed in cold water for 20min and dried at 65°C for 48h.

In vitro gas production

The gas production analysis was as carried out as described¹. Feed samples (500mg) without or with 500mg PEG 8000 (Sigma Chemical Co., U.K) were incubated in 40ml of incubation medium. Analyses were carried out in triplicate. Syringes were shaken twice up to 2h fermentation and once at every reading, which were taken after 2, 4, 6, 8, 12 and 24h. Net gas production data were corrected for 500mg substrate. A ryegrass grass hay was used as standard substrate to detect between-run effect. Incubation was terminated by immersing syringes in cold water.

Estimation of degraded substrate in vitro

Degraded substrate was estimated as the difference between the weight of substrate incubated and the weight of undegraded substrate. Undegraded substrate after 24h incubation was determined using either NDF-digestion method (Van Soest, and Robinson, 1985; Blümmel, M. and Becker, K. 1997) (NDS method) or nylon bag washing method (incubation in syringes followed by washing with nylon Bag; SNB method). The content of the syringe was completely transferred into a beaker by using of 50 ml NDF solution. The reaction mixture was refluxed for 1h the residue filtered through a pre-weighed sintered glass crucible No. 2 (Gallenkemp, U.K).

SNB method was carried out by completely transferring the entire content of the syringe into a pre-weighed nylon bag (8cmx16cm; pore size 40-60µm). The nylon bags were tied with rubber bands and then thoroughly washed. There was no significant difference (P>0.05) in the effective nylon bag surface area (30%, 50% and 75% of original surface area) on the 24h degradability data from *P. purpureum*. Therefore, 50% effective surface area (5mg incubated sample/cm²) was used for testing other samples.

METHODOLOGY

Substrates

Brownses, leguminous plants and grasses (as described in Tables 1 and 2) were hand-harvested, air-dried in-doors for 5 days and milled in a hammer mill to pass through a 1.0 mm sieve.

Chemical analysis

Dry matter and ash contents were determined according to AOAC. All analytical data were corrected and expressed on DM basis. Neutral (NDF) and acid (ADF) detergent fibre analysis were carried out as described by Goering and Van Soest (Goering, H.K. and Van Soest, P.J. 1970). Nitrogen (N) content in samples was determined by macro-N analysis (Foss Electric (UK) Ltd.) and N figures were multiplied by 6.25 to estimate crude protein (CP).

Extractable phenolic compounds

Analysis of phenolic compounds was carried out in four replicates (Khazaal, *et al*, 1993). Total extractable tannin (TETA; as tannic acid equivalent) was calculated by the difference between total extractable phenolics and phenolic compounds remaining after absorption onto poly-vinylpyrrolidone (PPVP) (Makkar, H.P.S. and Blümmel, M 1997). Total extractable condensed tannins, measured as catechin equivalent, were determined by the vanillin assay (TECTa) and expressed as absorbance read at 550nm (TEPAs) using the proanthocyanidins assay (Porter, *et al*. 1986).

Host animal diets and preparation of incubation medium

Sheep fitted with permanent rumen cannulae were used for *in sacco* degradation studies and as source of inoculum. Animals were fed at maintenance on diets consisted of 500g/kg DM grass hay, 300g/kg DM barley, 100g/kg molasses, 91g/kg DM fishmeal and 9.5g/kg mineral mix and vitamin mix. Feed was given twice a day at 08:30 h and 16:00h. Samples of rumen content were collected from at least two sheep prior to morning feeding and transferred into pre-warmed CO₂-filled thermo bottles. Rumen fluid was strained through three layers of gauze under continuous flushing with CO₂ gas. The temperature of the rumen liquor was maintained between 37-39°C throughout the preparation of the incubation medium. Substantial changes in the medium composition (Blümmel, M. and Becker, K. 1997) were made since the amount of substrate used in this study was increased from 200 mg to 500mg. Buffer volume was increased two fold to increase the buffering capacity of the incubation medium. Equal amount of distilled water was added to match the increase in buffer volume so that the medium had the same osmolality as that for the original method.

Validation of SNB method: effect of catechin+PEG on gas production and degradability of straw

Browse plants contain tannins that may affect microbial fermentation of substrate. The effect of condensed tannin (catechin; added at 5% or 10% of the substrate weight) on fermentation of straw *in vitro* was investigated using the

SNB method to demonstrate changes in wheat straw degradability as a result of addition of condensed tannin in the absence or presence of PEG.

Statistical analysis

The means of all treatments were compared using the least significant difference method²⁰. Standard errors (s.e.) were calculated using the pooled variances of each replication. Differences between treatments were considered statistically significant at the level of $P < 0.05$.

RESULTS

The chemical composition data of browse and leguminous plants are presented in Table 1. There was a wide variation in crude protein (7.6-24.3%), NDF (29.4-49.9) and ADF (21.5-69.8) contents for browses and leguminous plants. The condensed tannins, as estimated by three different methods, i.e. TETa, TECT and TEPA, ranged between 2.71 - 155.5 mg tannic acid equivalent/g DM, 3.2-39.8mg catechin equivalent/g DM and 34.4-438.8 absorbance at 550nm/g DM, respectively.

Estimation of degraded substrate: NDS digestion and nylon bag washing.

Differences in the amount of degraded substrate *in vitro* or *in sacco* are presented in Table 2. Degradability was higher for ryegrass hay, *P.purpureum*, and *Trypsacum sp.* when these were determined by NDS method and higher for *Paspalum sp.* when using the *in sacco* method. SNB method tended to give the lowest figures for degradability. When compared with the *in sacco* method it was found that SNB method gave a better

Table 1: Chemical composition of browse and leguminous plants

Species	%DM	%OM	%NDF	%ADF	%CP	TETa ¹	TECT ²	TEPA ³
<i>Acaliphahispida</i>	89.7	91.2	49.9	24.6	13.3	39.0	12.6	290.2
<i>Acacia mangium</i>	90.2	90.7	29.4	44.4	13.2	9.9	15.7	415.2
<i>Artocarpusheterophylus</i>	89.6	86.8	49.6	38.4	15.9	6.1	3.2	73.2
<i>Bixaarellana</i>	90.2	93.7	43.7	26.1	13.5	104.6	15.9	197.4
<i>Caliandracalothyrcus</i>	89.8	94.8	73.7	69.8	11.0	16.7	18.5	88.5
<i>Chromolaenaodorata</i>	88.7	89.0	36.5	35.6	20.8	25.8	7.5	34.4
<i>Cyperuskyllinga</i>	90.6	88.5	45.5	33.0	10.1	47.8	7.5	72.1
<i>Leucaena leucocephala</i>	92.1	91.0	31.8	21.5	24.3	14.8	39.8	264.8
<i>Manihot esculanta</i>	90.3	94.4	29.5	36.4	11.6	155.5	16.2	438.8
<i>Syzygiumaromaticum</i>	90.5	95.8	48.9	36.1	7.6	89.2	11.7	130.8
<i>Melastomamarabathricum</i>	91.0	91.2	67.2	38.5	12.4	2.71	10.7	158.9

¹Total extractable tannin (tannic acid equivalent; mg/g DM). ²Total extractable condensed tannin (catechin equivalent; mg/g DM). ³Total extractable proanthocyanidins (absorbance at 550 nm/g DM)

Analysis was carried out in duplicate and coefficient of variation was less than 5%.

Correlation ($y=1.06x + 0.29$; $r^2=0.98$) than that of NDF method ($y=0.90x + 2.88$; $r^2=0.68$). The SNB method was used in subsequent studies to estimate the degradability of browse plants.

Table 2: Estimation of substrate degraded after *in vitro* incubation using NDS-digestion or nylon bag washing (SNB method¹) and comparison with *in sacco* method 24 hr degraded substrate (%)

Species	GP24h	<i>in vitro</i> and 'washed' with NDS	<i>in vitro</i> and washed using nylon bag	<i>in sacco</i>	s.e.d.
Graminicae	68.3 ^a	54.2 ^x	51.5 ^x	52.1 ^x	1.1
Ryegrass Hay	44.5 ^b	43.9 ^x	38.3 ^y	36.6 ^y	1.4
Wheat Straw	50.1 ^c	55.5 ^x	41.9 ^y	48.7 ^z	3.2
P.purpureum	51.2 ^c	62.0 ^x	62.7 ^x	67.7 ^x	4.6
Paspalum sp	45.2 ^b	51.7 ^x	40.6 ^y	44.2 ^y	1.8
Trypsacum sp	3.0				
s.e.d.					

¹SNB = syringe-nylon bag method. ^{x,y,z} Means with different letters in the same row for 24 hr degraded substrate are statistically different, ($p<0.05$). ^{a,b,c} Means with different letters for the gas production (GP24h) column are statistically significant ($p<0.05$)

Effect of condensed tannin on wheat straw degradation

The inhibitory effect of added catechin was more pronounced on gas production (-6.9% and -29.8% respectively) than on the effect on substrate degradation (-1.7% and -22.2%), although the difference was significant ($P<0.05$) only at high levels of inclusion of catechin (see Table 3). As expected, addition of PEG suppressed the inhibitory effect of catechin as expressed by gas production and substrate degradability. The removal of the inhibitory effect of catechin by PEG however was not complete, even though PEG itself has no significant effect ($P>0.05$) on straw degradability *in vitro*. Addition of catechin increased marginally the PF values when compared to control (3.77 and 3.37 respectively), but such an effect was not significant ($P>0.05$).

In sacco degradability of browse plants was compared to *in vitro* DM degradability estimated the SNB method either in the absence or presence of PEG. The degradability *in sacco* ranged between 45.8 to 90.5%. The degradability *in vitro* using the SNB method was lower and varied between 27.3 and 70.9%. However, upon addition of PEG degradability was positively affected, i.e. an increase between 1.7% and 34.4%. The raise in degradability of browse plants due to the inclusion of PEG was positively correlated with changes in

Table 3: Effect of catechin ± PEG on straw degradation, gas production and PF in vitro .

Treatment	ml Gas/ 24h	%Substrate Degraded/ 24h	Partitioning Factor (mg DM/ ml Gas)
Straw	54.3 ^a	39.6 ^a	3.37 ^a
Straw+PEG	55.3 ^a	40.5 ^a	3.35 ^a
Straw+25mgCatechin	50.5 ^a	38.9 ^a	3.55 ^a
Straw+25mgCatechin+PEG	54.3 ^a	38.6 ^a	3.29 ^a
Straw+50mgCatechin	38.1 ^b	30.8 ^b	3.77 ^a
Straw+50mgCatechin+PEG	47.9 ^c	34.3 ^b	3.31 ^a
s.e.d.	4.5	3.7	0.24

^{a,b,c} Means with different letters in the same column are statistically different, (p<0.05)

Gas production, which ranged from +1.9% to +149.5%. The largest increase was observed for *C. calothyrcus*(149.5%) followed by *M. esculanta*(130.2%), *A.hispida* (98.5%), *S. aromaticum* (55.3%) and *C.kyllinga* (37.1%). The addition of PEG resulted in the reduction of PF values, which ranged between -0.8% and -92.3%. The greatest reduction in PF values due to PEG, -92.3% and -84.8%, were observed for *M. esculanta* and *C.calothyrcus*, respectively, which also had the highest PF values (11.04 and 11.05 respectively) in the absence of PEG. *L. leucocephala* responded differently to PEG; an increase in gas production (+50.5%) was associated with very small (+1.7%) and not significant (P>0.05) increase in dry matter degradability. Two plant species, *C. odorata* and *M.marabathricum* showed no significant (P>0.05) response in gas and dry matter degradability upon addition of PEG. No relationship was found between the changes in substrate degradability or gas production, upon addition PEG, and TETa, TECTa or TEPA.

Table 4: Gas production, degraded substrate and PF of browse plants ± PEG in vitro

Plant sample & Treatment	% DM degraded in sacco/24h	% DM degraded <i>in vitro</i> / 24h	Accumulated gas (ml)/ 24h	Partitionin g Factor (mgDM/ml Gas)
<i>M. esculanta</i>	68.7	46.7	19.2	11.04
<i>M. esculanta</i> +PEG		54.0	44.2	5.74
<i>C. calothrcus</i>	29.7	27.3	10.3	11.05
<i>C. calothyrcus</i> +PEG		34.2	25.7	5.98
<i>A.hispida</i>	85.7	55.8	27.0	8.94
<i>A.hispida</i> +PEG		75.0	53.6	6.32

Plant sample & Treatment	% DM degraded in sacco/24h	% DM degraded <i>in vitro</i> / 24h	Accumulated gas (ml)/ 24h	Partitioning Factor (mgDM/ml Gas)
S.aromaticum	57.3	47.1	32.0	6.85
S.aromaticum+ PEG		54.7	49.7	4.95
	79.8	71.7	41.8	6.03
L. leucocephala	53.2	72.9	62.9	5.25
L.leucocephala +PEG		50.1	31.6	7.49
	47.9	51.9	35.4	6.84
A. mangium				
A. mangium +PEG	45.8	44.4	28.2	7.09
		47.9	33.7	6.52
B.arellana		48.4	45.1	5.07
B.arellana+PEG	73.0	51.0	48.9	4.77
M.marabathricu m		54.3	64.4	5.05
	49.7	71.5	64.2	4.74
M.marabathricu m+PEG		38.8	35.6	4.94
	90.5	50.9	48.8	4.81
A.heterophylus				
A.heterophylus +PEG		70.9	52.6	6.10
		74.7	53.6	6.05
C. kyllinga				
C. kyllinga+PEG				
C.odorata				
C.odorata+PEG				
s.e.d	2.4	6.1	5.2	1.24

DISCUSSION

Estimation of in vitro degradability measured through nylon bag

Substrate degradability for grass species as estimated by SNB method is generally lower than those estimated by NDF-digestion. This may be explained by the greater amount microbial matter extracted from undegraded residue in the latter method (Blümmel, M. and Becker, K. 1997). Nevertheless, the degradability data estimate by the SNB method are very close to those from *in sacco*.

Addition of PEG and its effect on substrate degradability and gas production.

PEG was chosen as a tannin-binding agent because PEG complexes with tannin more efficiently than PVP (Khazaal, *et al.*, 1993; Makkar, 1995a). Additionally, due to its high solubility virtually all PEG was washed out from the nylon bag and thus it did not interfere with the residue weight. The use of SNB method to estimate substrate degradability after 24h incubation *in vitro* is important because the inhibitory effect of tannins on microbial fermentation and the degradability of substrate may be investigated simultaneously. For example, the SNB method developed allowed us to demonstrate changes in the substrate degradability resulting from addition of catechin and catechin plus PEG.

Inclusion of PEG had no significant effect on wheat straw degradability (Table 3), but its presence increased the degradability of browse plants (Table 4). Regression analysis between browse and leguminous plants DM degradability *in vitro* data with those determined by *in sacco* method was better when PEG was added to the incubation medium ($y=0.71x + 14.9$; $r^2=0.92$) as compared to in the absence of PEG ($y=0.59x + 15.0$; $r^2=0.72$). Better correlation in the former may be explained by the fact that most negative effects, which are intrinsic to browse plants tannins, were removed by PEG. It is interesting to note that the increase in degradability of browse plant, ranging from +1.7 to +34.4%, tended to be smaller than the changes in gas production, which ranged from +1.9 to 140.5%. This highlights the potential effect that addition of PEG to diets containing browse plants will enhance degradability of both soluble and insoluble fraction

PF values of substrates

End products of fermentation include short chain fatty acids (SCFA), fermentative gases and microbial cells. The more the substrate is being degraded by rumen microbes the more energy is made available as SCFA (measured partly as gas production) or to be used for the synthesis of microbial cells. Since the gas produced is inversely related to microbial yield (Blümmel, *et al.* 1997a), the PF value reflects variations in microbial biomass yield. In the present study, the addition of PEG generally resulted a much higher increase in gas production as compared to substrate degradability, and thus a reduction of PF values (PF_{+PEG}). Although it is desirable to have an increase in substrate degradability when the effect of tannin is reduced, a simultaneous much larger increase in gas production would simply result and lower partition of nutrients to microbial yield (Makkar, *et al.* 1998). Data from the present study provides further evidence that the presence of tannins in browse plants protect feed protein from extensive microbial degradation in the rumen, thus increasing protein availability post-*ruminally*. But, more importantly it has been shown that it may affect the partition of nutrients, which tend to enhance protein microbial synthesis as opposed to SCFA production.

The theoretical range for PF values for tannin-free plants was suggested (Blümmel, *et al.* 1997b) to range between 2.75 and 4.41mg truly degraded substrate/ml gas. PF figures greater than 4.41 is not theoretically possible (Makkar, *et al.* 1998) and if this occurred would simply indicate i) significant

loss of detached tannins from fermented substrate, which do not contribute to gas production, ii) non-utilisation of soluble fraction due to tannins, or combination of (i) and (ii). In the present study, calculated PF values for browse plants were much higher and ranged from 5.05 to 11.05. This could be attributed to combination of factors (i), (ii) and a third factor, i.e. feed particles, which escaped from the nylon bag during washing and did not contribute to gas, either due to inhibitory tannin effects on substrate fermentation or particles which are insoluble and undegradable. These factors stress the importance of being cautious when looking at absolute PF values. The values, however, may be used in relative comparisons and be particularly useful in the comparison of PF values within a tannin-containing plant (Table 4). It is interesting to note that the increase in gas production in the presence of PEG was not always contributed by a proportional increase in substrate degradability. This suggests that there may be differences in the way inhibitory tannins from different browse plants affect the activity of rumen microbes. Thus, the measurement of both gas production and substrate degraded in the presence and absence of PEG, and the changes in calculated PF would provide useful insight into the degree of inhibitory effects of tannins on substrate fermentation by rumen microbes. Further studies using greater variety and number as well as stage of growth of browse plants are necessary to establish a more robust relationship between browse plants intrinsic properties (chemical composition, voluntary dry matter intake, maximum inclusion in the diet), and the PF and PF+PEG values.

CONCLUSIONS AND RECOMMENDATIONS

In addition to the measurement of gas produced using the gas production *in vitro* the new validated method also enables the estimation of *in vitro* degradability. The method also provided the possibility of measuring PF values of browse plants in the absence or presence of PEG. Browse plants degradability *in sacco* after 24h in the rumen may also be mimicked *in vitro*, provided that polyethylene glycol (PEG) is also added in the incubation medium.

FURTHER STUDY

It is necessary to further test the effect of polyethylene glycol (PEG) in an *in vitro* method to produce gas and PF values in plants that have high tannin content in more than 24 hours

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