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FACTORS AFFECTING IN VITRO GAS PRODUCTION FROM FERMENTATION OF FORAGES AS DETERMINED BY PRESSURE TRANSDUCER TECHNIQUE

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ABSTRACT

A pressure transducer assembly connected to a digital read-out meter was used to determine the fermentation kinetics of temperate and tropical forages during in vitro digestion in batch cultures inoculated with rumen micro-organisms. The forages included straws and hays harvested as part of a forage evaluation program at different stages of growth from research centers in Ethiopia. Factors influencing the fermentation kinetics of these forages were investigated using parallel curve analysis. The factors included: (1) atmospheric pressure, (2) anaerobic status and composition of the culture medium, (3) the amount of microbial inoculum used, (4) the interval between successive pressure transducer readings, (5) the extent to which culture medium buffer contributed to the accumulating gas pool and (6) variability of the rumen microbial inoculum. Reproducibility and repeatability of gas accumulation measurements and the optimal construction of gas accumulation profiles were also investigated. All factors had significant ($p < 0.05$) influence on gas accumulation profiles emphasizing the need for standardized experimental conditions and procedures in evaluation of in vitro fermentation kinetics of ruminant feedstuffs. As a result of these experiments suggestions were made on routine procedure for in vitro determination of forage fermentation kinetics using the pressure transducer technique.

Introduction

Information on the kinetics of forage digestion is important as rate and extent of digestion of feeds in the rumen to a large extent, determine voluntary intake (Hovell et al. 1986, Ørskov et al. 1988). Several indirect methods have been used to estimate the extent of forage digestibility. The two-stage in vitro technique of Tilley and Terry (1963) has been widely used in predicting forage digestibility for ruminants and for screening large numbers of forages in plant breeding programs. However, the method provides no information on digestion kinetics and measures only an end-point of digestion after 48 hr. Grant and Mertens (1992) showed the method could be modified by using only the first stage of incubation to measure in vitro dry matter (DM) degradation pattern with time. However, this involved destructive sampling of the contents of digestion tubes thereby limiting the number of samples that could be tested at a given time.

The Dacron bag method, in which nylon bags are suspended in the rumen of fistulated animal and removed sequentially for DM determination, can serve as a tool in supplying information on rate and extent of DM disappearance of feeds (Ørskov and McDonald 1979). However, due to the cost and the difficulty of maintaining large numbers of fistulated animals the method is not convenient for concurrent evaluation of large numbers of samples. Given that in situ DM disappearance measurements reflect the rumen environment involving diet and animal differences, they are therefore inherently more variable than the corresponding in vitro measurements of digestion (Noeck 1985). The accuracy of the Dacron bag method is also influenced by certain technical aspects such as amount of sample in relation to bag size, bag pore size, sample particle size, the washing procedure for bags after removal from the rumen and the basal diet of the fistulate animals (Noeck 1985, Uden et al. 1974 and Van der Koelen et al., 1992). Thus, for these reasons it is often difficult to make comparisons of results from different research works or research centers.

Recently, it has been shown that measurement of rate of gas production during in vitro fermentation of forages with microbial rumen inoculum can be used to assess fermentation kinetics (Theodorou et al. 1991, Beuvink and Kogut 1993, Blümmel and Ørskov 1993, and Khazaal et al. 1993). Theodorou et al. (1991, 1994) developed the pressure transducer technique (PTT) for measuring in vitro gas production of forages. The method has been used to estimate rate and extent of gas accumulation of forages. The procedure is inexpensive and can handle large numbers of samples. In order to determine the precision of the results obtained, factors that may affect the kinetics of gas production need to be investigated. The present series of experiments were therefore undertaken on the factors that may affect gas accumulation of test forages as determined by the pressure transducer technique. The work was undertaken at Holetta Research Center, Ethiopian of Agricultural Research Organization, Ethiopia, Reading University, Reading, UK, and Institute of Grassland and Environmental Research, Aberystwyth, UK.

Materials and Methods

The list of experiments undertaken at different locations is as shown in Table 1

Forage Samples

Forages were grown in the highlands of Ethiopia as part of the national forage evaluation program of the Institute of Agricultural Research and straws were obtained from Holetta Research Center. They comprised of wheat straw, barley straw and Rhodes grass (*Chloris gayana* var "Massaba"), rye grass hay (*Lolium perenne*), pasture hay from permanent pasture (mixed species), Phalaris hay (*Phalaris aquatica* var. Sirroco), oat hay (*Avena sativa*), Panicum hay (*Panicum coloratum*), Tef straw, Pigeon grass (*Heteropogan whitei*), Molasses

grass (*Melinis minutiflora*), and Stylo hay (*Stylosanthes guianesis*). Except for Pigeon and Molasses grasses which were harvested at the post flowering stages, all forages were harvested at flowering stage. All forages were air-dried and milled through a 1.0-mm dry mesh screen using a Christy-Norris laboratory mill.

Microbial Inoculum

Samples of rumen digesta were taken as grab samples from three rumen fistulated sheep maintained on rye grass hay fed ad libitum with 200 g/day of concentrate (3.2% N), or four rumen fistulated steers fed grass ad libitum collected from permanent pastures and 3 kg/day of noug (*Guizota abyssinica*) seed cake. The digesta were transported to the laboratory in a warm (ca. 39 °C) vacuum flask . The digesta was squeezed through two layers of muslin and the strained rumen fluid collected in a flask while gassing with CO_2. The residual digesta solids were comminuted for 1 minute in a Kenwood electric blender after addition of anaerobic buffer equal in volume to the rumen fluid collected in flask. Fluid from the comminuted digesta was strained through muslin as above and combined in equal volumes with the rumen fluid. The microbial suspension was stirred and flushed with CO_2 during inoculation.

Table 1. List of experiments undertaken on factors affecting gas accumulation of forages as determined by the pressure transducer technique

Experiment no.	*Experiment title*	*Source of inoculum used in the experiment*	*Experiment location*
1	Effect of atmospheric pressure on gas production	-	IAR (Holetta Research Center) Ethiopia, and IGER , Aberystwyth , UK
2	Effect of type of medium on gas production profile of test forages	Three rumen fistulated sheep	Reading, UK
3	Effect of presence or absence of typrticase peptone and/or cystein in culture medium on gas production of test forages	Four rumen fistulated steers	IAR, Ethiopia
4	Effect of amount of rumen inoculum in culture medium on gas production of test forages	Four rumen fistulated steers	IAR, Ethiopia
5	Effect of gas reading interval on gas production profile of test forages	Four rumen fistulated steers	IAR, Ethiopia
6	Contribution of culture medium	Three rumen fistulated sheep	IGER, UK
7	Reproducibility and repeatability of gas production	Four rumen fistulated steers	IAR, Ethiopia

Culture Medium

The medium was composed of a basal solution, prepared by mixing together, (in numerical order), the following components: Trypticase peptone (Becton Dickinson Microbiology Systems, Cockeysville, MD 21030, USA), micromineral solution, buffer solution, macromineral solution, resazurin solution and distilled water. Each solution was prepared using glass distilled water and kept in the dark at 4 °C until required. The basal solution was pre-reduced by bubbling a stream of oxygen-free CO_2 through the medium for *ca.* 3 hr.

To complete the medium, a reducing agent , the forage sample to be fermented (1 g ± 0.5%) and 90 ml of basal solution were dispensed into serum bottles (Phase Separations Ltd., Clwyd, UK; nominally of *ca.* 160 ml capacity, but retailed as 125 ml bottles) (4–5 replicates per sample) using anaerobic procedures and sealed with butyl rubber stoppers and aluminium crimp seals (Bellco Glass Inc., Vineland, NJ, USA). The reducing agent was freshly prepared prior to use and had the following components: Cysteine Hcl, distilled water, 1 M NaOH and Sodium sulphide.

Sealed bottles were chilled to 4 °C (for not longer than 24 hr prior to inoculation), warmed to 39 °C and inoculated with 5 ml of microbial inoculum using a 10 ml syringe fitted with a 23 gauge x 1.5 inch needle (Sabre International Products Ltd., UK). Bottles were incubated at 39 °C until the end of the fermentation period.

In some experiments, the medium and culture procedures described above were slightly altered. Modifications included: (a) excluding Trypticase peptone and/or cystine HCl from the culture medium (Experiment 3), (b) varying the amount of microbial inoculum from 5 to 10 or 15 ml per 100 ml of culture medium (Experiment 4), (c) incubating test forages in medium above and in the medium of Tilley and Terry procedure (1963); the constituent and preparation of the medium used in Tilley and Terry procedure was as modified by Minson and McLeod (1972) (Experiment 2).

Acidification of PTT Medium (Experiment 6)

Culture medium with or without microbial 5 ml of incoculum (each replicated in 25 culture bottles), was incubated without forages and the volume of gas released measured 30 minutes after stepwise addition of 1–2 ml of a 2 M solution of acetic acid. Acetic acid was added stepwise to each bottle through the butyl rubber stopper, using a syringe and needle and after each measurement, two serum bottles each from medium with or without rumen inoculum were removed and used for pH readings.

Gas Accumulation Measurements

Measurement of gas accumulation from the fermentation serum bottles were as described

by Theodorou et al. (1994). In brief, a pressure transducer with a LED (light emitting diode) digital readout meter was used to measure the accumulation of fermentation gases in the head space of the culture bottles. Gas pressure was read from the display unit after insertion of the needle through the butyl rubber stopper in the head-space above the medium. The gas volume above the medium was transferred into the syringe barrel by withdrawal of the syringe plunger until the pressure in the transducer display unit became zero. The head-space pressure and volume of gas were recorded. The syringe was withdrawn from the bottle and the gas was discarded. Only a few bottles were taken (10–12 bottles) at a time from the incubator for readings in order to minimise the time that bottles were outside the incubator. The reading sequence followed the sequence of inoculation of the bottles. Gas readings were recorded at 3 to 4 hr intervals during the 12 hr incubation time and less frequently afterwards. However, in Experiment 5, five reading intervals (every 1, 3, 6, 8 or 12 hr) were set up during the 48 hr incubation time.

In the procedure outlined by Theodorou et al (1994), linear regression analyses of head-space gas pressure versus recorded volume was determined prior to summation and then cumulative gas production was calculated by the summation of the predicted (regression-corrected) gas volumes from the replicate serum bottles. This was done in order to correct possible slight differences between bottles caused by the pipetting procedure and the bottle size. However, constructions of cumulative gas production profile by summing up the measured volume was investigated in this study.

Statistical Analyses

The model proposed by France et. al. (1993) was fitted to the exponential profile to estimate values after subtraction of the mean control profiles . In the model, rate of gas production is expressed by two fractional rates. The equation is in the form:

$$Y = A\{1-ev^{[-bf(t-T)-cf(\sqrt{t}-\sqrt{T})]}\}$$

The model was transformed and fitted with the functional form:

$G = A-BQ^{t}Z^{\sqrt{t}}$; where,

G is the cumulative gas volume (ml),

A is the asymptotic gas pool value (ml),

$B = A\, e^{(bfT+cf\sqrt{T})}$,

T is the lag time (hr),

$Q = e^{-bf}$,

$Z = e^{-cf}$ with b_f (h^{-1}) and c_f ($h^{-0.5}$) being the two rate constants.

A combined rate of gas production (m) (rates vary with time) was calculated as:

$m\ (/hr) = b_f+(c_f/2\sqrt{t})$ where,

b_f and c_f are rate constants as defined above and t is incubation time.

Differences in estimated parameter due to treatments were analysed using parallel curve analysis (MLP; Ross 1987).

Variance components of repeatability errors (between replicates of a feed in a single run) and reproducibility errors (between runs), repeatability and reproducibility of gas production from the mean of 10 forages over 5 runs were calculated based on the statistical procedure of ISO, 1981 using the REML of Genstat (1987). The number of culture bottles used to incubate each test forage was regarded as replicates within a run.

Gas production and pH data from the acidification of the culture medium after stepwise addition of acetic acid were analysed using one node linear spline curve analysis (MLP; Ross 1987).

Results and Discussions

Construction of Cumulative Gas Production Profile of Test Forages

Gas accumulation data from 25 culture bottles were selected at random and linear regression analyses performed for each culture bottle between head-space gas pressure and volume. The relationship obtained between head-space gas pressure and gas volume was highly significant ($p < 0.01$) and the error from the predictive equation (RSD) was small (< 1.0 ml) indicating that the corrected gas volume data were not significantly ($p > 0.05$) different from that of the original data of measured gas volume. Provided the dispensing solutions and recording of volume for each serum bottle is done as accurately as possible, the use of measured volume of gas and not regression corrected volume would not affect the final results of cumulative gas production. Thus, the measured volume of gas was used to calculate cumulative gas production and the data were corrected for the controls.

Experiment 1: Effect of Atmospheric Pressure on Gas Measurements

This study was conducted to assess the relationships between gas-pressure and volume at the two locations viz UK (IGER, Aberystwyth, 100 m asl, atm. 752 mm Hg) and in Ethiopia (Holetta Research Center, 2400 m asl, atm 587 mm Hg). The relationship between pressure (x, psi) and volume of gas (Y, ml) at each location was:

- **V (ml) = 10.48 x (psi) (R^2 = 0.99, RSD = 0.293)** in UK and
- **V (ml) = 13.32 x (psi) (R^2 = 0.98, RSD = 1.145)** in Ethiopia.

The result shows that for the same gas pressure reading, the volume of gas measured at Holetta (Ethiopia) was 21% higher than the volume of gas measured at Aberystwyth (UK). The observations made above were also shown for gas production measurements from forage samples fermented in 100 ml medium. At Aberystwyth, for each psi (head-space gas pressure) reading the corresponding gas volume was 4.5 ml. However, in Ethiopia the volume of gas for each psi was about 5.9 ml.

It is necessary to record the atmospheric pressure of the site when measuring gas production of forages. Gas production results measured at different locations can be standardised to 1 atmospheric pressure (760 mm Hg) (V') by calculating the quotient using Boyles' Law:

$V' = P/P'^* V$; where,
P is the atmospheric pressure at the site of measurement,
V is the volume of gas measured and
P' is the standard atmospheric pressure.

The same quotient can also be used to standardise the estimated parameters A and B fractions representing the gas pool obtained using the France et al (1993) equation.

Experiment 2: Effect of type of medium on gas production profile of test forages

Five forages (wheat straw, barley straw, Rhodes grass and two samples of rye grass hay) were each incubated either in medium used in the pressure transducer technique (PTT) or medium used in the Tilley and Terry (TT) procedure and gas production from each of the fermented forages was measured during a 122 hr incubation period. Each forage had significantly ($p < 0.05$) higher rates of gas production when fermented in the PTT than when incubated in the TT medium. Wheat straw, Rhodes grass and rye grass II had smaller gas pool (A, B) when fermented in the PTT medium than in TT medium.

The higher rates of gas production of forages fermented in PTT medium as compared with TT medium could result from provision of nutrients for micro-organism or maintaining anaerobic conditions in the medium or a combination of the two. Grant and Mertens (1992) compared the effect of two media on the kinetics of forage fiber digestion. They reported that maintaining anaerobic conditions had a major effect on the rate of digestion and that this was more important than differences in media compositions. Poor anaerobisis (Grant and Mertens 1992) resulted in reduced rate of fiber digestion and increased lag time which is consistent with these results obtained here. According to Leedle and Hespell (1983), the effect of aerobic conditions in TT medium would lead to a substantial loss of cellulolytic as compared with amylolytic bacteria. Thus, the lower rate of gas production in TT could be caused by a decreased number of cellulolytic micro-organisms since these bacteria are responsible for digesting fiber of forages.

Experiment 3: Effect of Presence (or Absence) of Trypticase Peptone and/or Cystein in Culture

Four culture media were prepared, with the presence and/or absence of Trypticase peptone

and/or Cysteine HCl. Four test forages (barley straw, pasture hay from permanent pasture (mixed species), Phalaris hay (*P. aquatica* var Sirroco) and oat (*A. sativa*) hay) were incubated in each medium and gas production was measured over a 96-hr incubation period.

The presence or absence of Trypticase peptone and Cysteine HCl did not affect the rates f gas production (b, c) of each forage significantly ($p > 0.05$). However, the presence of Trypticase peptone and Cysteine in medium increased estimated parameters of gas pool ($P < .05$). On average, the inclusion of Tyrpticase peptone increased the gas pool (A fraction) by 20.0 (± 0.2) ml while inclusion of Cysteine HCl resulted in an increase of 11.5 (± 0.5) ml.

It was reported that inclusion of Trypticase peptone in medium would stimulate the growth of rumen micro-organisms and increased forage digestion (Grant and Mertens 1992). The present study demonstrated that gas pool was more affected by the presence or absence of Trypticase peptone than was the rate of gas production. It may be argued that inorganic nitrogen (ammonium) and nitrogen source in the rumen inocula might have provided enough N for the rumen micro-organisms. The increased gas pool with forages could be from the deamination of peptone.

Inclusion of sodium sulphide and flushing the medium with CO_2 for 3 hr seemed to be enough to reduce the PTT medium to the same extent as with the addition of Cysteine HCl. As a routine procedure, it is desirable to remove sodium sulphide from the medium as sodium sulphide is potentially toxic. Inclusion of sodium sulphide and flushing the medium with CO_2 for 3 hr seemed to be enough to reduce the PTT medium to the same extent as with the addition of Cysteine HCl.

Experiment 4: Effect of Amount of Rumen Incoulum in Culture Medium on Gas Production Profile of Test Forages

Three levels of inoculum (5, 10 or 15 ml per 100 ml of the medium) were used to study the effect of estimated parameters of gas production of grass hay (mixed species) from grazing pasture, Rhodes grass (*gayana* var Massaba), oat (*sativa*) hay and Panicum (*coloratum*) hay. Increasing the amount of inoculum increased gas production from each of the test forages, although the differences in gas production between bottles inoculated with 10 or 15 ml of inoculum were small. Increasing the volume of rumen inoculum in the medium did not increase the rates of gas production (b, c) of forages significantly ($p > 0.05$).

The volume of rumen inoculum in medium had a significant ($p < 0.01$) effect on the gas pool size (A and B parameters) of each of the test forages. Forage fermented in 5 ml inocula had a lower gas pool ($p < 0.01$) than when fermented either in 10 or 15 ml of inoculum. The gas pool from fermentations conducted using 10 or 15 ml of rumen inoculum were not significantly different ($p > 0.05$).

The absence of significant differences in rate of gas production due to increasing the level of rumen inoculum from 5 to 10 or 15 % of the medium does not agree with the

results of Pell and Schofield (1993) where it was shown that an inoculum of less than 20% of the medium decreased the rate of gas production of forages. However, although Pell and Schofield (1993) showed increased rate of gas production with increased amount of inocula in medium, their data were not subjected to statistical analysis. The lack of a significant effect on rate of fermentation in the current study could be as a consequence of the method of preparation of the microbial suspension used in the PTT method. The homogenisation step, for example would have increased the microbial population required to saturate the system. Forsberg and Lam (1977) reported that ca. 75% of the bacteria population are associated with solid particles of the feed. Other researchers (Fay et al, 1980 and Senshu et al, 1980) have also recommended the procedure adopted in this study as a method of choice to obtain representative microbial species at a satisfactorily high concentration in vitro cultures.

Experiment 5: Effect of Gas Reading Intervals on Gas Production Profile of Test Forages

The effect of five reading intervals (1, 3, 6, 8, or 12 hr) during a 48 hr fermentation period on the pattern of gas production of tef (*Eragrostic abyssinica*) straw and three hays viz Rhodes grass (*C. gayana*), oat hay (*A. sativa*), and Phalaris hay (*P. aquatica*) was investigated. Gas production from each forage was higher when read at 1 hr interval as compared with either 3, 6, 8 or 12 hr reading intervals. After 48 hr of incubation, when considering the mean of the four forages, the volume of gas measured using a 1 hr reading interval was only 2% higher than that measured using a 3 hr reading interval. However, for the other reading intervals the volume of gas measured decreased in greater proportions as compared with the 1 h reading interval. Percentage decreases in cumulative gas production as compared with the 1 hr reading interval were 15, 19 and 30% for reading intervals of 6, 8, and 12 hr, respectively.

The data have limitations in fitting to the model of France et al. (1993) as the incubation was only done for 48 hr. The gas production asymptotes of each forage were not reached within this time. The model of France et al. (1993) has a weakness of predictive capacity when the substrate does not reach the asymptote. Therefore, some of the estimated parameter values may not be realistic. Analysis continued, however, to show the effect of reading intervals on the estimated parameters of gas production. Reading intervals influenced ($p < 0.05$) the estimated parameters of gas production of each forage sample. Increasing the reading interval tended to increase the lag time. The estimated parameters of gas production of forages read at 1 hr were different from the parameters derived from reading at 3 hr interval. There was no difference ($p > 0.05$) between the estimated parameters of gas production of forage sample read at either 6 or 8 hr interval. As it can be seen from the mean gas production at each hour of the four forages, there were substantial differences in the volume of gas measured using the four reading intervals within the first 21 hr of incubation. The magnitude of the differences between the intervals depended on the incubation time.

Experiment 6: Contribution of Culture Medium to Gas Production

This experiment intended was to quantify the CO_2 released from buffered cultured media with or without microbial inoculum. With progressive addition of acetic acid, gas production from the media increased linearly although the rate of increase tended to decline during the later steps of addition, but failed to reach a plateaux within the present level of acetic acid addition (16 mmol). Thus the data was analysed using single node linear spline curve analysis.

For both media (with or without microbial source), the break point at which the rate of gas production tended to decrease with increased addition of acetic acid was after 10.2 mmol. The estimated parameters were the same in the two media. After the break point in each case, values for the slope of the line were 10.8 and 11.7 ml, respectively, for medium without and with rumen inoculum.

At 39 °C, the molar gas volume is 25.62 ml, and this was used to calculate the gas production in mmol from media with and without microbial inoculum for each mmol of acetic acid added. Thus, from 0 to 11 mmol acetic acid/100 ml, the addition of 1 mmol of acetic acid released 0.66 and 0.63 mmol of gas from the medium and medium with microbial inoculum, respectively.

In the case of pH data, the break point for the two lines for medium with rumen inoculum was after the addition of 5.9 mmol of acetic acid at a corresponding pH of 6.3. The slopes of the two lines before and after break point were -0.045 and -0.134, respectively. Although the inflection points for the two media were not different, the slope of the lines for medium alone were higher than corresponding slopes for the medium with microbial inoculum medium. The inflection point for medium alone was at 6.0 mmol of acetic acid addition with slopes of -0.085 and -0.154 before and after the break point, respectively. The volume of CO_2 released from PTT medium with rumen inoculum (16.8 ml/mmol of VFA) was similar to that reported by Beuvink and Spoelstra (1992) (20.8 ml gas per mmol VFA production or 0.87 mmol gas). The volume of medium used by Beuvink and Spoelstra (1992) was 60 ml and gas measurements were recorded at 20 °C. In the current experiment, however, gas measurements were made at 39 °C and from 94 ml medium. When these two differences are taken into consideration results from the two experiments were similar.

In the rumen, VFAs are absorbed through the rumen wall into the blood, or removed by passage from the rumen with rumen fluid to the omasum. Absorption by diffusion is influenced by both pH and VFA concentration, with low rates of absorption at low pH and high VFA concentration (Tamminga and van Vuuren 1988). In the in vitro method, since there is no mechanism for the absorption of the VFAs produced during fermentation, their accumulation would ultimately exhaust the buffering capacity of the medium. The results of this study demonstrated that in the PTT medium, after the production of 6 mmol (pH 6.34) of VFA, the pH of the medium declines at a faster rate. Thus, in the PTT medium VFA production should not exceed more than 6 mmol per 100 ml PTT medium with microbial inoculum (5%).

Experiment 7: Reproducibility and Repeatability of Gas Production

This experiment used the statistical procedure of ISO (1981) to determine reproducibility and repeatability of gas volume from the fermentation of 10 feeds (Tef straw, Pigeon grass, molasses grass, native hay, *P. aquatica* "Sirroco" hay, oat hay, wheat straw, Rhodes grass, Panicum hay and Stylo hay) measured in five consecutive runs. Estimated parameters of gas production of each forage at five runs were determined by fitting the data to the equation of France et al (1993). For each forage, parallel curve analysis was used to test differences in the estimated parameters of gas production obtained from the five runs.

Both repeatability (W) and reproducibility (B) errors depended on the volume of gas production from the forage samples which in turn were affected by incubation period. The linear functional relationship between repeatability (W) and volume of gas production (V, ml) from a forage sample was:

$$W = 6.4\ (\pm 0.47) + 0.070\ (\pm 0.0031)\ V\ (R^2 = 0.98;\ RSD = 0.78).$$

The linear relationship between reproducibility (B) and volume of gas production (V, ml) from a forage substrate was:

$$B = 5.8\ (\pm 0.46) + 0.099\ (\pm 0.0030) V\ (R^2 = 0.99;\ RSD = 0.76).$$

The reproducibility error was much higher than the repeatability error and both varied more in the first 20 hr incubation time. After 30 hr incubation time, the repeatability and reproducibility errors constituted 10 and 12% of gas productions of forages.

Variations occurred in the rates of gas production (b, c) and gas pool (A, B) for each forage estimated from each of the five runs. Differences in rates of gas production (b, c) between the five runs were significant ($p < 0.05$) for Rhodes grass and hay from pasture but not significant ($p > 0.05$) for the remaining eight forages. The rates of gas productions of these two forages measured in the fifth run were different ($p < 0.05$) from the rates of gas production determined in the previous four runs.

Gas pool for each forage showed significant differences ($p < 0.05$) between the five runs. For some of the forages, the highest gas pool was determined in the second run while for others the highest gas pool was in the fifth run. However, in the majority of cases forages showed their highest gas pool estimates in the second and their lowest in fifth run.

Pell and Schofield (1993) recommended that reproducibility of gas production of forages among runs can be improved by following strict schedules in collecting rumen inoculum for each run. However, Beuvink et. al, (1992) used the same procedure as Pell and Schofield (1993).

Beuvink et.al (1992) reported that the within-days mean square variances of gas production for glucose, rice starch and cellulose were 181, 458 and 139, respectively. The mean square variances between days for the same samples, respectively, were 1374, 1546 and 5072. The variances within and between runs of gas production after 34 hr incubation

reported in the current experiment were lower as compared with these values. coefficient of variation between runs was 4%.

General Discussion and Conclusion

The results showed that results from gas production experiments could be affected by several factors, notably constituents and the method of preparation of the culture used to incubate test forages, size and variability of microbial inoculum and the reading intervals used in recording gas pressure and volume from the fermentation bottles. In addition to these factors, it was shown that gas production volumes were influenced by atmospheric pressure Variation in PTT results caused by different experimental procedures of PTT could be reduced by using standard procedures. Sample size, constituents and method of preparation of PTT medium were as described by Theodorou et al (1991). However, based on the results of this study, certain procedures need to be modified. Recommendations on experimental procedure for PTT are outlined in Table 2.

Table 2. Recommended experimental procedures for Pressure transducer, technique (PTT)

Variable	*Recommendation*
Construction of cumulative gas production	Determine cumulative gas production by summation of gas volumes experimentally read (not regression corrected) from the set of replicate cultures. Gas production profile is determined after subtraction of gas amounts which accumulated in control cultures (inoculated bottles incubated in the absence of forage substrate)
Amount of rumen inoculum	10 ml/ 100 ml medium
Preparation of microbial inoculum	Collect rumen digesta, squeeze through two layers of muslin. Comminute the residual digesta solids for 1 minute in a - Kenwood electric blender after addition of anaerobic buffer equal in volume to the rumen fluid.
Medium	As described in Theodorou *et al* (1991, 1994) except that there is no need to add trypticase peptone and Cysteine HCl
Preparation of medium	Maintain anaerobic condition during preparation of the medium as well as during inoculation
Reading interval	Every 3 hr for the 15 or 18 hr incubation period
Incubation period	At least 72 hr
Correction factors	Use of standard samples in each run and correct results for blanks. In comparing with other experimental results, the results have to be corrected for site elevation (atmospheric pressure).

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