



## Calibration of In Vitro Gas Production Technique Using Digital Pressure Gauge: Air Versus Fermentative Gases

Seyed Hadi Ebrahimi<sup>1</sup> Pankaj Jha & Madhu Mohini

Division of Dairy Cattle Nutrition,  
National Dairy Research Institute (NDRI), Karnal, Haryana -132001, India.

### ABSTRACT

This experiment was aimed to compare air with fermentative gas for calibrating an in vitro gas production system using digital pressure gauge. Serum bottles (100 ml) containing 45 ml of in vitro medium were injected with air and fermentative gas produced from incubation of starch (S gas), starch and cellulose (S + C gas) and cellulose (C gas). The pressures in the bottles were read after equilibrating the vials overnight at 39 °C for each gas, calibration line was constructed by linear regression of gauge reading (in psi) versus volume fraction (Fv) of added gas, where Fv = milliliters added gas/milliliters bottle headspace volume. For all four gases (air, S gas, S + C gas and C gas), there was a linear relationship between volume and pressure ( $r^2 = 0.99$ ). There was no significant difference between slopes of calibration curve obtained from air, S gas, S + C gas and C gas. Results of present work indicated that calibration of in vitro gas production system using air can give reliable regression equation applicable for measurement of total gas volume of wide ranges of substrates.

**Key words:** Calibration, In vitro gas production technique, Digital pressure gauge

### INTRODUCTION

Studies with live animals (in vivo) for nutritional evaluation of feeds are time-consuming, laborious, and expensive and require large quantities of feed. The in vitro gas production technique has gained general recognition over the last decades, being used increasingly for feed evaluation, to investigate mechanisms of microbial fermentation and for studying the mode of action of anti-nutritive factors, additives and feed supplements. The technique is based on the assumption that the gas produced in batch cultures is just the consequence of the fermentation of a given amount of substrate (France et al. 2000).

There are basically two approaches for measuring gas volumes. The first approach implies measurement of gas collected at atmospheric pressure and its volume determined directly (Menke et al. 1979; Menke and Steingass 1988). Calibrated glass syringes of 100 ml capacity were used for incubating 200 mg substrate with 30 ml buffered rumen liquor (BRL) and gas volume were determined by difference between the positions of piston initially and after 24 h incubation. However, calibrated glass syringes are expensive and assuming 30 ml of BRL needed for incubation, there is always limitation for incubation of 200 mg substrate because gas produced from incubation of concentrate and compound feed might be higher than the capacity of syringes. Therefore, to

estimate in vitro nutrient digestibility precisely, incubation of higher amount of substrate is favored.

Alternatively, gas-tight culture bottles are used for the incubations, measuring changes in gas pressure in the headspace using pressure transducer (Theodorou et al. 1994; Lopez et al. 2007). In this system, it is important to define the relationship between pressure and gas volume. According to ideal gas law, there is linear relationship between pressure measurements and estimated gas volume hence, higher pressure reading by pressure transducer represents greater gas volume at constant temperatures. Typically, ruminal fermentation produces a mixture of gases mainly CO<sub>2</sub>, CH<sub>4</sub> plus small amount of H<sub>2</sub>, H<sub>2</sub>S and N<sub>2</sub> (Moate et al. 1997). The proportion of CO<sub>2</sub> and CH<sub>4</sub> differs depending upon the type of substrate and presence or absence of methanogens inhibitors (Trei et al. 1972; Getachew et al. 1998).

Conversion of pressure readings after incubation of substrate in culture bottles to the gas volume is based on calibration of system with defined conditions such as volume of bottles and volume of BRL however, gases have different solubility and therefore changes in gas composition may affect pressure obtained from similar volumes of different gases. In the present experiment air and gases obtained from the incubation of different substrates were used to determine whether or not type of gases used for calibration of system can influence pressure measurements and subsequently regression equations for estimation of gas volume.

## MATERIALS AND METHODS

### Gas pressure sensor and calibration

Gas pressure measurements were made with a digital pressure gauge (Durck Incorporated, DPI 700,

20 Psi, UK). A 'B Braun plastic stopcocks-3 way' modified to give two male spin-lock connectors and one female luer lock port. The female side was permanently closed and a disposable needle was connected and screwed to one male spin-lock connector. The second male portion connected to the male terminate of pressure gauge through a 5 cm rubber tube (ID = 1 mm). The rubber tube was tied in both sides using Self-locking nylon cable tie to avoid gas leakage (Figure 1).

Several serum bottles (500 ml capacity) were used for incubation of starch, starch and cellulose (50:50) and cellulose to provide enough gas for calibrating in vitro system. Rumen contents were collected from two male cattle, fitted with permanent rumen fistula, maintained on a roughage based diet (2.0 kg concentrate mixture in equal amount at 10.00 am and 4.00 pm and wheat straw ad lib.) before morning feeding. Rumen contents were mixed and strained through two layers of cheesecloth into pre-warmed thermo-flask with an O<sub>2</sub>-free headspace. Feed particles were allowed to settle to the bottom (5 min) and finally the fluid was strained through two layers of nylon cloth (50 μm pore size). Particle-free fluid was mixed with the buffer solution of Menke and Steingass (1988) in a proportion of 1: 2 (v/v) at 39°C under continuous flushing with CO<sub>2</sub>. Samples (2g) of each substrate were accurately weighed into bottles. Bottles were warmed (39°C) prior to the dispensing of 300 ml buffered rumen contents into each one and incubated in a water bath at 39 ± 0.5°C for 24 h. Suitable aliquot of gas was withdrawn from the tip of the incubation bottles using gas tight syringe and composition of gas in the headspace of bottles determined using gas chromatograph (Nucon 5700, Nucon Engineers, New

**Table 1. Volume percentage of gas components in fermentative gases produced after 24 h incubation of starch (S gas), starch and cellulose (S + C gas) and cellulose (C gas)**

Attribute	S gas	S + C gas	C gas
H <sub>2</sub> (%)	0.13	0.23	0.01
CH <sub>4</sub> (%)	37.24	33.32	30.60
CO <sub>2</sub> (%)	62.64	66.46	69.40

Delhi) equipped with thermal conductivity detector (TCD) and stainless steel column packed with Porapak Q (length 1.5m; o.d. 3.2 mm; i.d. 2 mm; mesh range 80-100). Oven, detector and injector port temperature were same at 45 °C. Argon was used as carrier gas with flow rate of 30 ml/min. Samples of air and gas obtained from fermentation of starch (S gas), starch and cellulose (S + C gas) and cellulose (C gas) further used for calibration as described below:

The sensor was calibrated using serum bottles (100 ml) of known volume, measured to 0.01 ml by filling the tared vials to the brim with water and determining the net weight of water (assuming a density of 1.00 ml per g). Headspace volumes were adjusted for the volume displaced by the flanged butyl rubber stopper (1.87 ml) and the volume of included substrate, buffer and inoculums. The calculated volume of the headspace was 83.76 ml (weight of filled bottles up to brim - weight of filled bottles with 45 ml buffered rumen fluid). Bottles containing 45 ml Menke and Steingass (1988) CO<sub>2</sub> saturated buffer were sealed with new stoppers and injected with varying amounts (5 - 120 ml) of i) air, ii) S gas, iii) S + C gas and iv) C gas obtained from in vitro incubation of substrates in triplicate as explained earlier. The pressures were recorded after equilibrating the vials overnight at 39 °C. A calibration line was constructed by linear regression of gauge reading (in psi) versus volume fraction (F<sub>v</sub>) of added gas, where F<sub>v</sub> = milliliters added gas/ milliliters bottle headspace volume.

**Statistical analysis**

Pressure measurements were fitted against volume fraction (F<sub>v</sub>) of added gas by linear regression using GraphPad Prism software version 3. The slopes of regression lines were compared using same software by procedure described by Motulsky (1999).

**Table 2. Calibration of pressure gauge.**

Attribute	Air	S gas	S + C gas	C gas	P
Slope	12.74	12.94	13.08	13.10	0.45
R <sup>2</sup>	0.999	0.999	0.999	0.999	

**RESULTS AND DISCUSSION**

Constituents of gas in headspace of bottles after 24 h incubation of different substrates with BRL have been presented in Table 1, where figures are volume percentage of hydrogen, methane and CO<sub>2</sub> and not total volume. Cellulose as substrate yielded higher CO<sub>2</sub> percentage than starch alone and starch and cellulose, which normally occurs when substrate produces more acetate and butyrate (Getachew et al. 1998). It was also apparent that CO<sub>2</sub> represented predominant gas after incubation of different substrates with mixed ruminal microorganisms.

When know amount of air or different fermentative gases were injected to the 100 ml bottles containing 45 ml in vitro medium and pressure measurements were



Figure 1. Pressure gauge and modified B Braun plastic stopcocks-3 way used for pressure measurements

Figure 2. Regression lines obtained from fitting of pressure measurements in headspace of bottles filled with air, S gas, S + C gas and C gas against volume fraction. Slopes and r<sup>2</sup> values are presented in Table 2.

fitted against fraction volume, four calibration curves obtained for four substrates and Figure 2 shows similarity of these lines. Slopes of regression lines and their goodness of fit (R<sup>2</sup>) have been summarized in Table 2. The pressure gauge showed the expected linearly increasing pressure to increasing amounts of air and fermentative gases. Pell and Schofield (1993) calibrated an automated gas production system using either CO<sub>2</sub> or air and they also found a linear relationship between volume and pressure in the headspace of serum bottles. When a mixture of 36% CH<sub>4</sub> and 64% CO<sub>2</sub> was used for calibration of in vitro system, it also resulted in a linear calibration curve (Weimer et al. 2005). There was no significant difference between slopes of calibration curves (Table 2). Because in vitro digestion produces a mixture of a water-soluble gas CO<sub>2</sub> and an essentially water-insoluble gas CH<sub>4</sub>, the calibration must measure the sensor response curves to both types of gas. The solubility of CH<sub>4</sub>, CO<sub>2</sub>, O<sub>2</sub> and N<sub>2</sub> is 0.023, 1.7, 0.043 and 0.019 g/kg water at 20 °C ([http://www.engineeringtoolbox.com/gases-solubility-water-d\\_1148.html](http://www.engineeringtoolbox.com/gases-solubility-water-d_1148.html)). In the present experiment, air (containing mainly Nitrogen and Oxygen) was used as a low soluble gas whereas fermentative gases contained 62-69 % CO<sub>2</sub> that had relatively greater solubility. The result of calibration with gases differ in solubility with air

indicated that increase in proportion of soluble fraction caused a slight increase in the slopes however, differences between fermentative gases and air were not significant. These results were in agreement with those of Pell and Schofield (1993) who used air versus CO<sub>2</sub> and found a greater slope for calibration curve obtained using CO<sub>2</sub> than air.

It can be concluded that at a particular temperature, using air for calibration of in vitro gas production by pressure gauge and serum bottles can give reliable equation, which can be applied for measuring the volume of total gas production from wide ranges of substrates in vitro.

## REFERENCES

- France, J., J. Dijkstra, M.S. Dhanoa, S. L'opez, and A. Bannink. 2000. Estimating the extent of degradation of ruminant feeds from a description of their gas production profiles observed in vitro: derivation of models and other mathematical considerations. *Br. J. Nutr.* 83: 143-150.
- Getachew, G., M. Blümmel, H.P.S. Makkar, and K. Becker. 1998. In vitro gas measuring techniques for assessment of nutritional quality of feeds: a review. *Anim. Feed Sci. Technol.* 72: 261-281.
- L'opez, S., M.S. Dhanoa, J. Dijkstra, A. Bannink, E. Kebreab and J. France. 2007. Some methodological and analytical considerations regarding application of the gas production technique. *Anim. Feed Sci. Technol.* 135: 139-156.
- Menke, K.H. and H. Steingass. 1988. Estimation of the energetic feed value obtained from chemical analysis and in vitro gas production using rumen fluid. *Anim. Res. Dev.* 28: 7- 55.
- Menke, K.H., L. Raab, A. Salewski, H. Steingass, D. Fritz, and W. Schneider. 1979. The estimation of the digestibility and metabolizable energy content of ruminant feedingstuffs from the gas production when they are incubated with rumen liquor in vitro. *J. Agric. Sci.* 93: 217-222.

- Moate, P.J., T. Clarke, L.H. Davis and R.H. Laby. 1997. Rumen gases and bloat in grazing dairy cows. *J. Agric. Sci.* 129: 459-469.
- Motulsky, H.J. 1999. *Analyzing Data with GraphPad Prism*. GraphPad Software Inc., San Diego.
- Pell, A.N., and P. Schofield. 1993. Computerized monitoring of gas production to measure forage digestion in vitro. *J. Dairy Sci.* 76:1063-1073.
- Solubility diagrams of gases in water. [http://www.engineeringtoolbox.com/gases-solubility-water-d\\_1148.html](http://www.engineeringtoolbox.com/gases-solubility-water-d_1148.html).
- Theodorou, M.K., B.A. Williams, M.S. Dhanoa, A.B. McAllan and J. France. 1994. A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. *Anim. Feed Sci. Technol.* 48: 185-197.
- Trei, J.E., G.C. Scott, and R.C. Parish. 1972. Influence of methane inhibition on energetic efficiency of lambs. *J. Anim. Sci.* 34: 510-515.
- Weimer, P.J., B.S. Dien, T.L. Springer and K.P. Vogel. 2005. In vitro gas production as a surrogate measure of the fermentability of cellulosic biomass to ethanol. *Appl. Microbiol. Biotechnol.* 67: 52-58.

(Received on 20-10-2010 and Accepted on 24-12-2010)