## Importance of accurate and correct quantitative measurements in a new volumetric gas measuring technique for *in vitro* assessment of ruminant feeds

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### Introduction

*In vitro* rumen incubation analysis have already been used for years to evaluate the nutritive qualities of feeds, originally employing end-point measurements focusing on feedstuff digestion. The relation between accumulation of fermentation gases and metabolisable energy content of the feed was established in the 1970s. Since then, measurement techniques based on *in vitro* gas production have been further developed for feed evaluation experiments. Much of these early reports rely on a manometric gas measurement principle. Not many publications on liquid displacement systems based on a volumetric gas measuring principle are available due to the limitations of the instrumentation setup.

This work describes a new volumetric gas measuring technique specially developed for monitoring production of ultra low gas volumes, with various applications in batch fermentation tests. The technique has been successfully applied and validated for quantifying biochemical methane potential from various biodegradable organic matters. Its high measurement accuracy and precision, as well as its unique feature on gas volume normalisation can potentially match the analysis demand for *in vitro* digestibility test. An automated measuring system based on this volumetric measuring technique can offer continuous monitoring of gas production from *in vitro* digestibility tests with high throughput and significant reduction of labour and time intensity. A wide range of applications of such an automated system is expected in ruminant feed evaluation, including continuous monitoring of gas production for extracting process kinetic information, determination of feed digestibility and its metabolisable energy content, comparing different technological pre-treatments of feed compounds, optimisation of feed composition and nutrient content for livestock, screening a large range of feeds or additives before testing these *in vivo*, etc. In this study, the effect of various parameters on the measurement technology are discussed, and the method is used for a short term *in vitro* digestion test. Preliminary results from a long term validation tests are discussed as well.

### Background

Ruminants contribute substantially to the human food supply, accounting for almost all of the milk and a large part of the meat production world wide. A lot of research has therefore been completed on the digestive system of ruminants, increasing the understanding of its functioning and optimising feed efficiency for increased production. The close association between rumen fermentation and gas production was recognised several decades ago and has since been well studied. The gas production measurement technique has been widely used to determine organic matter digestibility, protein degradation, or to predict metabolisable energy content and feed digestion rate (Cone et all., 1996; Getachew et all., 1998; Getachew et all., 2008; Murray et all., 2014). Other applications include testing the use of dietary supplements in support of, for instance, greenhouse gas mitigation (Martínez-Fernández et all., 2014;

Romero-Perez et all., 2014). There are many protocols available on how to perform *in vitro* digestibility tests. Some of them are adapted for the utilisation of the gas measurement technique, but they differ in the experimental set up and are generally modified and adapted to the specific researcher´s purpose. Because of this, it is often difficult to evaluate results from different studies and values can vary substantially. Thus, there is a need for a test standard and general procedure, but also for a measurement quality standard of the gas measurement technique for *in vitro* digestibility tests.

One issue that is not fully addressed in the current protocols is the equipment and experimental set up that is used for these kinds of tests. Many times these are developed in house and specific for each laboratory. A solution to minimise the differences is the use of a complete lab platform such as the Gas Endeavour (Figure 1). The Gas Endeavour is specially designed for low gas volume and flow analysis and includes everything needed to perform *in vitro* digestibility tests; i.e. temperature controlled continuously stirred test vessels, optional vessels for carbon dioxide removal when methane analysis is performed, and a robust and reliable gas measuring system with a resolution of approximately 2 ml. In a study where three different ways of measuring the biochemical methane potential of cellulose were tested, the Gas Endeavour´s predecessor, AMPTS (Automatic Methane Potential Test System), provided the highest accuracy and repeatability (Esteves et al., 2011). Examples of studies where the AMPTS has been used are: investigation of methane potential from algae farming on available sludge streams from a waste water treatment plant (Rusten & Sahu, 2011); evaluation of different pre-treatments of sugarcane bagasse (Badshah et al., 2012) and evaluation of the effects from different chemical and biological additives on a substrate mixture (Strömberg et al., 2011).

A common problem when comparing results from different sources is the various ways of presenting the quantitative gas measurements (Walker et al., 2009; Wulf et al., 2011). Corrections to standard conditions for temperature and pressure are often poorly described or presented using different standard values which could lead to differences of up to 10% in the corrected volume. Another factor, that is not addressed in many corrections, is the water content of the gas. At 20°C and 1 atmosphere, roughly 2.3% of the gas volume consists of water and thus, should not be considered as gas in the reported values. It is also important to get an accurate value of the instant temperature and pressure throughout the test period in order to adjust gas volumes correctly in real time. Many times a fixed room temperature and pressure is assumed but in fact, these values can vary substantially. In a 30-day long experiment, carried out in a well sealed and shielded lab in Lund, Sweden, the pressure varied between 99-106 kPa, which could lead to a difference of up to almost 7% if the extremes were used. Clearly, there are many factors that need to be addressed in order to present quantitative gas measurements in a correct way. For the Gas Endeavour, all these issues are addressed and fully automatic.

**System and Calculations**

The Gas Endeavour has been used to perform a number of methane potential tests for biogas production on various types of substrates. As can be seen in Figure 1, the Gas Endeavour consists of two major parts, of which the first part is the temperature controlled water bath with 15 reactors of 500 ml, which can be seen on the left hand side of Figure 1. Each bottle has a mixer with a motor that can be run in either continuous or intermittent mode. The second part, which can be seen on the right hand side of Figure 1, is a gas measurement unit

where gas is collected in a cell through water displacement. When a pre-defined gas volume has been accumulated, the cell opens and releases the gas which is registered in the embedded CPU. Every opening corresponds to roughly 2 ml of gas and for each opening the ambient temperature and pressure are registered for calculations of normalised values (0°C, 1 atmosphere and zero moisture content).

**Figure 1** The Gas Endeavour

*Ambient pressure*

The variation of ambient pressure can significantly influence gas volume and flow measurement. To minimise the influence of ambient pressure difference and variation among different testing sites and labs, the gas volume is usually corrected to standard conditions using the ideal gas law. However, it should be considered that there are two common standard conditions which differ from each other on reference temperature (i.e. 0ºC or 20ºC). This can lead to differences of up to 10 % (Walker et al., 2009).

In order to meet demands for high accuracy and precision, it is not sufficient that pressure is measured by off line spot checks. It should be measured continuously at each measuring point in real time, to be sure that a correct value is registered. The ambient pressure can vary from day to day which will impact on both the dynamic profile and the accumulated volume.

*Temperature*

As with pressure, temperature at the measuring point will affect the volume of the gas and should be adjusted to standard conditions using the ideal gas law. Equation 1 shows how to adjust a gas volume to standard volume and pressure based on the ideal gas law.

$$V_{STP} = \frac{p_{STP}}{p_{gas}} * \frac{T_{gas}}{T_{STP}} V_{gas} \quad (1)$$

In Equation 1, $V_{STP}$ is the volume adapted to standard temperature and pressure, $p_{STP}$ is the standard pressure, $p_{gas}$ is pressure of the measured gas, $T_{gas}$ is the temperature of the measured gas, $T_{STP}$ is the standard temperature (which is 0ºC for the Gas Endeavour) and $V_{gas}$ is the measured volume.

*Water content*

Gas produced from anaerobic digestion and *in vitro* digestibility tests is assumed to be saturated with water vapour and, in order to give accurate and correct gas measurements, this water should be removed (Walker *et al.,* 2009). At the ranges where an anaerobic digestion test and *in vitro* digestibility tests normally are performed (i.e. 0.9-1.1 bar and 10-40°C), the vapour pressure of water can satisfactory be approximated using the Antoine equation

(Equation 2). In Equation 2, $p_{vap}$ is the fraction of water in the gas and $T_{gas}$ is the temperature of the gas in °C.

$$p_{vap} = 10^{8.1962-\frac{1730.63}{233.426+T_{gas}}} \quad (2)$$

*Relative error*

The error introduced by assuming either the temperature or pressure constant or including water vapour in the gas volume measurement, is calculated according to Equation 3.

$$Relative\ error = \frac{V_{mL} - V_{NmL}}{V_{NmL}} * 100\% \quad (3)$$

In Equation 3, $V_{mL}$ is the measured accumulated volume at a certain time in mL, for a scenario where either the pressure or temperature is assumed constant or where the water content is included in the measurement. $V_{NmL}$ is the measured accumulated volume in normalised mL.

**Materials and Methods**

*Biochemical methane potential test*

The sample (banana stems) was mixed with an inoculum in 500 ml bottles to reach a liquid volume of 400 ml with an inoculum to substrate ratio of 2:1 (based on volatile solids content). The inoculum was collected from a sewage treatment plant in Sweden (Ellinge sewage plant, Sweden), which receives municipal wastewater and vegetable residues from the food industry, and was stored at room temperature for five days to reduce as much of its organic content as possible. Triplicates of each sample were used and the bottles was incubated, at 37°C with continuously mixing of approximately 80-100 rotations per minute. Triplicates with only 400 ml of inoculum was included to remove background production from fermentable material contained in the inoculum. No additional external nutrients or trace elements were added to the reactors. Before starting the test, the headspace was flushed with nitrogen gas for 1 minute to achieve anaerobic conditions. The produced biogas was led through 80 mL of 3 M sodium hydroxide solution to remove carbon dioxide and hydrogen sulphide to allow measurement of only methane. The test was performed for 35 days during which the gas volume, together with temperature and pressure, was continuously recorded with the Gas Endeavour´s predecessor AMPTS II.

*Methods short-term incubation*

Samples were incubated with 200 ml rumen fluid and 200 ml of VOS buffer (Lindgren, 1979), containing per litre: 8.50 g $NaHCO_3$, 5.80 g $K_2HPO_4$, 0.50 g $(NH_4)_2HPO_4$, 1.00 g NaCl, 0.50 g $MgSO_4$•7 $H_2O$, 0.01 g $FeSO_4$•7 $H_2O$ and 0.10 g $CaCl_2$. Rumen fluid was from a maintenance fed non-lactating cow and collected at about 17:00 h, after last feeding. The rumen fluid was transported to the lab and strained through a kitchen strainer (approx. 1-mm openings).

Wheat starch and urea (both from Kebo, Stockholm) were incubated at three different levels: 3 g starch + 200 mg urea, 6 g starch + 400 mg urea and 9 g starch + 600 mg urea. In addition, a grass sample with in vitro digestible organic matter (IVDOM) concentration of 840 g/kg OM and a blank (only rumen fluid and buffer) were incubated. All incubations were in triplicate.

Feed samples were added first to the incubation bottles and VOS buffer was added after flushing the bottles with $CO_2$. Rumen fluid was thereafter added to a triplicate of incubation vessels, tubing and stirring motors were connected and gas measurement started for that triplicate. About 5 minutes elapsed from rumen fluid addition until gas production was being logged for a triplicate of vessels.

*Methods 96 h incubation*

This incubation involved both gas measurement and gravimetric determination of organic matter disappearance in the incubation vessels. It was performed in conjunction to the lab's weekly routine IVDOM determination of forage samples according to the 96 h VOS procedure (Lindgren 1979; Åkerlind et al., 2011). Proportions of rumen fluid, buffer and sample were similar to the VOS procedure with 10 ml rumen fluid, 290 ml VOS buffer and 4 g of air-dry sample.

A set of six calibration samples with IVDOM 686-901 g/kg OM that are included in each IVDOM batch at the lab were incubated in duplicate and so was a barley straw sample with IVDOM 505 g/kg OM.

The rumen fluid was from a maintenance fed non-lactating cow and collected in the morning. Handling of rumen fluid and buffer was similar to the lab's IVDOM procedures with straining of rumen fluid through a 1 mm screen, mixing with buffer and dispensing into incubation bottles without previous $CO_2$ flushing. Incubation was conducted over 96 hours.

After incubation termination, each bottle was split into three glass filter tubes with porosity P1 (100-160 μm) and rinsed according to the VOS procedure with hot water and acetone. The samples were then dried overnight at 103°C and ashed for 3 h at 500°C according to the standard procedures for VOS to get a measure of remaining organic matter amount and hence organic matter digestibility in vitro.

**Results and Discussion**

In this section, the results from the biochemical methane potential test are presented. Focus is directed on showing the effect of the different factors influencing the results, and a feed digestibility test performed using standard samples.

Three different aspects that influence the recorded gas volume were studied: assuming a fixed ambient temperature (22ºC) and pressure (1 atm) *vs* continuous measurements of the two parameters as well as considering or not considering the water vapour content of the gas. The recorded variation of pressure and temperature from the biochemical methane potential test can be seen in Figure 2. Pressure varied considerably, whereas temperature remained more stable due to the temperature controlled environment inside the lab.

Figure 3 shows the difference in the measured accumulated volume for the three different scenarios. The right hand side figure shows the variation in the relative error *vs* a reference case (considering variation in temperature and pressure as well as removal of the water vapour content). It can be seen that the introduced error varies for the scenario where the pressure is assumed constant. The errors introduced by fixing the temperature or including water vapour are more constant in time.

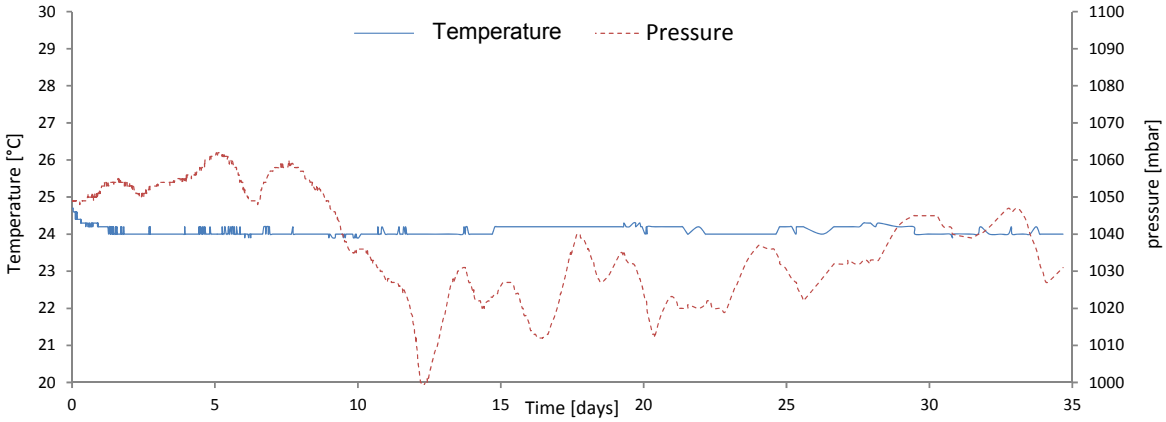


**Figure 2** Dynamic profile of temperature and pressure during the biochemical methane potential test.

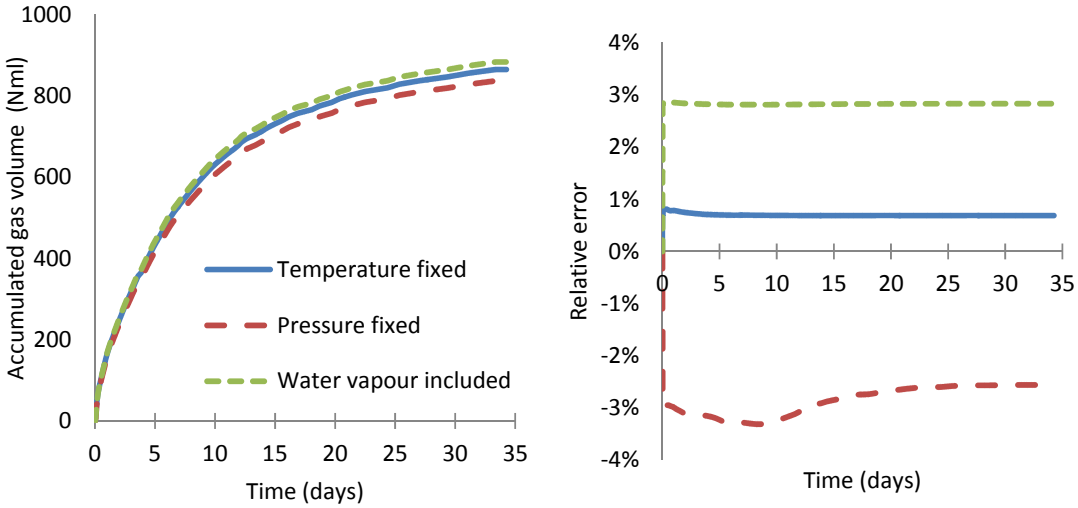


**Figure 3** Recorded accumulated volume of a biochemical methane potential test when different factors have been assumed constant, and the resulting error percentage over time for each situation.

The *in vitro* digestibility test was performed together with the Feed Science Division, Department of Animal Nutrition and Management, SLU, Uppsala. The accumulated gas volume was monitored over time, and pH was measured at the end of the incubation, after *circa* 14 hours. The average results are plotted in Figure 4.

Figure 4 shows that the standard deviation of measured gas volumes within triplicates was in general very low, with exception for the highest concentrations of starch and urea (respectively, 9 g and 600 mg). This suggests that an accumulation of volatile fatty acids and the resulting low pH of 5.5 was limiting fermentation. This could also be a sign of substrate overload in the test vessels, as well as the reason for the higher standard deviation within triplicates with high concentrations of starch and urea. A clear correlation can be seen between starch level and gas production. Fermentation of the grass resulted in a relatively low final gas volume but a rapid onset of fermentation, probably as a result of easily fermentable sugars present in the grass.

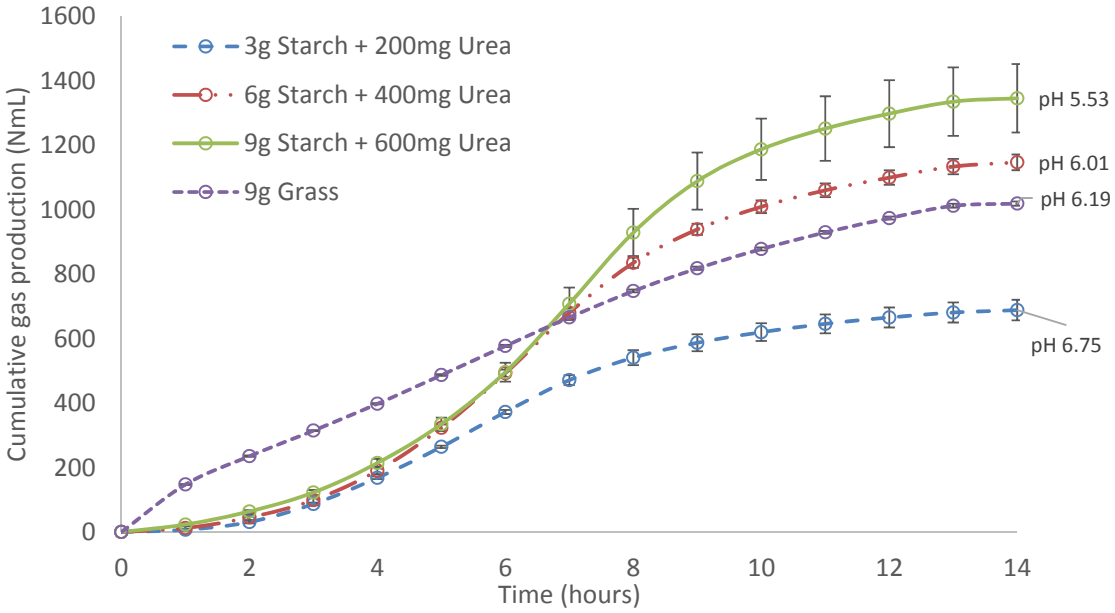


**Figure 4** Accumulated gas volume in normalised mL over time, with final pH value.

To further validate the instrument for *in vitro* feed digestibility tests, a long term incubation of 96 hours was also performed. The method was compared to the gravimetric standard Swedish in vitro analysis (VOS). The Gas Endeavour resulted in a slightly higher remaining organic matter amount whereby the relative error compared to the VOS analysis was circa 3% (results not shown), except for the blank sample where the relative error was higher. Overall, the results from the two methods were well correlated.

## Conclusions

In this work, results from various biochemical methane potential tests are reported, highlighting the importance of a correct adjustment of quantitative gas measurements. It was shown that variable ambient pressure and temperature can have a significant effect on the measured accumulated gas volume. Some preliminary result of an *in vitro* feed digestibility test was also performed with the Gas Endeavour, showing a clear correlation between the measured accumulated gas volume and starch concentrations used. In general, the variation between triplicates was minimal. More long and short term incubations are currently performed to further validate the instrument for feed digestibility tests.

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**Evaluation of bio-forage feeds developed through gene silencing, modification and inserting techniques for ruminant livestock systems: progress update**

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## Introduction

Alfalfa (*Medicago sativa L.*) is one of the most important forages in the world (Wang et al., 2006). Alfalfa is a relatively winter hardy and drought tolerant legume with good longevity (Popp et al., 2000), high nutrient levels, high digestibility, unique ratio of structural to non-structural components (Yu et al., 2003a) and high dry matter intake (Thornton and Minson, 1973). A major disadvantage of alfalfa is its excessively rapid initial rate of ruminal protein digestion and degradation (Boderick, 1995; Yu et al., 2004). This results in digestive disorders like frothy bloat (Popp et al., 2000; Wang et al., 2006) and low protein use efficiency with consequent release of up to 25% of alfalfa nitrogen from the ruminant into the environment (Boderick, 1995). Proanthocyanidins (PA) are oligomeric and polymeric secondary plant products which share the early and middle steps of the flavonoid pathway with the plant pigments anthocyanins. The flavonoid pathway arises from the phenylpropanoid pathway (4-coumaroyl CoA + 3 malonyl CoA) via naringenin chalcone (Marles et al., 2003). The building blocks of most PA are the monomer flavan-3-ol (e.g. (+) catechin and (-) epicatechin) and a flavan 3,4-diol (Winkel-Shirley, 2001). The composition of PA varies in linkage between the flavan monomers (C4 to C6 or C4 to C8), stereochemistry at carbons 2, 3 and 4 and the number of hydroxyl groups on the A and B rings. These differences in PA composition affect its molecular structure and influence the capability to interact with other molecules like protein. PA can form complexes not only with protein but also with starch, essential amino acids, carbohydrates and digestive enzymes (Aron and Kennedy, 2008). It provides a strong defense mechanism to plants, especially protecting them against herbivores (Zucker, 1983).

PA-protein complexes are formed in the rumen, reducing protein degradation and consequently increasing ruminal escape protein (Aerts et al., 1999; Broderick, 1995) and reducing foam stability (Fay et al., 1980; Tanner et al., 1995). PA-protein bonds are broken in the abomasum due to low pH (Jones and Mangan, 1977), which may result in increased amino acid (AA) digestion in the small intestine (Aerts et al., 1999). PA are also found to reduce the production of methane (Woodward et al., 2004). Flavonoid structural gene chalcone synthase (CHS) is active in alfalfa leaves but there is no expression of flavanone 3-hydroxylase (F3H) and dihydroflavanol 4-reductase (DFR). These latter genes stimulate intermediate steps in the flavanoid pathway leading towards the accumulation of PA. Instead of PA, alfalfa leaves accumulate upper pathway flavones. In western Canada Three Lc alfalfa genotypes were crossed with hardy Western Canadian varieties to facilitate the movement of the Lc gene into a broader spectrum of alfalfa breeding germplasm. To our knowledge, no forage analysis has been conducted on these crossed progeny of transgenic Lc alfalfa. Transgenic alfalfa germplasm has also been developed by expressing a PA regulating bHLH from lotus uliginosis and with a bHLH gene from alfalfa. This material has not been analyzed yet for the presence of PA. There is a need to develop and evaluate these populations of PA