MEASURING METHANE PRODUCTION FROM RUMINANTS

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Edited by

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FOREWORD

The world's livestock sector is amidst a major transformation, fuelled by high demand for meat and milk, which is likely to double over the next two decades in developing countries. The major driving force behind this demand for livestock products is a combination of population growth, urbanization and income growth, especially in developing countries. The challenge is to enhance animal productivity without adversely affecting the environment. A key to this is reducing methane emissions from ruminants.

The major limitation to ruminant production in many tropical regions of Africa, Asia and Latin America, where a large proportion of the global ruminant population is located, is poor nutrition. The productivity of animals is restricted by the low nitrogen and high fibre content of the native grasses and crop residues, which form the basis of the diets in these regions. Animals on these types of diets emit more methane than animals fed better quality temperate forages. These methane emissions represent a loss of digestible energy to the animal (up to 15%) as well as a threat to the environment. Reducing methane production by ruminants could improve their productivity, provided the efficiency of ruminal metabolism is not compromised, and reduce their contribution to greenhouse gas emissions. The challenge is to devise nutritional strategies and identify dietary components, particularly from locally available plant resources, that reduce methane emissions. However, before new strategies can be developed, it is essential to be able to measure methane emissions accurately to establish baseline values for current practices. Without this, there is no way to establish whether specific plants or new management systems improve methane emissions by lowering them under the baseline.

This publication stems from a training workshop that was organized as part of an FAO/IAEA Coordinated Research Project entitled 'Development and use of rumen molecular techniques for predicting and enhancing productivity'. It contains a comprehensive account of the key techniques for measuring methane *in vivo*. These techniques provide a variety of options for measuring methane emissions ranging from respiration chambers to the tunnel method, which can be used with animals in the field. These techniques will allow baseline estimates of methane emissions to be established in Member States and the extent to which ruminants contribute to greenhouse gas emissions in these countries. This information will be of interest to national agricultural research organizations, extension officers and government officers responsible for formulating environmental policy. They also

provide the opportunity to test new plants for their potential to reduce methane emissions and to help design new management and feeding strategies based on these measurements. The aim of the book is to better equip the readers to measure and monitor methane emissions accurately and meet the challenge of improving productivity from ruminants without damaging the environment.

INTRODUCTION

Methane is one of the most important greenhouse gasses and approximately 70% of methane emissions are linked to human activities. Domestic ruminants are responsible for 25% of these emissions, which are derived mainly from their gastrointestinal tract but also from their manure. Importantly, the methane produced by ruminants is an energy loss to production and reducing emissions would benefit ruminant production and the environment. The demand for meat, milk and fibre from ruminants is increasing but there is also pressure from society to reduce the impact our production systems have on the environment. Under these circumstances, developing systems for reducing methane emissions seems an appropriate and logical problem to address.

Methane emissions derived from the gastrointestinal tract of ruminants are approximately 10 times higher than the emissions from manure. These emissions are a direct result of fermentation processes performed by ruminal microorganisms and, in particular, the archael methanogens, which scavenge hydrogen and use it to produce methane. The amount of methane that is produced is determined by a number of factors including for example, diet, the number of protozoa in the rumen and the number and type of methanogens present; these are not mutually exclusive. The production of methane is a direct energy loss to the animal as well as a source of pollution. Manipulating any or all of these factors could help to reduce methane emissions and achieve our objective of improving productivity without damaging the environment. However, to establish baseline estimates of methane emissions from current practices and assess the impact of new practices introduced to reduce methane emissions, it is essential to be able to quantify methane accurately under different circumstances.

Methane emissions can be measured using both *in vitro* and *in vivo* techniques. Using and maintaining animals for experimental purposes is expensive. Consequently, *in vitro* techniques are often the initial choice for investigating strategies for reducing or inhibiting methane production. The *in vitro* techniques are relatively inexpensive ways to test many different diets, combinations of diets, potential inhibitors and additives for their effects on methanogenesis, simultaneouslyand

quickly. In these tests, a sample of rumen fluid is used to help simulate normal rumen fermentation in batch or continuous culture. Methane production can be measured in these simulated systems by sampling the gas and analysing its composition, using for example, gas chromatography. Diets, additives and inhibitors that reduce methane production *in vitro* can then be examined in more expensive *in vivo* experiments for their potential to mitigate methane emissions in practical feeding situations. The *in vivo* experiments involve measuring methane in live animals, either in animal houses or under production conditions. The animals, or the head of the animals, are often enclosed in respiration chambers, where the gases that are emitted can be collected and analysed. There are also systems where methane production is measured using isotopic tracers or by inserting inert tracer gases into the rumen. All of these techniques are addressed in this manual.

The chapters in this manual have been written by experts with a keen interest in developing systems for reducing methane emissions to improve livestock production without damaging the environment. The techniques they have described are the best and most up-to-date techniques available for quantifying methane in the laboratory and from animals under both production and experimental conditions. The readers are provided with a variety of alternatives for quantifying methane and will find that one technique may suit their circumstances better than the others. For example, attempting to measure methane emissions from ruminants in an enclosed chamber in the heat and humidity associated with a tropical environment will be different to attempting a similar experiment under European conditions. The 'head box' technique described in Chapter 8 and being used in Thailand is a good example of this. Using this technique, only the head of the animal is contained within a respiration box minimizing the effects of the heat and humidity in comparison to retaining the entire animal in an enclosed chamber. The chapters are written in the detail used normally for standard operating procedures and provide detailed descriptions on the assembly and construction of equipment. Additional photos are provided in some chapters that help the reader to visualize what is described in the text, in easy step-wise guides. All of the chapters include literature that is relevant to the technique. The first two chapters deal mainly with *in vitro* techniques and the following 6 chapters provide an array of *in vivo* techniques that are used to quantify methane emissions.

This manual will enable researchers in both developed and developing countries to choose and establish the most appropriate techniques for quantifying methane emissions accurately for their situations. It will enable researchers to screen plant resources and by-products for their potential to reduce methane emissions and assess the impact of new strategies for reducing methane under production conditions. It will also enable better feeding practices and management systems to be designed that improve productivity while reducing methane emissions. By encouraging more research institutes around the world to establish accurate methods for quantifying

methane emissions, the inventories of the contribution ruminants make to total methane emissions and the impact our efforts to reduce these emissions are having will improve substantially.

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CHAPTER 1

ANALYSIS OF METHANE

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INTRODUCTION

Methane is one of the end-products of ruminal fermentation, formed autotrophically by methanogenic archaea from CO_2 and H_2 derived from the fermentation of carbon sources, in particular sugars (5, 9, 14, 27). Methane is finally eliminated by belching, representing a loss of between 5 and 8% of the gross energy contained in the feedstuffs consumed by the animal (1). Methane is also considered a major source of greenhouse gas emissions from agriculture (13, 20).

Methane has been measured *in vivo* by a number of techniques (1, 12, 13, 18). *In vitro* cultures allow for determination of methane production when different diets are fermented alone or in the presence of additives to study their responses on rumen fermentation (15, 19). The aim of this section is to describe the measurement of methane production from *in vitro* incubations inoculated with mixed ruminal microorganisms. Methane measurement from the following two *in vitro* systems will be described: i) batch cultures in sealed serum bottles (19) and ii) the fermenter called Rumen Simulation Technique (RUSITEC) that is a semi-continuous culture (3). The measurement of the methane production follows several steps:

- Measurement of total gas production
- Sampling of gas
- Analysis of gas composition
- Calculations

1. MEASUREMENT OF TOTAL GAS PRODUCTION

Different procedures can be used to measure the total gas produced after incubation in a buffered medium containing rumen fluid. As the final goal is to determine methane production, it is important that the system enables gases to accumulate as the fermentation proceeds.

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An approach could be the use of gas-tight culture bottles for the incubations (26). Briefly, a culture medium containing macro- and micro-mineral solutions, resazurin and a bicarbonate buffer solution is prepared as described by Goering and Van Soest (11). The medium is kept at 39°C and saturated with CO_2. Oxygen in the medium is reduced by the addition of a solution containing cysteine-hydrochloride and Na_2S, as described by Goering and Van Soest (11). Rumen fluid is then diluted into the medium in the proportion 1:4 (v/v). The substrate (500 mg) is placed in the bottle and diluted rumen fluid (50 mL) is dispensed anaerobically. Once filled up, the bottles are closed with rubber stoppers and crimped with aluminium caps, and finally placed in an incubator adjusted at 39°C.

Once the incubation is finished, the gas accumulated in the headspace of the bottles is measured. One option is to use a lubricated glass syringe connected to a needle that is inserted through the stopper into the headspace, recording the volume in the calibrated syringe after the plunger displacement (17). The procedure followed in our laboratory is based on the use of a pressure transducer (Bailey & Mackey Ltd., Birmingham, UK, www.baileymackey.com) connected to a digital readout voltmeter (Bailey & Mackey Ltd.) and a gas-tight syringe assembly (26). The transducer is connected to one outlet of a disposable Luer-lock three-way stopcock (Discofix®; B. Braun Melsungen AG, Melsungen, Germany). The other two outlets of the stopcock are connected to a disposable hypodermic needle and to a disposable plastic syringe, respectively. The needle is inserted through the bottle closure and headspace gas pressure is recorded on the digital display. The corresponding gas volume is then measured by displacing the syringe plunger until the headspace pressure returns to ambient pressure, as indicated by a zero reading on the digital display unit. The Luer-lock of the stopcock is rotated to close the outlet to the syringe, the assembly is withdrawn from the bottle, and the volume of gas in the calibrated syringe is recorded. The gas collected in the syringe can then be discarded, or a sample can be taken if required.

In the RUSITEC, the gas produced during incubation is collected in an expandable container connected to the effluent output (3). The container used is a gas-tight plastic-coated aluminium bag. To check the existence of leaks, bags are filled up with air and allowed to stand overnight. A deflated bag indicates a leak.

A precision wet-test gas flow meter drum-type (RITTER Series TG, Dr.-Ing. Ritter Apparatebau GmbH, Bochum, Germany; www.ritter-app.com) is used to quantify gas volume in the collection bags. Wet-test meters measure the actual volume of gas flow directly, functioning upon the principle of positive displacement. The sampling bag is connected to the inlet of the meter, and then emptied so the gas flows through some rigid, fixed volume chambers placed inside the meter, and finally is released through an outlet. The sample gas stream rotates a measuring drum within a packing fluid. Coupled to the rotating drum, a needle-dial and counting mechanism record the volume of gas flow.

2. SAMPLING OF GAS

A representative sample of the gas produced has to be taken for subsequent analysis. Sampling is conducted using special gas-tight glass syringes provided with removable needles (pressure-lock gas syringes Series A-2 with matching needles; Valco Instruments Co. Inc. (VICI) Precision Sampling; Baton Rouge, LA, USA; www.vici.com/contact.htm). These syringes are fitted with a push-button valve allowing for sample locking and storage within the syringe.

In the batch cultures, a sample can be taken directly from the headspace, after inserting the needle through the septum of the stopper, taking a sample in the syringe and closing the valve. This is collected after measuring total gas production, assuming that gas composition will be the same in the gas released as that remaining in the headspace.

In the RUSITEC, samples of gas are taken from the collection bags, using these gas-tight syringes. The sample can be taken as the bag is emptied to measure the gas volume in the flow meter. This procedure is followed provided the samples can be analysed immediately after the sample collection. If this is not the case, samples can be collected in Vacutainer® (Becton, Dickinson & Company, Franklin Lakes, NJ, USA; www.bd.com) or Venoject® (Terumo Europe, Leuven, Belgium; www.terumo-europe.com) 10 mL glass vacuum air-tight tubes. In this case, a sample is collected in a syringe connected to a three-way stopcock. From batch cultures in serum bottles, the same syringe used for gas measurement will be used to take the sample. From RUSITEC bags, a syringe will be filled as the bags are emptied to measure total gas volume. Then, the syringe contents can be injected into the vacuum tube.

It is important to inject a volume of gas greater than the capacity of the sampling tube, so that contents in the tube are pressurized. This will avoid the entry of air from outside. It is important to take samples in duplicate, to check for any leak of gas from the air-tight tube during storage. At the time of analysis, a sample of gas is taken from the sampling tube using the special gas-tight syringe, inserting the needle through the rubber septum of the vacuum tube.

Finally, the needle of the syringe can be inserted into the injection port of the GC, the valve opened and the sample injected.

3. ANALYSIS OF GAS COMPOSITION

Methane can be determined by different analytical methods (12, 13):

- Infrared photoacoustic spectrometry – trace gas analyser (TGA)
- Fourier transform infrared absorption spectroscopy
- Mass spectroscopy
- Tuneable Diode Laser Absorption Spectroscopy (TDL)
- Gas chromatography (GC)

The first four methods are theoretically more sensitive than the GC method for methane; but are also much more expensive and less extensively used. The most

commonly used instrument for measuring CH_4 is a GC. It relies on the individual partitioning characteristics of different gases in the sample between a mobile phase (an inert gas such as He) and a stationary solid phase packed in a column. Thus the components in the gaseous mixture are separated, with each component being identified by its retention time on the column and quantified by a subsequent detector.

The conditions used in our laboratory to measure methane will be outlined, but it must be stressed that before putting the GC analyser into routine operation, the operational conditions have to be optimized according to the manufacturer's specifications to provide good resolution and minimum analysis time. Therefore, the final settings to be used should be established in each laboratory. Some of the options to be examined will be discussed.

3.1. Detector

A key part of a GC system is the detector. Two types of detector are commonly employed for measuring methane (12, 22):

- Thermal conductivity detector (TCD) – The TCD detects the difference in thermal conductivity between the effluent (carrier gas + sample) and a reference flow of carrier gas, which produces a voltage proportional to this difference. It is sensitive to both CO_2 and CH_4.
- Flame ionization detector (FID) – The FID detects compounds that produce ions when burned in the H_2-air flame, and hence is sensitive to CH_4.

The FID is capable of detecting CH_4 from ppm to low percentage levels, while the TCD can detect CO_2, H_2, O_2, N_2, and CH_4 from low ppm to high percentage levels. Detection limits below 200 ppb are possible with CH_4.

The detectors may be connected individually to GC systems, or fitted in combination, thus allowing the simultaneous analysis of several gas species.

3.2. Column

Columns packed with different solid adsorbents have been used for the analysis of methane. Some of the absorbents used are porous polymers (Porapack, Chromosorb, Hayesep), carbon molecular sieves (Carboxen, Carbosieve) or synthetic metal aluminasilicate molecular sieves (commonly referred to as 13X) (25). All of them can be used, but the final choice will depend on the chromatograph and some of the analytical conditions (compounds to be measured together with CH_4), time of analysis for each sample, type of detector, etc.

One of the stationary phases most commonly used is Carboxen-1000 particles with a mean pore diameter of 70Å, a surface area of more than $1200\,m^2\,g^{-1}$ and a distribution of macro- (diameter >500Å), meso- (20–500Å diameter), and micropores (diameter <20Å) optimized to provide effective kinetics for GC analyses and high efficiency (24). Carboxen-1000 packing of either 60/80 mesh or 45/60 mesh can be used depending on the compounds to be separated.

Prior to use of a column, it should be conditioned following manufacturer recommendations to reduce column bleed.

3.3. Temperatures and Gas Flows

Before analysing any sample it is important to establish the appropriate carrier gas flow; adjust the column, injector and detector temperatures to the recommended levels; and allow sufficient time for temperature stabilization. This may require 1 h for each change in temperature.

Standard configuration settings will be provided by the column and GC manufacturers. However, these can be tested in each particular laboratory and slightly modified to improve the efficiency of analysis, in particular to find the best compromize between sensitivity (separation of compounds, repeatability, accuracy) and time required for the analysis.

The limited resolution of nitrogen/air from methane requires low starting temperatures (about 40°C), but this increases analysis time and may affect the accuracy of the analysis. Isothermal configurations are used by most laboratories, but ramps of oven temperature increasing to values of up to 240°C can be also used (24). Different carrier gases can be used as the mobile phase, such as nitrogen, helium or argon. Helium is the most commonly used, although when H_2 has to be determined, argon should be the choice because the TCD detects all gas components except the carrier, and interferences between H_2 and He may occur (thermal conductivity of both gases is similar).

3.4. Injection Port

In most cases, the sample is injected directly from the gas-tight syringe. It is also possible to use a multiple-port valve with a sampling loop to introduce the gas sample onto the column in split mode. This latter option may improve precision but it is obviously more sophisticated and expensive than the simple manual injection.

3.5. GC Parameter Settings (Configuration in Our Laboratory)

Instrument: Shimadzu GC-14B

Column 2.3 meters length × 2.1 mm internal diameter stainless steel column packed with 60/80 mesh Carboxen™-1000 stationary phase [Supelco (Bellefonte, PA 16823–0048 USA) sells a stock packed column with the same properties (Cat. No.: *12390-U*) but with a nominal length of 4.5 m; the 2.3 m column is manufactured on request; the Supelco catalogue can be accessed at www.sigma-aldrich.com].

Injection volume 300–500 μL of gas are injected directly into the GC using the special gas-tight syringe. It is important that the gas contained in the syringe is at atmospheric pressure before injecting the sample. Tight control of the sample size should minimize the variation in the peak area ratios.

Carrier gas Helium, flow rate (30 mL/min; 100 kPa), constant flow mode

Temperatures Depend upon the gases to be analysed and hence the type of detector:

a) if only methane is to be determined

Detector FID. Temperature 200°C, Synthetic Air flow rate (400 mL/min), H_2 flow rate (50 mL/min)

Injector Temperature 200°C

Column-oven 170°C (isothermal)

b) if other gases (H_2, CO_2) apart from methane are to be determined

Detector TCD. Temperature 170°C

Injector Temperature 150°C

Column-oven 120°C (isothermal)

Both configurations are simple, give satisfactory separation and good reproducibility of the analysis.

All gas cylinders with carrier or standard gases were acquired from "Carburos Metálicos S.A." (Barcelona, Spain; www.carburos.com), a Spanish Company integrated into the International group Air Products and Chemicals, Inc. (Allentown, Pennsylvania, USA; www.airproducts.com).

4. CALCULATIONS

Tests of linearity and appropriate calibration are performed before sample analysis. Using standard gas mixtures, the detector linearity is verified over the range of likely concentrations in the samples, with at least three points per gas of interest. In our laboratory this calibration is performed every time the GC is initialized to analyse a set of samples and thereafter every day. Subsequent calibrations within the day may be carried out using the standard gas at a single concentration (single-point calibration) provided the calibration point is within 20% of the component concentration in samples being tested. The standards used for calibration can be pure gases, a mixture of the gases to be analysed diluted into the same gas used as carrier (He) or a mixture of gases of known and certified composition.

The standard curve can be established by plotting a linear regression of the standard quantities injected of each gas versus the area of the peaks to obtain the response factor for each compound. Using the regression equation, the amount of gas in a sample can be estimated from the area integrated for the peak of that particular compound.

4.1. Example

Pure methane supplied by the company Carburos Metálicos (Spain) is used as a standard. Several volumes of methane taken directly from the cylinder using a gas-tight syringe were injected in the GC fitted with a FID. The results obtained are presented in Table 1.

With these data, the ensuing relationship was derived by linear regression (Fig. 1): $CH_4 = 0.00188 \times \text{Area} + 5.64$ ($R^2 = 0.997$, RSD = 0.561)

Table 1. Example of a Standard Curve for Methane

Volume of methane (μL)	Peak area
20	7684
25	10444
30	12782
35	15224
40	18645
45	20814

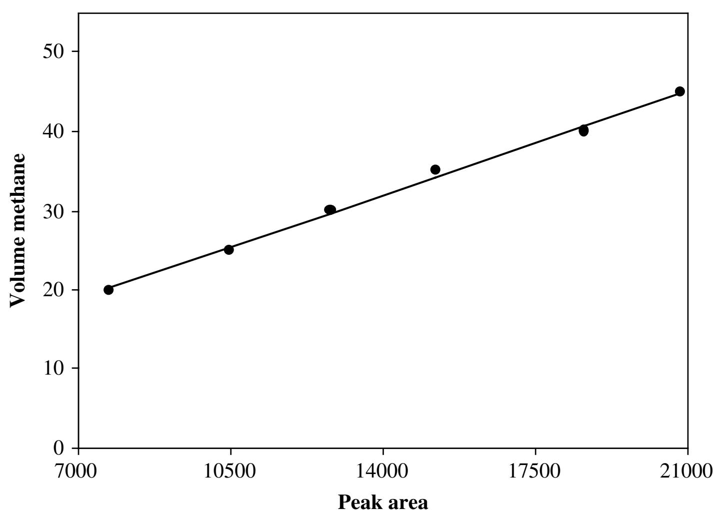

Figure 1. Example of a standard curve for methane

Then, 0.4 mL of a test sample was injected into the GC. The observed chromatogram (Fig. 2) and the peak area (Table 2) are presented below.

Using the regression equation, the amount of methane injected with 400 μL sample was $0.00188 \times 11347 + 5.64 = 27$ μL, thus the percentage of methane in the sample is $27 \times 100/400 = 6.75\%$.

If several gases are determined, the relative proportions of all of them in a mixture can be calculated, taking one of them (CO_2) as internal standard (or reference compound). In this case, a response factor for each component of the mixture is calculated as:

$$(1) \qquad RF_i = \left(\frac{CC_i}{Area_i}\right) \times \left(\frac{Area_{ref}}{CC_{ref}}\right)$$

where:

$\boldsymbol{RF_i}$ is the Response Factor for gas $\boldsymbol{i}$

$\boldsymbol{CC_i}$ is the proportion of gas $\boldsymbol{i}$ in the calibration sample (standard mixture)

$\mathbf{Area_i}$ is the area of gas $\boldsymbol{i}$ peak

$\boldsymbol{CC_{ref}}$ is the proportion of the reference gas (CO_2) in the calibration sample (standard mixture)

$\boldsymbol{Area_{ref}}$ is the area of the peak for the reference gas (CO_2)

From this RF, the proportion of a gas in a sample can be estimated as:

$$(2) \qquad Conc_i = \frac{RF_i \times Area_i}{\sum_{i=1}^{n} RF_i \times Area_i}$$

where:

$\boldsymbol{Conc_i}$ is the proportion of component $\boldsymbol{i}$ present in the analysis sample

$\boldsymbol{RF_i}$ is the Response Factor for gas $\boldsymbol{i}$ calculated in the calibration run

$\boldsymbol{Area_i}$ is the area of gas $\boldsymbol{i}$ peak measured in the analysis run

$\sum_{i=1}^{n} RF_i \times Area_i$ is the summation of all of the detector response corrected areas in the chromatogram, one for each gas component $\boldsymbol{I}$

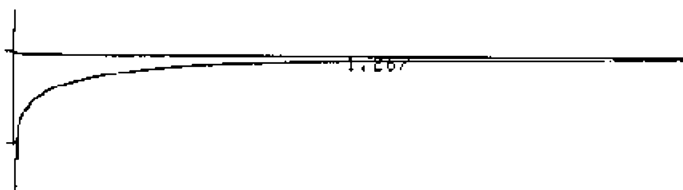

Figure 2. Chromatogram with the peak for methane after injecting 0.4 mL of a sample of fermentation gas (detector FID)

Table 2. Retention Time and Integrated Area of the Peak Depicted in Fig. 2

Gas	Retention time	Peak area
CH_4	1.267	11347

4.2. Example

A mixture of gases supplied as requested by the company Carburos Metálicos (Spain) is used as standard. The composition of the mixture is: 5% hydrogen, 35% nitrogen, 10% methane and 50% carbon dioxide. When a sample of this gas was injected using a TCD, the results obtained are given in Table 3.

The response factors for each gas in the last column were calculated using Eqn. 1.

Then, 1 mL of a test sample is injected. The observed chromatogram (Fig. 3) and the peak area (Table 4) are presented here.

In this example, $\sum_{i=1}^{n} (RF_i \times Area_i) = 21883.5$, and therefore the percentage of each gas in the sample is calculated using Eqn. 2.

Each sample is analysed in duplicate, calculating the average sample area. The results are acceptable when the peak areas for two consecutive injections agree within 5 percent of their average. If they do not agree, additional samples should be run until the area data obtained are consistent.

Table 3. Results of the Chromatogram Observed when a Sample of the Standard Gas Mixture is Analysed

Gas	Percentage	Retention time	Peak area	Response factor
H_2	5	0.94	14619	0.074
N_2	35	1.15	10197	0.744
CH_4	10	1.75	7698	0.282
$C0_2$	50	3.06	10835	1.000
Total	100		43348	

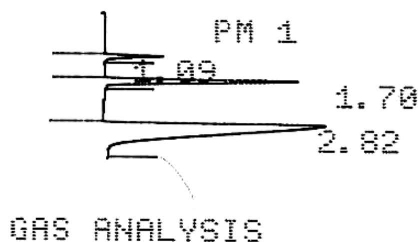

Figure 3. Chromatogram with the peak for methane (at retention time 1.70 min) after injecting 1 mL of a sample of fermentation gas (detector TCD)

Table 4. Retention Times, Peak Areas and Estimated Gas Percentages After Integrating the Peaks of the Chromatogram Depicted in Fig. 3

Gas	Retention time	Peak area	Response factors	Peak × RF	%
H_2		0	0.074	0.0	0.00
N_2	1.09	599	0.744	445.5	2.04
CH_4	1.70	5442	0.282	1532.0	7.00
$C0_2$	2.82	19906	1.000	19906.0	90.96
Total		25947		21883.5	100.0

Finally, the volume of methane (mL) produced at the end of the incubation can be calculated from the volume of gas and the gas composition data as:

$$CH_4 = [GP + HS] \times Conc$$

where:

CH₄ is the volume (mL) of methane

GP is the volume (mL) of total gas produced at the end of the incubation

HS is the volume (mL) of the headspace in the serum bottle or the gas space remaining between the fermentation vessel and the effluent flask in the RUSITEC (this is not gas produced, but the composition of this gas at end of the incubation is the same as that of the gas released, whereas at the beginning of the incubation no methane was present in this headspace)

Conc is the percentage or proportion of methane in the analysed sample

The approximate amount of methane produced (in mmol) can be calculated applying the ideal gas law:

$$pV = nRT \quad \left(n = \frac{pV}{RT}\right)$$

where ***n*** is the amount of gas (in mmol); ***p*** is the atmospheric pressure (in atm); ***V*** is the volume of gas (in mL); ***R*** is the gas constant ($0.082\,L \times \mathrm{atm}/[\mathrm{mol} \times {}^\circ\mathrm{K}]$); and T is the absolute temperature (°K). If $\boldsymbol{p} = 1$ atm and $\boldsymbol{T} = 273$°K, then one mol of any gas occupies 22.415 L of volume.

4.3. Stoichiometric Calculations of Methane Production

We have often found it useful to compare our measured values of methane production from both batch cultures and RUSITEC with methane production calculated based on a stoichiometric consideration of volatile fatty acid production. While the resultant values are not always suitable for publication they have provided us with reassurance that our measured values are biologically possible and that no undetected leaks or analytical errors are likely to have occurred. On occasion the stoichiometric considerations have also been useful in expanding our thinking of the pathways involved in the production of the precursors for methane synthesis (16, 17)

A number of stoichiometric models of ruminal volatile fatty acid (VFA) production have been published (see for instance Forbes and France, 1993) (10); however we have in general found those proposed by Demeyer and Van Nevel (6) to be simple and functional. In summary it is necessary to calculate the VFA production within the incubation of interest; in batch culture this would require an estimate to be made of the concentration of VFA at both the start and end of the incubation, while in RUSITEC the collection of the total daily effluent into a suitable preservative (acid or HgCl) can facilitate the measurement of VFA production in a single sample where production equals concentration times outflow volume. VFA concentration can be readily determined by either HPLC (7, 8) or GLC (2, 21, 23).

Considering the hydrogen transfer reactions in carbohydrate fermentation and assuming the absence of other hydrogen donors or acceptors, 2H released and accepted can be calculated from the amounts of acetate (A), propionate (P), butyrate (B) and methane (M) formed in a fermentation. Both VFA and methane production should be expressed on a molar basis. Electrons will be represented as reduced protons (H) and referred to as hydrogen (2H), 1 mol of 2H representing an electron pair. With these considerations, the equations suggested by Demeyer and Van Nevel (6) are:

$$2\text{H released} = 2\text{A} + \text{P} + 4\text{B}$$

while

$$2\text{H accepted} = 4\text{M} + 2\text{P} + 2\text{B}$$

The above are clearly simplistic as they make no attempt to account for transfer of H into other fermentation products or indeed microbial cell mass, although revised equations attempting to account for these variables have been published (4). However, the equations above are simple and readily applied. For example using some of our recent data we observed that addition of fumarate in batch cultures both decreased methane and altered VFA production (Table 5).

Using the equations above, 2H released in the control was

$$2 \times 789 + 259 + 4 \times 138 = 2389\,\mu\text{mol}$$

while 2H accepted was

$$4 \times 360 + 2 \times 259 + 2 \times 138 = 2234\,\mu\text{mol}$$

Table 5. Effect of Fumarate on Methane Production *In Vitro*

	Control	Fumarate
Methane produced (μmol)	360	342
Acetate produced (μmol)	789	881
Propionate produced (μmol)	259	471
Butyrate produced (μmol)	138	151

The percentage recovery of hydrogen was thus 93.5%. The equivalent values in the fumarate treatment were 2837 μmol 2H released, 2612 μmol 2H accepted, percentage recovery of hydrogen = 92%

Based on our experience hydrogen recoveries in both batch culture and RUSITEC tend to range between 80 and 110% (with some of the deviation from 100% being due to the inadequacy of the equations used to fully describe rumen stoichiometry), and we would suggest that values outside this range might lead the experimenter to consider the possibility of leaks (in the case of low recoveries) or analytical error (in case of higher values).

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CHAPTER 2

MEASURING METHANE EMISSION OF RUMINANTS BY *IN VITRO* AND *IN VIVO* TECHNIQUES

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INTRODUCTION

One of the problems our planet faces today is global warming due to the augmentation of the greenhouse effect which arises from accumulating gases that trap heat in the atmosphere. Methane is considered the second most problematic greenhouse gas emitted from anthropogenic sources (23) and its role in the destruction of the stratospheric ozone layer (17) is uncontested. Worldwide, approximately 81 Tg methane per year are emitted from the gastrointestinal tract of domestic ruminants and an additional 7 Tg of methane per year originate from the manure of these animals (7). Related to total anthropogenic methane emission, this means that domestic ruminants are responsible for 25% of total anthropogenic methane emission (9). Methane production in the rumen not only represents an ecological but also an economic problem. It was estimated that methane formation during fermentation represents a loss of 7–10% of the ruminant's gross energy intake (15), depending on the level of feed intake, diet composition and apparent digestibility of dietary energy. According to Johnson and Johnson (8), cattle can produce 250 to 500 L of methane per day.

There is still a great lack of quantitative data on the efficacy of mitigation strategies, particularly in tropical regions where a very high proportion of domestic ruminants are kept, due to the difficulties in measuring ruminal methanogenesis in conjunction with variations in feeding. Therefore, the purpose of the present chapter is to describe extensively different techniques suitable for quantitative measurement of methane emission from ruminants. These *in vitro* and *in vivo* techniques allow the estimation of the efficiency of strategies in mitigating ruminal methane formation.

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1. HOHENHEIM GAS TEST FOR MEASURING METHANE FORMATION

The Hohenheim gas test (HGT) apparatus to estimate the digestibility and the metabolizable energy content of ruminant feedstuffs was developed by Menke *et al.* (13) and the operation of the HGT system is described in detail by Menke and Steingass (14). Few modifications are necessary to enable analysis of the composition of the fermentation gases such as methane, hydrogen and carbon dioxide. These are described by Soliva *et al.* (18).

Hohenheim gas test equipment

a) Incubator with rotor (Fig. 1a) or a water bath (Fig. 1b) maintained at 39°C (±0.5°C). In the original method, Menke *et al.* (13) used an incubator; however, lately many workers have started using a water bath.
b) Incubator containing a polyvinylchloride rotor (Fig. 1a, 49 cm in diameter) with spaces for holding 55 to 60 HGT glass syringes, activated by a motor (about 1 round per minute). When the water bath is used for the incubation, a thick (6 mm) sheet with holes to hold the syringes is placed on the water bath (Fig. 1b).
c) Modified HGT glass syringes (Fig. 2a, b; 100 mL, graduated cylinder, with two inlets/outlets attached) to enable the analysis of fermentation gas composition (Häberle Labor technique, Lonsee-Ettlenschiess, Germany).
d) Elastic tube (about 5 cm in length per HGT glass syringe; pushed onto the inlet, Fig. 2a, No. 4, attached) fixed with a plastic clamp.

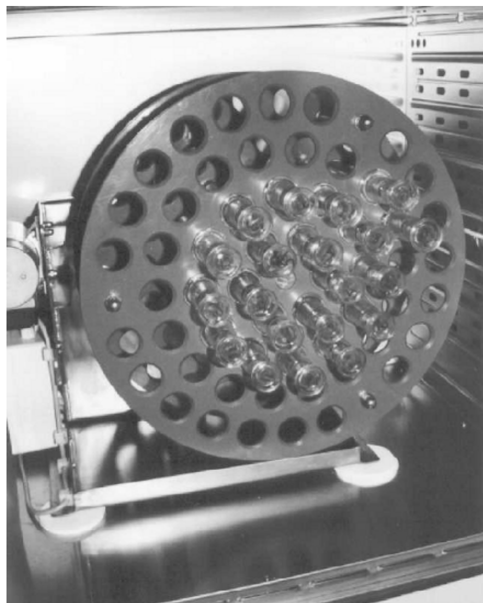


Figure 1a. Incubator with rotor

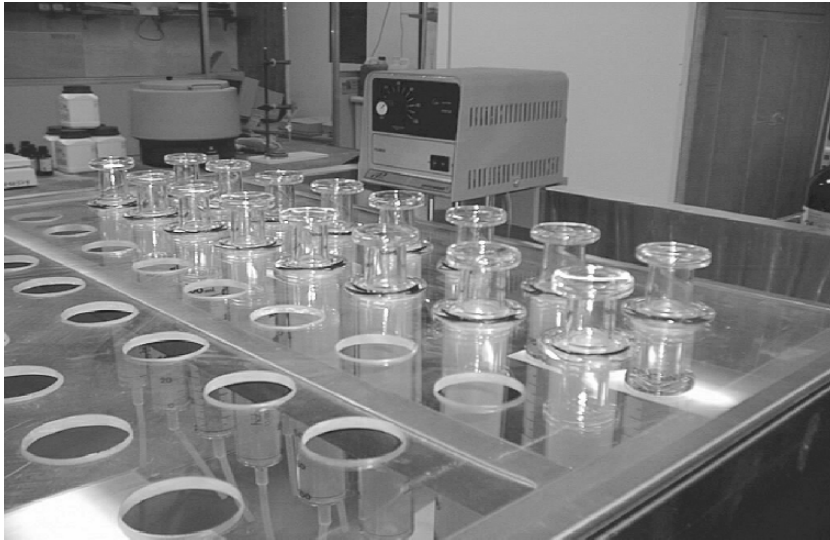


Figure 1b. Water Bath

e) Polytetrafluoroethylene-layered seals (screwed into the outlet attached, Fig. 2a, No. 1).
f) Filling equipment for rumen fluid (Fig. 3) consisting of:
   - Semi-automatic pipette or a dispenser (50 mL capacity)
   - Woulff flask (2 L capacity)
   - Thermostat with circulation pump
   - A glass bowl (10 L)
   - A magnetic stirrer

Reagents

1. Disodium hydrogen phosphate (Na_2HPO_4)
2. Potassium dihydrogen phosphate (KH_2PO_4)
3. Magnesium sulphate ($MgSO_4.7H_2O$)
4. Sodium hydrogen carbonate ($NaHCO_3$)
5. Ammonium hydrogen carbonate (NH_4HCO_3)
6. Calcium chloride ($CaCl_2.2H_2O$)
7. Manganese chloride ($MnCl_2.4H_2O$)
8. Cobalt chloride($CoCl_2.6H_2O$)
9. Iron chloride ($FeCl_3.6H_2O$)
10. Resazurin (0.1%)
11. Sodium hydroxide (1N NaOH)
12. Sodium sulphide ($Na_2S.H_2O$)

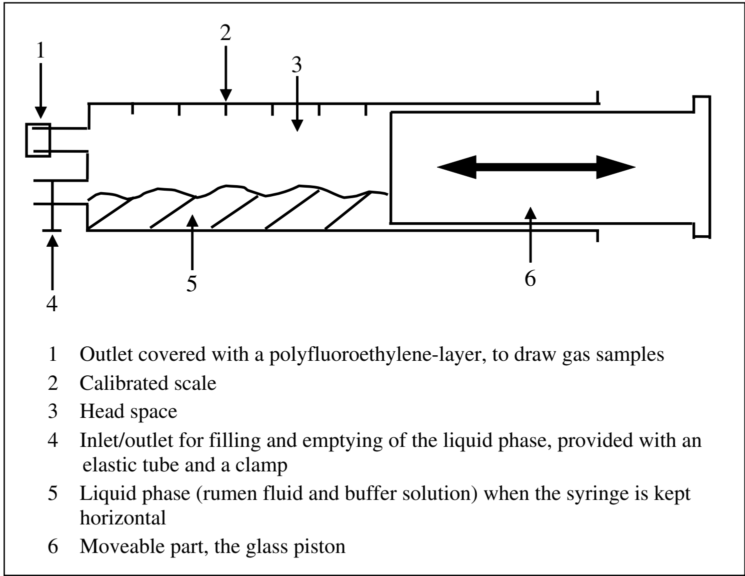


Figure 2a. Schematic illustration of the modified HGT glass syringe to analyse fermentation gases

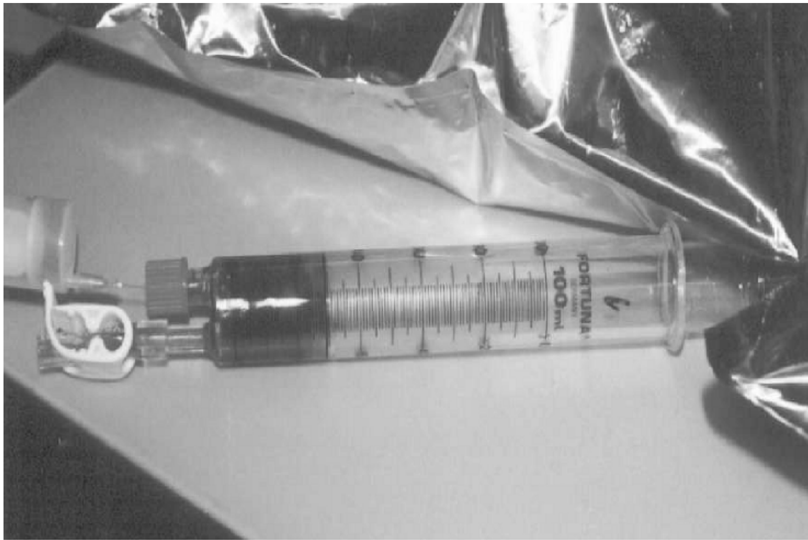


Figure 2b. Modified glass syringe

Figure 3. Assembled filling equipment for rumen fluid

Solutions

Main element solution	5.7 g – Na_2HPO_4
	6.2 g – KH_2PO_4
	0.6 g – $MgSO_4.7H_2O$
	make up to 1000 mL with distilled water (dH_2O)
Trace element solution	13.2 g – $CaCl_2.2H_2O$
	10.0 g – $MnCl_2.4H_2O$
	1.0 g – $CoCl_2.6H_2O$
	8.0 g – $FeCl_3.6H_2O$
	make up to 100 mL with dH_2O
Buffer solution	35 g – $NaHCO_3$
	4 g – NH_4HCO_3
	make up to 1000 mL with dH_2O
Resazurin solution	100 mg resazurin
	make up to 100 mL with dH_2O
Reducing solution	Add 285 mg of $Na_2S.7H_2O$ to 50 mL of 0.04 N NaOH
	(2 mL 1N NaOH + 48 mL dH_2O)

The reducing solution must be prepared fresh, just before use, but other solutions can be stored.

1.1. Preparation of the Buffer Medium

The solutions are mixed in a Woulff flask in the following proportions:
- 474 mL dH_2O
- 0.12 mL trace element solution

- 237 mL buffer solution
- 237 mL main element solution
- 1.22 mL resazurin solution
- 50 mL of reducing solution

The Woulff flask is placed in a container (e.g. a glass or plastic bowl) filled with water maintained at 39°C using a thermostat with a circulating pump. A magnetic stirrer is placed beneath the container and in alignment with the Woulff flask containing the medium and a magnetic bar. The medium is kept stirring using a magnetic stirrer. While adding the reducing solution gaseous carbon dioxide is flushed through the Woulff flask to replace oxygen and make the medium anaerobic. In addition, the purpose of carbon dioxide is to bring the pH to 6.8 from 8.4 and to saturate the medium with carbon dioxide so that all the fermentative and buffering carbon dioxide is quantitatively released. Because of the reducing solution the incubation solution turns to a reddish color and then becomes colorless. Rumen fluid is added only when the indicator (resazurin solution) changes to colorless.

1.2. Setting up the Glass Syringes

Before the buffer medium containing rumen fluid (the incubation medium) is filled into the HGT glass syringes, the syringes are prepared in the following manner. The feedstuff to be evaluated should be dried and milled to pass through a 1 mm screen. The feedstuff, between 200 mg (easily degradable feed material) and 300 mg (poorly degradable feed material) dry matter is weighed and placed into each HGT glass syringe. Since the HGT glass syringes have only a volume of 100 mL, the amount of feed added should not produce more than 70 mL of gas after 24 h of incubation. The movable part of each glass syringe, the piston, is greased with vaseline to avoid gas diffusion or the leak of incubation medium and also to prevent the piston from becoming stuck in the HGT glass syringe during the incubation.

1.3. Sampling and Preparation of Rumen Fluid

Rumen fluid is collected from rumen-fistulated animals before morning feeding, since at this time rumen fluid is more constant in its microbial composition and activity. It is advisable to take rumen fluid from two donor animals if available, which would provide even greater homogeneity and reproducibility. Rumen fluid should be kept anaerobic and warm during the period of transport to the laboratory. About 600 mL rumen fluid is needed to set up 55 HGT glass syringes.

Before adding the rumen fluid to the buffer medium, which still is under gaseous carbon dioxide flow, it is strained through three layers of gauze (1000 μm pore size, Type 17, MedPro Novamed AG, Flawil, Switzerland) and 474 mL of rumen fluid is added to the buffer medium (rumen fluid is added in a proportion of 1:2, v/v). Keep flushing the solution with carbon dioxide for 10–15 min before starting

to fill up the syringes. Through the inlet of the HGT glass syringe (Fig. 2, No. 4) 30 mL incubation medium is then dispensed into the pre-heated HGT glass syringe (39°C) with the help of a semi-automatic pipette or a dispenser. Care is to be taken to avoid the inclusions of air bubbles in the HGT glass syringe. This is achieved by keeping the HGT glass syringe in upright position and pushing the piston until all the air is removed from the syringe. Then the clamp on the tube is closed. The elastic tube (Tygon R3603, Type E, 6.4 mm ID, Ismatec SA, Glattbrugg-Zurich, Switzerland) fitted on the inlet of syringes consisted of a material with an extremely low gas diffusion rate, as carbon dioxide is known to diffuse through most rubbers and rubber-like materials. The exact volume (V_0) of the incubation medium is read from the calibration scale. The HGT glass syringes are placed in the rotor or water bath and incubated for 24 h at 39°C.

In our laboratory the donor animals are fed a diet consisting of 60% forage and 40% concentrate to ensure that the microbial population is composed of fibrolytic as well as amylolytic and proteolytic bacteria.

2. DATA COLLECTION

Three HGT glass syringes containing just the incubation medium and no feed are included in each test. The mean gas production in these glass syringes is the blank value (GP_0). It indicates the amount of gas volume originating from the fermentation of the residual feed in the rumen fluid, and is subtracted from the gas volume recorded for test samples to obtain the net gas produced.

The gas production is read after 4, 8, 12 and 24 h after starting the incubation. After 24 h, incubation is stopped by decanting the liquid phase from each glass syringe through the outlet (Fig. 2, No. 4), making sure that the gaseous phase is still completely present in the HGT glass syringe. Afterwards the remaining gaseous phase can be analysed for the concentration of methane, hydrogen and carbon dioxide by gas chromatography (GC).

2.1. Gas Chromatography Apparatus and Conditions Used in Our Laboratory

GC model	e.g. 5890 Series II (Hewlett Packard, Avondale, PA, USA)
GC column	Carboxen-1000, 15’ (4.5 m) × 1/8” SS (2.1 mm ID), mesh size 60/80, (Fluka Chemie AG, Buchs, Switzerland)
Gases analysed	Methane, hydrogen, carbon dioxide (Fig. 4)
GC Detector A	TCD (Thermal Conductivity Detector)
GC Detector B	FID (Flame Ionization Detector)
Initial temp. A	80°C
Detection temp. A	250°C
Initial temp. B	230°C
Detection temp. B	250°C

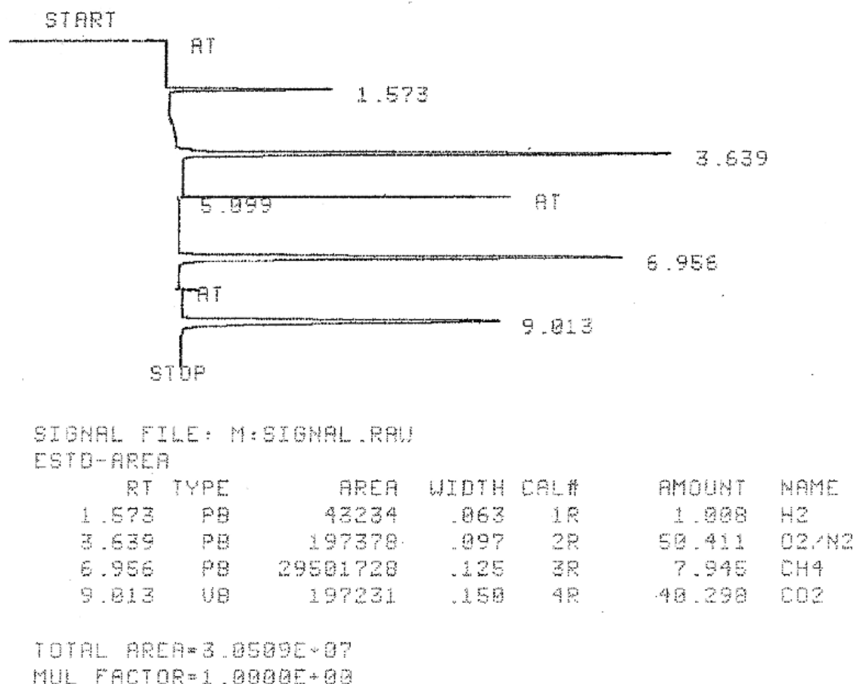


Figure 4. Gas detection profile of hydrogen (H_2), oxygen/nitrogen (O_2/N_2), methane (CH_4) and carbon dioxide (CO_2)

Carrier Gas	Argon (28 mL/min)
Injection quantity	150 μL of sample gas
Injection tool	Gas-tight syringe, 250 μL, 1725 RN (Hamilton AG, Bonaduz, Switzerland)
Operation time	15 min (cooling time included)
RT (Fig. 4)	Individual retention time of the specific gases
Calibration gas	H_2 1%
	O_2 1%
	CH_4 8%
	CO_2 40%
	N_2 50%
	(PanGas, Dagmersellen, Switzerland)

Gas samples can be taken with the help of a gas-tight Hamilton syringe (Fig. 5, 1725 RN, Hamilton AG, Bonaduz, Switzerland) after the liquid phase has been removed from the HGT glass syringe after the incubation. Therefore exchangeable needles (Hamilton needle RN (22s/51/5) L, Fisher Scientific, Wohlen, Switzerland) with rounded tops and an opening at the side of the needle are used.

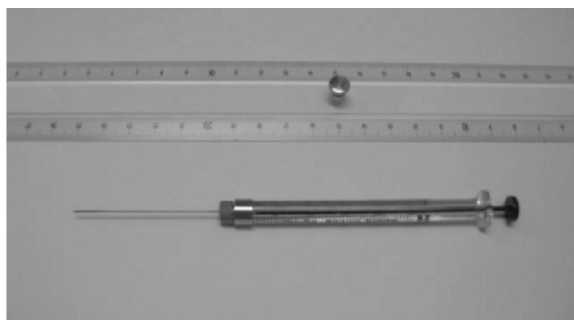


Figure 5. Gas-tight Hamilton syringe

Gas samples are taken through the polytetrafluoroethylene-layered seals screwed on the second outlet attached on the modified HGT glass syringe. A 150 μL portion of the gas is drawn from the HGT glass syringe and injected into the gas chromatograph. The amount of methane, hydrogen and carbon dioxide produced can be calculated from the volume of the gas produced in the HGT glass syringe, after correcting the values found with the blanks (GP_0). If it is not possible to measure the gas composition on the same day the liquid is drained off as mentioned earlier and the syringes can be stored at room temperature. Make sure that the syringes are gas tight (that there is still enough grease on the piston) and that there is no incubation medium left in them.

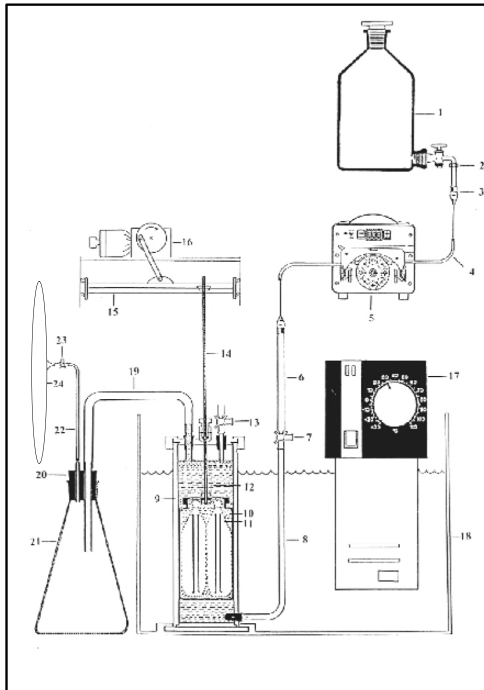
2.2. Measurement of Other Rumen Fluid Properties

Incubation medium is analysed for pH, ammonia, and redox potential with the respective electrodes connected to a pH meter (model 713, Metrohm, Herisau, Switzerland). For the determination of the volatile fatty acids 1.8 mL portion of the rumen fluid samples are stabilized with 0.2 mL of a 46 mM $HgCl_2$-solution and frozen until analysis by gas chromatography (GC Star 3400 CX, Varian, Sugarland, TX, USA) as outlined by Tangermann and Nagengast (20). Counts of ciliate protozoa (entodiniomorphs and holotrichs) and bacteria are obtained daily with Bürker counting chambers (0.1 mm and 0.02 mm depth, respectively; Blau Brand®, Wertheim, Germany).

2.3. Rumen Simulation Technique for Measuring Methane Formation

The rumen simulation technique (RUSITEC, Fig. 6) was developed by Czerkawski and Breckenridge (5) to maintain a normal ruminal microbial community under strictly controlled conditions over an extended period of time. A slightly modified version of the RUSITEC system is described in Machmüller *et al.* (10, 11).

RUSITEC equipment



1	Vessel for artificial saliva (5 L capacity)
2, 3, 4	Connection between vessel and pump
5	Pump for artificial saliva
6, 7, 8	Connection between pump and fermenter, inlet of the artificial saliva
9	Fermenter, gas-tight (1 L capacity)
10	Perforated feed container
11	Nylon bags
12	Incubation mixture (rumen fluid/buffer solution)
13	Sampling three-way valve
14	Driving shaft (stainless steel)
15	Bearings
16	Electric motor
17	Thermostat
18	Water bath (kept at 39°C)
19	Outlet of effluent trough overflow
20	Seal
21	Collection flask for effluent
22	Connection to gas-collection bag
23	Valve
24	Gas–collection bag (8 L capacity)

Figure 6. Schematic illustration of the RUSITEC system

2.4. Rusitec Equipment

Reagents

1. Sodium hydrogen carbonate ($NaHCO_3$)
2. Di-sodium hydrogen phosphate ($Na_2HPO_4.2H_2O$)
3. Sodium chloride (NaCl)
4. Potassium chloride (KCl)
5. Calcium chloride ($CaCl_2.2H_2O$)
6. Magnesium chloride ($MgCl_2.6H_2O$)

2.5. Buffer Solution (McDougall Buffer)

Solution A 49.00 g – $NaHCO_3$
23.38 g – $Na_2HPO_4.2H_2O$
4950 mL dH_2O
Solution B 47.0 g – NaCl
57.0 g – KCl

5.3 g – $CaCl_2.2H_2O$
12.8 g – $MgCl_2.6H_2O$
make up to 1000 mL with dH_2O

The buffer solution consists of solution A (4950 mL, freshly prepared daily) and 50 mL of solution B which can be prepared in advance and stored in a cool place for months. The addition of solution B to solution A is done with the help of a titration pipette (in drops) while the solution is stirred continuously with a magnetic stirrer.

2.6. Sampling of Rumen Fluid

Rumen fluid is collected from at least one (preferably two) rumen-fistulated animal (note: sheep may yield too little rumen fluid). Generally, we feed the donor animal with a diet consisting of 60% hay and 40% concentrate to make sure that the microbial population is composed of fibrolytic as well as amylolytic and proteolytic bacteria. Rumen fluid should be kept anaerobic and warm during the period of transport to the laboratory. About 8000 mL rumen fluid is needed to set up an eight fermenter RUSITEC system. Prior to the incubation, rumen fluid is strained through four layers of gauze (1000 μm pore size, Type 17, MedPro Novamed AG, Flawil, Switzerland).

2.7. Setting up and Handling of the Fermenters

Each fermenter is filled with 900 mL of strained rumen fluid and 100 mL McDougall buffer (12) achieving a final pH of the incubation mixture between 6.9 to 7.1, which has to be controlled. The fermenters are placed in a water bath maintained at 39°C. On the first day of each experimental run two nylon bags, one filled with solid rumen content and the other filled with one of the respective treatment diets, are put in a perforated feed container (see Fig. 6., No. 10) and placed in the respective fermenter. The nylon bags (70 mm × 140 mm) have a pore size of 100 μm as recommended by Carro *et al.* (4).

Using a shaft fixed to the feed containers on one end and to an electric motor on the other end, feed containers are moved up and down in the fermenters eight times a minute. After 24 h of incubation, the nylon bag containing solid rumen content is replaced with a nylon bag containing the fermenter-specific treatment diet. Each feed bag is therefore incubated for 48 h. To immediately re-establish anaerobic conditions in the gaseous phase of the fermenters, gaseous nitrogen or carbon dioxide is flushed through the incubation units for 3 min (3 L/min), each time after closing the system. Before opening the system the incubation units are flushed again with gaseous nitrogen for 30 s to ensure the collection of the whole amount of fermentation gases in gas-tight bags (TECOBAG 8 L, PETP/AL/PE: 12/12/75 quality, Tesseraux

Container GmbH, Bürstadt, Germany) which is especially important when the amount of methane or hydrogen is to be determined.

During the experimental runs, artificial saliva is infused continuously into every fermenter at an average buffer flow rate of 500 mL per day. To guarantee a constant buffer flow an electronic pump and small tubes (Tygon LFL, 0.48 mm ID, Ismatec SA, Glattbrugg-Zurich, Switzerland) are used. Since buffer solution is flowing constantly into the fermenters, liquid effluent is automatically transferred through an overflow tube into the respective collection flasks. The nylon bags, after removal from the fermenters, should be rinsed under running water to wash away the microbes attached to the feed particles until the outflow from the bags is clear. An alternative procedure, which can be better standardized, is washing the bags gently in a washing machine. The washed nylon bags should be frozen and lyophilized (Lyophilisator Beta 1–16, Christ®, Osterode, Germany) for later analyses.

In the original method (5), fermentation activities in the effluent were stopped by adding mercury solution to the effluent collecting flasks, which results in environmentally hazardous waste. A modified method (11) can also be used for stopping the fermentation activity. In this method flasks containing the incubation effluents are immediately chilled to −20°C using a freezing machine (Werner Kuster AG, Zurich, Switzerland). The flask (one per fermenter) is embedded into an aluminium block insulated with expandable polyethylene. To accelerate the freezing process, a small amount of coolant (Nigrin, Tegro AG, Schwerzenbach, Switzerland) with a freezing point of −40°C can be poured between the aluminium block and the flasks.

2.8. Measurement of Methane Produced

The gases produced during 24 h of incubation are collected quantitatively since all gases are transferred into the gas-tight bags (8 L volume) upon flushing the system with gaseous nitrogen for 30 s before the system is opened. The fermentation gases can be analysed for the concentrations of methane, hydrogen and carbon dioxide, e.g. by a Hewlett Packard gas chromatograph (model 5890 Series II, Avondale PA, USA) equipped with a TCD and FID detector and a Carboxen-1000 column (mesh size 60/80, Fluka Chemie AG, Buchs, Switzerland) as described earlier for the HGT system. The volume of the fermentation gases produced is quantified by water displacement (Fig. 7). Total amount of methane can be determined by multiplying its concentration with the total amount of fermentation gases. Although solubility of carbon dioxide is very high in water (0.76 mL/mL water at 25°C) a loss of carbon dioxide due to its solubility and, consequently, the underestimation of fermentation gas volume is not to be expected when following the recommended procedure. In this procedure, the vessel water is not exchanged during the whole period of the experiment and therefore gets saturated with carbon dioxide after the first 3–4 days of each experimental run. The data from these 4 days are not considered in the statistical evaluation since the microbes in the RUSITEC system need 4 to 5 days of adaptation. The water in the vessel can also be saturated by flushing with

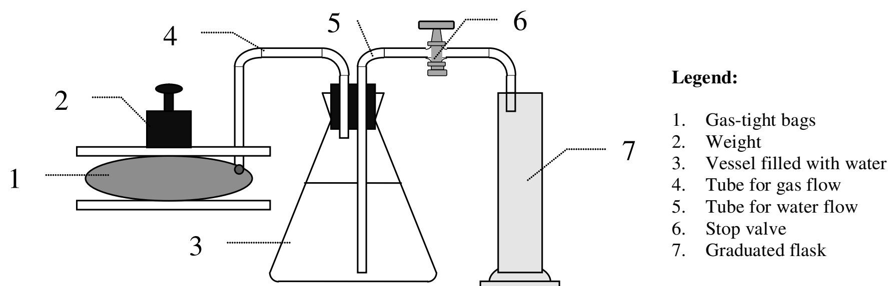


Figure 7. Schematic illustration of water displacement

carbon dioxide. Another option would be to apply an entirely different approach by measuring the gas volume with a gas flow meter.

2.9. Measurement of Other Rumen Fluid Properties

Incubation medium is analysed for pH, ammonia, and redox potential (to control the anaerobic conditions) daily, with the respective electrodes connected to a pH meter (model 713, Metrohm, Herisau, Switzerland). The volatile fatty acids determination and counts of ciliate protozoa (entodiniomorphs and holotrichs) and bacteria are obtained as described earlier for the HGT.

Dietary ingredients and fermentation residues are lyophilized and analysed for dry matter, total ash, ether extract, and N (Dumas method; Leco-Analyser Type FP-2000, Leco Corporation, St. Joseph, MI, USA), following standard procedures (2). Crude protein is calculated as $6.25 \times N$. The neutral detergent fiber (NDF) is analysed after incubation with α-amylase (Termamyl 120L, Type S, Novo Nodirsk A/S, Bagsværd, Denmark), but without sodium sulphite, according to the protocol of Naumann and Bassler (1997) (16). Non-NDF carbohydrates are defined as the organic matter other than ether extract, crude protein, and NDF. Hydrogen balance can be calculated by the equation of Demeyer (6) considering the VFA acetate (A), propionate (P), butyrate (B), valerate (V) and iso-valerate (iV), and methane (M). Thereby hydrogen recovery (%) is calculated as $2H_u/2H_p \times 100$, H_u representing hydrogen utilized and H_p representing hydrogen produced, with $2H_u = 2P + 2B + 4M + V$ and $2H_p = 2A + P + 4B + 2iV + 2V$.

2.10. Open-Circuit Respiratory Chambers for Measuring Methane Emission

Methane release from ruminants can be measured continuously with a dual chamber as elements of an open-circuit indirect respiration calorimetric system (Fig. 8). In the open-circuit respiratory chamber, outside air is supplied to the chamber continuously and chamber air is removed.

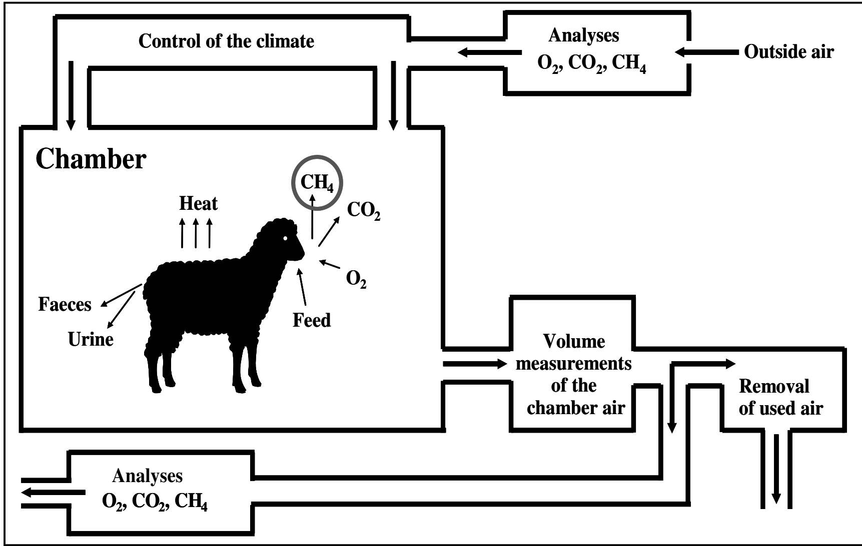


Figure 8. Open-circuit indirect respiration calorimetry

2.11. Respiratory Chamber

The respiratory chambers, in our case with volumes of 5.44 m^3 for sheep, are constructed using aluminium and glass, allowing sight contact between the sheep in the adjoining chamber compartment. The chambers are air-conditioned with an average ambient temperature of 18°C, a relative humidity of 64% and an air flow of 8.3 m^3 h^{-1}. In-line electronic flow meters (Type 8GD-LRM, Fluid Inventor AB, Stockholm, Sweden) continuously record the air volume leaving the chambers.

Before experiments with the respiratory chamber, the functional efficiency of the chamber is conducted and the instruments for gas analyses are tested. Therefore simulation measurements are conducted by burning propane gas and evaporating water (21, 22). The average recovery rates for oxygen, and carbon dioxide, and the respiratory quotient for the chambers are evaluated. A schematic diagram of the airflows and electronic control equipment of the respiratory chamber is provided (Fig. 9).

TK_x	Chamber for the Animals
LU	Air agitation in the chamber
F_1–F_3	Air filter
SW	'Swing whirl', air flow measurement instrument (14–40 m^3)
H	Heating
I	Fresh air channel
I_S	Branching for collecting gas samples for the analysis of the outlet air
K	Cooling aggregate

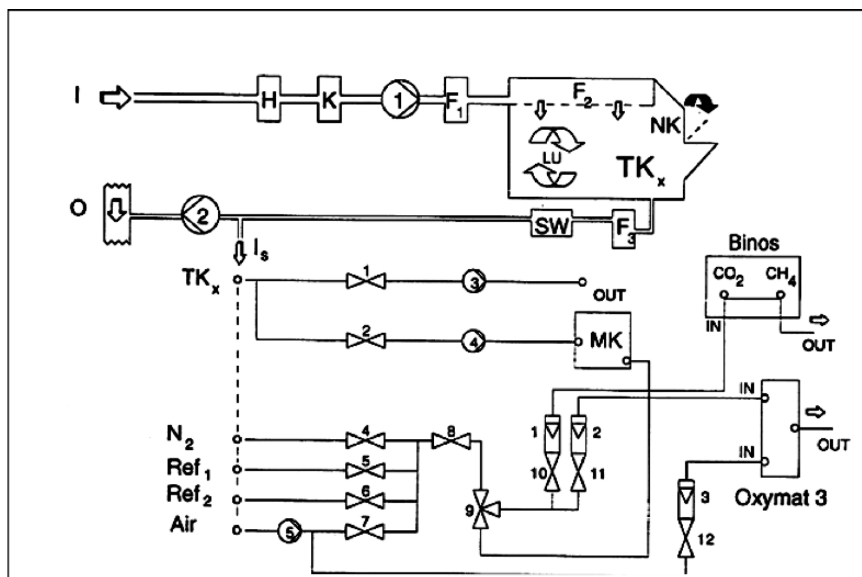


Figure 9. Schematic illustration of the airflows and electronic control equipment of the respiratory chamber (19)

O	Outlet air channel
MK	Condenser for measurement gas (Typ PC 14 Leybold–Heraeus AG)
N_2	Calibration gas inlet: Nitrogen for the calibration of the instruments for gas analyses
Ref_1	Calibration gas inlet 1: reference gas for the calibration of the instruments for gas analyses
Ref_2	Calibration gas inlet 2: reference gas for the calibration of the instruments for gas analyses
Air	Fresh air channel: (Calibration and for permanent reference gas Oxymat 3)
NK	Emergency shutter, electronically regulated, opens when CO_2-concentration > 1.95%
(1)	Fresh air ventilator
(2)	Outlet air ventilator
(3)	Membrane pump for suction cleaning of the next control point
(4)	Membrane pump for measurement gas
(5)	Membrane pump for reference gas Oxymat
⋈	1–12, Electronically magnetic valve
▽	1–3, Flow rate display

Table 1. Gases Used for the Calibration of CH_4, CO_2, O_2 and NH_3

Calibration of	Lower calibration point	Upper calibration point
CO_2 and CH_4	N_2	0.1007% CH_4, 1.06% CO_2, 19.56% O_2
O_2	0.1007% CH_4, 1.06% CO_2, 19.56% O_2 (outside air)	20.628% O_2
NH_3	N_2	7.98 ppm NH_3

2.12. Measurement of Chamber Gases

The concentration of oxygen is measured either with an Oxymat 3 (Siemens-Albis AG, Dietikon-Fahrweid, Switzerland) or an Oxymat 6 (Siemens AG, Karlsruhe, Germany) system. Carbon dioxide and methane are measured with a Binos 1001 (Fisher-Rosemount, Baar-Walterswil, Switzerland). Ammonia concentration from manure is measured with an NO-NO2-NOy-Analyser (Model 42 C, Thermo Environmental Instruments Inc, Franklin, USA).

Prior to each measurement period, the detectors are calibrated manually with standard gases (Table 1). During the measurement periods, within an interval of 90 min, one automatic calibration and four measurements of the gas concentrations in the air flowing into the chambers and 24 measurements of the gas concentrations in the outgoing air from each chamber are performed.

3. CALCULATIONS

For the calculation of methane energy and energy expenditure (heat) the equation of Brouwer (3) is used: Energy expenditure (kJ) = 16.18 $\times$ O_2-consumption (l) + 5.02 $\times$ CO_2-emission (l)–2.17 $\times$ CH_4-emission (l)–5.99 $\times$ Urine-nitrogen (*g*)

Energy retention is calculated as metabolizable energy (ME) minus energy expenditure. Retained protein (intake less excreted with faeces and urine) is assumed to contain 23.8 kJ g^{-1} for calculating energy retention in protein ($RE_{Protein}$). Energy retention in fat is derived as: ME less energy expenditure less $RE_{Protein}$. The efficiency of utilization of ME for maintenance (k_m) is estimated using the AFRC (1) equation. For calculation of the efficiency of utilization of ME for growth (k_f), the individual energy requirements for maintenance are estimated considering fasting metabolism and activity assumptions for housed fattening lambs and wethers, respectively (1).

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CHAPTER 3

THE SF_6 TRACER TECHNIQUE: METHANE MEASUREMENT FROM RUMINANTS

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OVERVIEW

The purpose of this document is to describe in detail the sulfur hexafluoride (SF_6) tracer technique. Included within are instructions to construct and use the equipment needed to make accurate measurements of methane emissions from ruminants. However, as with any technique, improvements and modifications are needed and will occur with continued use. As long as the basic principles behind this technique are followed, modifications to improve ease of use will not compromise measurements. The list of sources and part numbers are included only to help users understand what is needed and not to imply that other sources are not acceptable. The authors of this document are very interested to hear of the successes, problems and solutions that users encounter with the technique.

This document is divided into four sections:

Section 1: This section is intended to introduce the reader to the SF_6 technique and to give background information as to its development.

Section 2: Specific instructions regarding construction and assembly of the different parts needed to measure methane are included in Section 2.

Section 3: An example sampling sequence, a calculations summary and a Quality Assurance document are included in this section.

Section 4: Appendices include a troubleshooting guide, assembly directions, parts list and the steps for preparing an SF_6 permeation tube.

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1. INTRODUCTION

Methane (CH_4) is a greenhouse gas whose atmospheric concentration has increased dramatically over the last century. Next to carbon dioxide, methane is the largest potential contributor to warming of the earth. The increase in tropospheric methane level correlates closely with global expansion in the human population. Consequently, it is believed that approximately 70% of methane emissions are associated with human activities. Landfilling of waste, fossil fuel usage and agricultural practices generate and release methane to the atmosphere in amounts that will increase as the number of people in the world grows. Methane released to the atmosphere by domestic ruminant livestock is considered to be one of the three largest sources on a global scale (1).

The emission of methane by cattle and sheep represents a carbon loss pathway that results in reduced productivity. If the energy that is lost in generating methane could be rechanneled into weight gain or milk production, it would be cost effective to the producer as well as provide a means of reducing methane emissions to the atmosphere. Past studies with ruminant animals have shown that methane production is dependent on the quantity and quality of the diet. Generally, highly digestible feeds yield lower methane emissions when compared to poor quality diets. Thus, with feedlot cattle on high grain diets, about 2% of their energy intake is converted to methane, while as high as 12% of the gross energy intake is lost as methane from cattle on low quality roughage diets. Dietary manipulation may provide a mechanism for reducing methane emissions from domestic livestock.

To develop strategies to mitigate livestock methane emissions, it must be possible to quantify cattle emissions under a wide range of circumstances. Many techniques exist to quantify methane emissions from individual or groups of animals. Enclosure techniques (e.g. respiration calorimetry) are precise but require trained animals, reduce animal movement and have a high labor input and expense, which may limit the number of animals that can be measured. Prediction equations based on fermentation balance of feed characteristics have been used to estimate methane production. These equations are useful, but the assumptions and conditions that must be met for each equation limit their ability to accurately predict methane production, particularly under production conditions. Isotopic tracer techniques have also been developed and are useful under controlled conditions, but are of limited usefulness under production situations. A measurement technique that makes use of an inert tracer gas (SF_6) has been developed for determining methane emission rates from ruminants under production conditions.

The sulfur hexafluoride tracer technique method was developed to circumvent the limitations of the other techniques. In this technique, a small permeation tube containing sulfur hexafluoride (SF_6) is inserted into the rumen. The release rate of the SF_6 from the permeation tube is known prior to placement in the animal. A halter fitted with a capillary tube is placed on the animal's head and connected to an evacuated sampling canister (Fig. 1). A sampling valve is opened and as

Figure 1. Cow outfitted with halter and collection canister

the vacuum in the sampling canister slowly dissipates, a sample of the air around the mouth and nose of the animal is collected at a constant rate. The collection vessel is allowed to fill to a pressure of about 0.5 atm. By varying the length or diameter of the capillary tube, the duration of sampling may be regulated. After collecting a sample, the canister is pressurized with nitrogen to slightly more than one atmosphere. Methane and SF_6 concentrations are then determined by gas chromatography. Methane emission rate is calculated as the product of the permeation tube emission rate and the ratio of CH_4 to SF_6 concentration in the sample. This technique eliminates the necessity to restrain or enclose the animal, thus allowing the animal to move about and graze. Also it is not necessary to sample directly from the animal's rumen or throat because the use of the tracer accounts for changes in dilution associated with head or air movement. Since the majority of methane is eructated and respired in ruminant animals, collection around the mouth and nose will result in accurate estimation of the methane production by the animal. Most hindgut methane is absorbed into the blood stream and respired; therefore the SF_6 technique will measure it, as well.

1.1. Validation

The key assumptions associated with the SF_6 technique were examined during the development of this procedure. For the tracer to be effective in measuring CH_4 emissions it had to meet several conditions: 1) the release rate of the permeation tube must be constant and predictable; 2) the tracer must have no impact on ruminal fermentation; 3) the tracer must be detectable in low concentrations; and, 4) the tracer must be inert and nontoxic. Sulfur hexafluoride meets these qualifications because it is an odorless, colorless gas used in pulmonary function tests and is detectable at 1 ppt. To test the assumption that the release rate of SF_6 from the permeation tube was constant and predictable, permeation tubes were prepared and then release rates of SF_6 were measured gravimetrically for several months. These tubes were then placed in 39°C water baths and continuous culture fermentors containing rumen microorganisms. Sulfur hexafluoride release rates were measured under both of these conditions and were found to be constant and predictable.

The next requirement was that SF_6 itself would not impact the rumen microorganisms and alter the fermentation process. There is ample evidence in the literature of the use of halocarbons as methane inhibitors. To examine the effects of SF_6 on ruminal fermentation, continuous culture fermentors containing ruminal microorganisms were used. After establishment of a stable population of bacteria and protozoa (determined by examining volatile fatty acid production, ruminal pH and microscopically) methane production measurements were taken over the course of two days. Following methane sampling, SF_6 tubes were placed in the fermentors and the cultures were allowed to stabilize for an additional three days. Daily examination of the cultures continued through this period as well. Methane production was then measured for an additional two days. There were no differences seen in volatile fatty acids, pH, methane production or bacteria and protozoa due to addition of the SF_6 permeation tube. Following this study, an *in vivo* examination of the impact of SF_6 addition to the rumen was conducted using two ruminally fistulated cows. Again there was no significant effect on volatile fatty acids or ruminal pH due to the addition of SF_6.

After concluding SF_6 had no impact on the ruminal fermentation, a series of studies were conducted to compare the estimates of methane production from the tracer technique to measurements made using indirect open-circuit respiration calorimetry. Methane emissions from eleven beef cattle fed various diets at different levels of intake were measured using both techniques. In most cases, the tracer technique was employed followed immediately by two days of methane measurements in the chambers. After the chamber measurements were complete, the animals were returned to the barn and further tracer measurements made. After 55 measurements using the tracer method and 25 chamber measurements, no significant difference could be detected between the two methods. The tracer technique

resulted in estimates of 11.53 ± 0.41 L/h, while the chamber measurements averaged 12.36 ± 0.33 L/h. Three other groups have also compared the tracer technique to calorimetric measurements and reached similar conclusions (Boadi *et al.* (2); O'Mara *et al.*, personal communication; Terada *et al.*, personal communication). Leuning *et al.* (3) in New Zealand compared the SF_6 tracer technique to a micrometeorological technique and concluded the SF_6 technique resulted in similar methane production estimates. Pinares-Patino (4) and Ulyatt *et al.* (5) compared the SF_6 technique to chamber techniques and did not find good agreement between the techniques. These results may be a function of the inability to account for recycling of the tracer when the animal rebreathes the air in the chamber.

2. MATERIALS AND METHODS

2.1. Permeation Tube

The permeation tube body is constructed from a 3.2 cm × 1.1 cm OD (outer diameter) brass rod. The SF_6 cavity is made by drilling a 4.77 mm hole to a depth of about 25.4 mm in one end of the rod. On the open end, the outside of the rod is threaded to allow attachment of a 6.35 mm Swagelok nut along with a thin Teflon window and a stainless steel frit (Fig. 2). The thickness and type of Teflon dictate the permeation rate. TFE Teflon of 12 mm thickness and a 2 micron frit will normally provide SF_6 permeation rates in the range of 1000–2000 ng/min at 39°C. The function of the frit is to stabilize and protect the Teflon membrane.

Note: We have found large variation in the thickness of TFE Teflon from purchase to purchase from the same supplier. Therefore we recommend that each batch of Teflon purchased needs to be tested prior to use.

When all the parts have been acquired, the brass body is cleaned thoroughly and the permeation tube is assembled. Each tube is given a number, which is stamped into the bottom of the permeation tube. The tare weight of the assembled tube is then determined to the nearest tenth of a microgram.

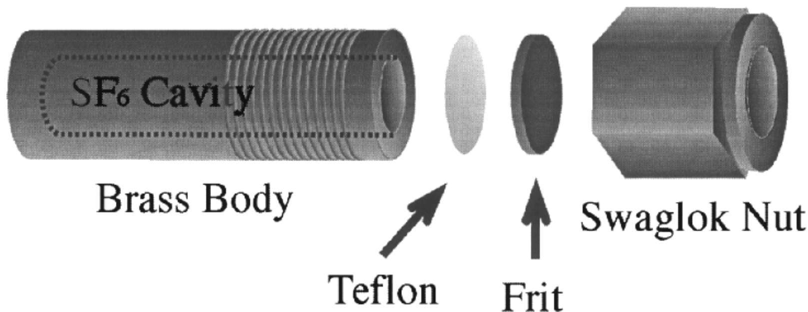


Figure 2. Schematic representation of a SF_6 permeation tube

To charge the tube with pure SF_6, the cap, Teflon window and frit are removed followed by immersion of the tube body in a cryogen such as liquid nitrogen. After the tube has reached the cryogen temperature, it is removed and any liquid is poured out of the cavity.

The cavity is then quickly filled with pure SF_6. The filling is accomplished by withdrawing about 60 cc of gaseous SF_6 in each of two plastic syringes fitted with wide bore needles (18 gauge). The needle is placed in the cavity of the cold permeation tube body and the plunger is slowly depressed to transfer the gaseous SF_6 into the permeation tube cavity. The tube is quickly capped with Teflon window, frit and Swagelok nut following completion of the SF_6 transfer. The permeation device is then weighed. This procedure should provide a tube containing about 600 mg of SF_6.

The tube should be placed in a glass receptacle, in a 39°C water bath. A small flow of clean N_2 gas is maintained to purge the glass receptacle of SF_6 emissions. Weights of each tube should be taken weekly to determine the release rate of SF_6. Five to six weeks is generally required to gain a good idea of the release rate.

Permeation tubes can be re-used after recovery from the animal. In most cases, a new Teflon membrane is all that is required for assembly of another permeation tube. The steps required to prepare a permeation tube are illustrated in Appendix A, Section 4.

Note: Sulfur hexafluoride is itself a greenhouse gas. Care should be taken to prevent release of large amounts of SF_6 *to the atmosphere. Permeation tubes should be made in a hood to allow ventilation of the* SF_6 *from the room and permeation tube construction and incubation should not be done where this* SF_6 *source could contaminate the gas chromatographs or sampling apparatus. A separate room with a different ventilation system should be used.*

2.1.1. Halter construction

The sampling apparatus consists of the collection canister (PVC yoke) and a modified halter. The size of the halter is a very important consideration as the location of the inlet over the nostrils is critical to the success of sampling. Generally, the best successes have been obtained with larger halters that have adjustable chinstraps. Punch new holes in the halter straps for smaller animals to ensure a snug fit to the noseband. Rivet or sew a leather flap to the halter noseband (Fig. 3) to provide support for the capillary tube inlet and filter.

2.2. Capillary Tubing

The length of the capillary tubing regulates the sampling rate. Stainless steel tubing with an inside diameter of 0.127 mm and an outside diameter of 1.59 mm serves as the flow restrictor and transfer line. Short sample integration periods (e.g. 1 h) may require only a short piece of tubing while longer periods (e.g. 24 h)

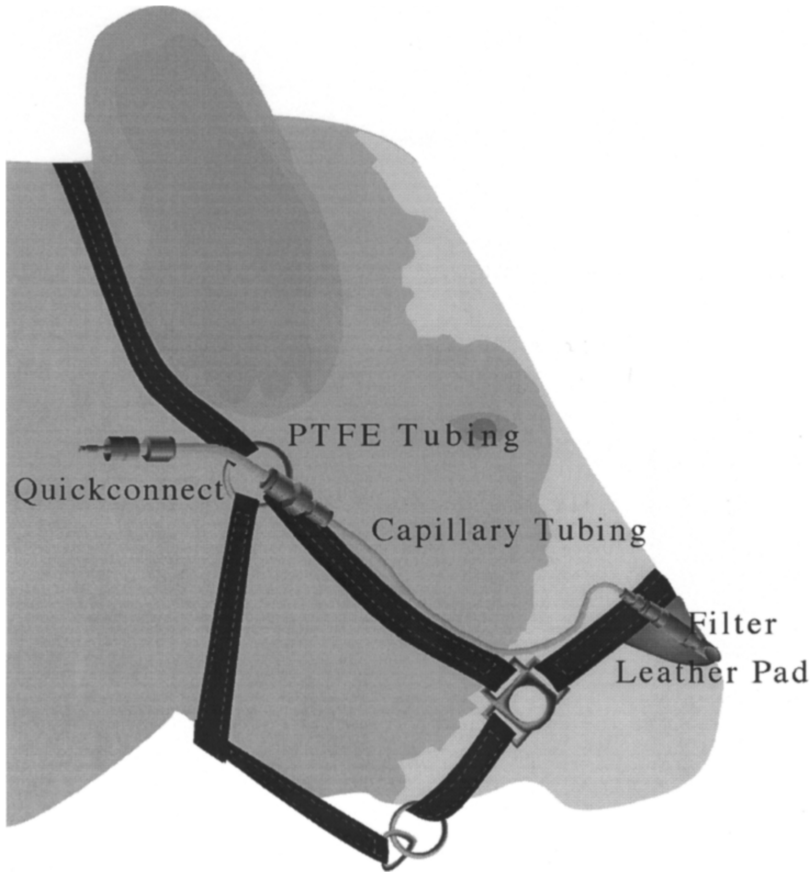


Figure 3. Schematic representation of sampling halter assembly

Parts required: 1 – Horse or Cow halter with adjustable chin strap; 1 – Piece of leather with rivets; 1 – 2.0 cm piece of Tygon tubing; 1 – 1.0 cm piece of 3.17 mm PTFE tubing; 1 – 50 micron filter; 1 – 3.17 mm to 1.59 mm reducing unions; Length of 1.59 mm (0.127 mm ID) stainless steel capillary tubing; Length of 3.17 mm PTFE tubing; 1 – Male Quickconnect; Assorted ferrules and Swagelok nuts; 3 – Velco straps (50 mm width); Teflon tape for fittings; Tape for halter; Strap ties

require much longer lengths of tubing. If short lengths of capillary tubing are used, then 1.59 mm × 0.76 mm stainless steel tubing may be employed to connect the remainder of the transfer line. It is worth the investment to purchase a tube cutter. Use of this tool makes certain the small opening of the tube remains open and the cut is smooth. To determine the sampling rate of a piece of capillary tubing, connect it to an evacuated yoke and allow it to fill for several hours while periodically checking the pressure. Calculate the fill rate and compare it to the desired length of

time. The canister should fill to approximately 1/2 atm during the desired collection time. Filling to 1/2 atm ensures the fill rate will be constant.

Note: Capillary tubing has been found to be very different both within and among different lots. It is necessary to check all fill rates prior to sampling.

After the appropriate length of capillary tubing has been selected and tested, attach a 50-micron filter to the upstream (nose) end of the capillary tube (Fig. 4). This will also require a 3.17 mm to 1.59 mm reducing union and appropriate Swagelok fittings. The purpose of the filter is to protect the capillary tubing from filling with dust and debris. The size of the filter is less important than its presence. To prevent water from clogging the filter and capillary tube, fit a short piece (1.0 cm) of 3.17 mm PTFE tubing on the inlet end of the filter using a set of ferrules and Swagelok nut, then slide a short piece of Tygon tubing (2.0 cm) over the PTFE tubing and onto the 7.95 mm nut. Attach the filter to the leather noseband so that the filter and tubing on the end will be located above the nostril of the animal. The halter should fit so the filter is on the hair of the cow's nose.

To the downstream end of the tubing, install another 3.17 mm to 1.59 mm reducing union and connect it to 3.17 mm PTFE tubing. To the other end of the 3.17 mm tubing, attach a male Quickconnect. The length of the 3.17 mm PTFE tubing is variable and depends on the length of the capillary tubing and the size of the cow. Check all fittings for fit and tightness. Run the capillary tubing up the side of the halter and tape into place. In the event that the capillary tubing is longer than the side of the halter, carefully coil it up and tape the coil into place.

To support the canister, three Velcro straps are constructed. One strap is used on each side of the canister and connects the canister to the halter sides. The final strap supports the connection from the halter to the canister (Fig. 3).

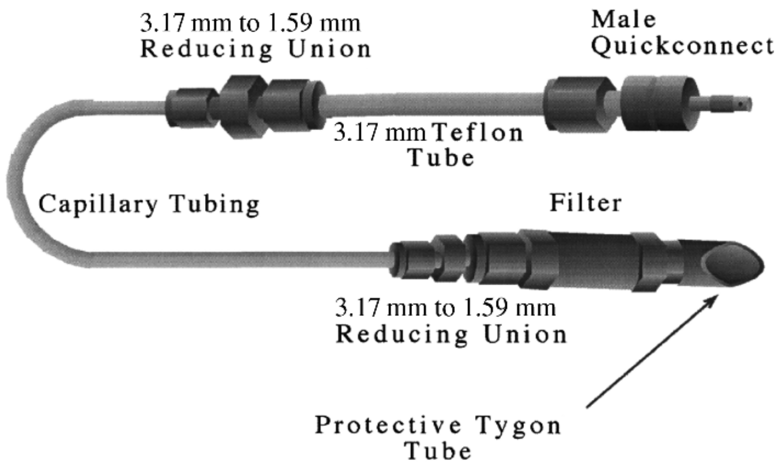


Figure 4. Capillary tubing assembly

More illustrations of the halter construction and attaching the capillary tubing are provided in Appendix B, Section 4.

2.2.1. Collection canister (PVC)

The sampling apparatus consists of a PVC canister that is formed to fit around a cow's neck and a modified cow halter. A PVC pipe of 5–6 cm ID (internal diameter) and 200 psi pressure rating is satisfactory for canister construction. PVC end caps and a 90° elbow are used to seal the sample container. To construct a canister (Fig. 5), cut two 51 cm pieces of PVC pipe and wash the inside and outside of the

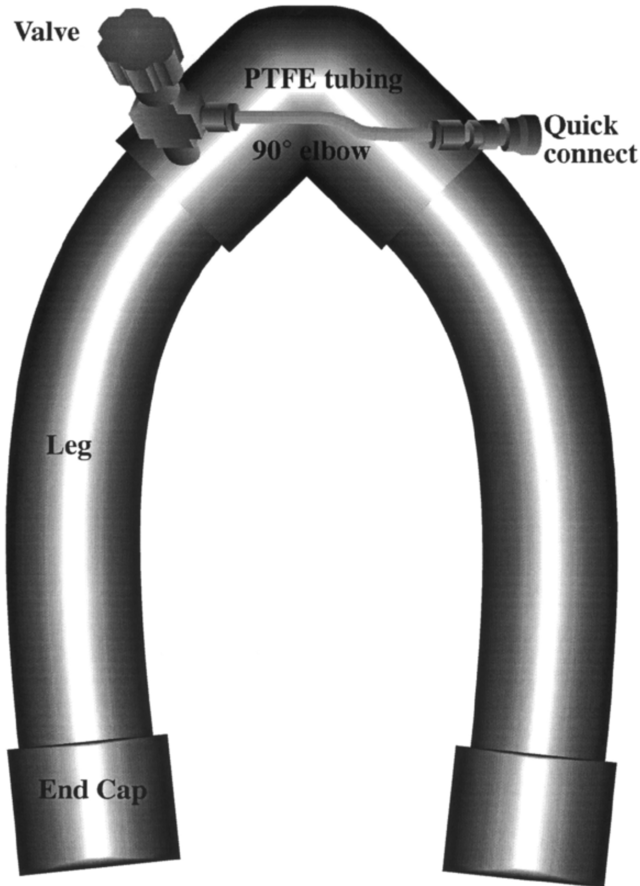


Figure 5. PVC Collector Canister (Yoke)

Parts required: 2 – 51 cm pieces of 5 – 6 cm, 200 psi PVC pipe; 2 – End caps (5 cm) (5 cm); 1 – 90° elbow (5 cm); 1 – can PVC primer; 1 – can PVC glue; Oven (120°C–135°C); 1 – Gas sampling valve; 1 – 100 mm Teflon tubing piece and Swagelok fittings (6.35 mm); 1 – Female Quickconnect; Packaging Tape; Compressed air or nitrogen.

pipe and fittings with warm soapy water and allow them to dry. Using appropriate PVC primer, prime the ends of the pipe and the inside of all fittings and allow them to dry. The priming step is essential for the glue to be effective. Glue the two pieces into the 90° elbow and the end caps to the open ends of the PVC pipe using PVC glue. Be very generous with the glue. After the glue has dried (12–24 h), place the entire assembly into a 120–135°C oven for 5 to 10 min. Keep checking the pipe by squeezing it until it is soft and pliable. Remove the pliable pipe and bend the legs into the desired position by applying pressure to the top while bowing the legs. When the desired shape is obtained, cool the canister with cool water applied by sponge or towels. The final dimensions of the canister depend on the size of the animal being sampled. For average sized cows, an 203 mm span between the ends of the pipe is adequate. If the canister does not have the desired shape, re-heat it and begin again. If the pipe collapses on itself, it is not hot enough. Illustrations of the steps involved in constructing the collection canister are provided in Appendix C, Section 4.

To install the gas-sampling valve, tap the canister's elbow with 6.35 mm pipe thread. Make the hole on one side and centered in the 90º elbow (Fig. 5). The valve will point to the cow's back when sampling. This location prevents the animal's poll from being hit by the valve. Install the gas-sampling valve by gently screwing it into the threaded hole. Take care to seat the valve gently and do not crack the PVC while inserting the valve. A short 10.1 cm piece of 6.35 mm Teflon tubing is attached to the valve with a female 6.35 mm. Quickconnect on the upstream end to allow attachment to the halter. Wrap the legs in tape for safety purposes against the possibility of implosion and shattering of PVC shards.

After assembly, the canister can be checked for leaks by pressurizing it with compressed air or nitrogen to 40 psi and then submerging it under water to watch for bubbles. Emphasize care in handling the canisters when training the staff. Most canisters are damaged beyond repair when they are dropped on the valve.

2.2.2. Dilution system and vacuum pump

A vacuum pump is required to evacuate the canisters, a pressure gauge is necessary to measure the pressure after filling with a gas sample, and a dilution system is necessary to pressurize the sample with nitrogen gas. Prior to sampling, a collection canister must be evacuated. Any vacuum pump capable of reducing the pressure in the canister to a few Torr is acceptable. After closing the valve on the canister, it is then ready to use. After the collection period has been completed, the canister is connected to the dilution system (Fig. 6) and the final pressure recorded. Nitrogen is then added slowly until the pressure in the canister is increased to about 1.2 atm. The exact pressure reading is recorded to calculate the dilution factor. With the contents of the canister under positive pressure, it is easy to transfer an aliquot of sample to the GC systems. The canister can be attached directly to the gas-sampling valve in the GC via Quickconnect fittings. Simply opening the canister valve will then allow sample transfer to the fixed volume loop on the GC (Fig. 7).

Diluting and pressurizing the canister after sampling

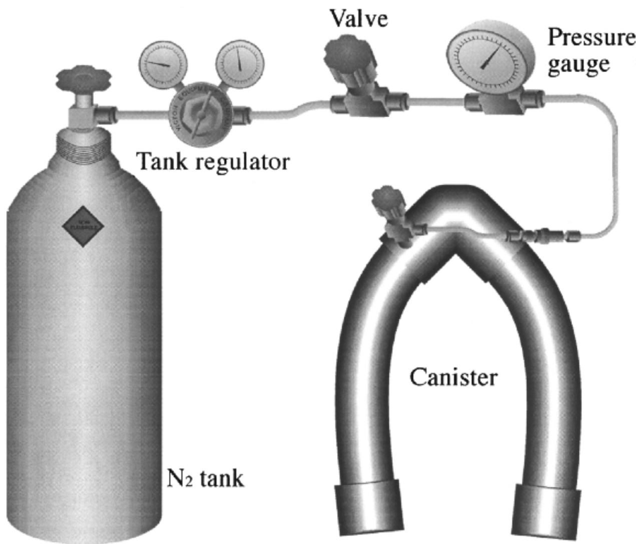


Figure 6. Dilution apparatus
Required Parts: 1 – Tank of pre-pure nitrogen; 1 – Tank regulator; 1 – Regulator valve; 1 – Pressure gauge; 1 – Length of copper tubing; 1 – Male Quickconnect; 1 – Vacuum pump.

3. GAS ANALYSIS

3.1. Methane Analysis

Methane concentration in the sampling canister is determined by gas chromatography (GC). The GC system consists of a 1.0 cc sample loop attached to a low dead volume gas sampling valve, a 3.17 mm × 1.22 m stainless steel column packed with Porapak N, and a flame ionization detector. The GC oven is maintained at about 50°C for CH_4 analysis and then conditioned at 150°C for several hours (overnight)

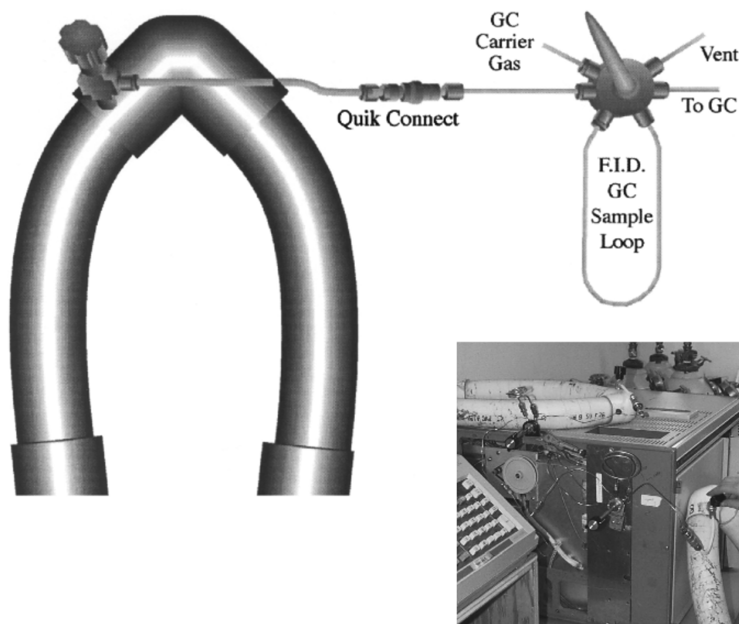
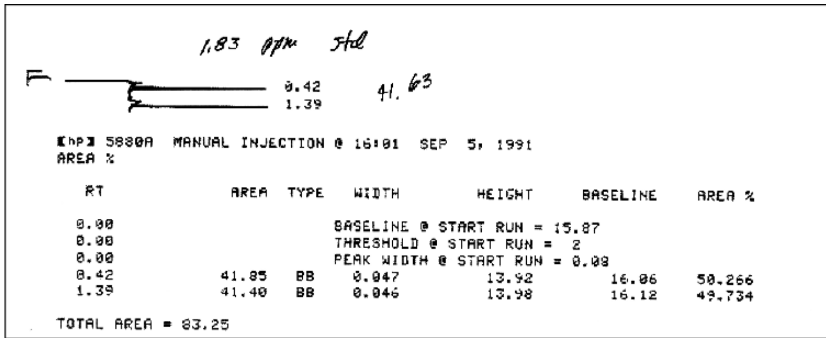


Figure 7. Canister connection to gas chromatograph

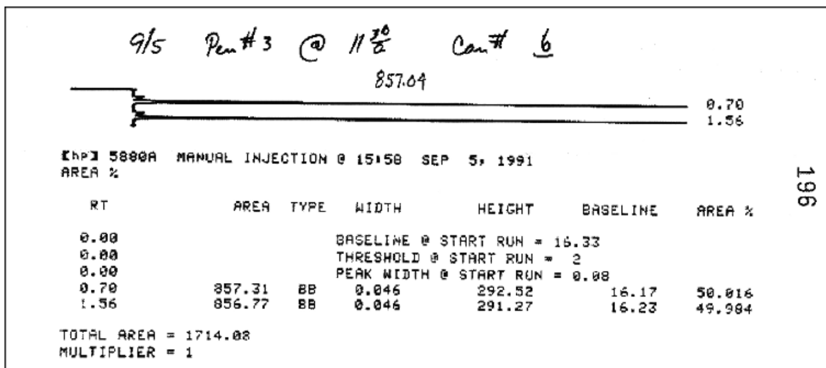
prior to the next analysis period. Each analysis can be completed in less than one minute. Duplicate or triplicate analyses should exhibit reproducibility of 2% or better. The GC calibration is based on a NIST methane standard reference material (SRM). A less costly certified standard available from several gas suppliers can be employed for routine span checks. Examples of the chromatographs are provided in Fig. 8.

3.2. SF_6 Analysis

Sulfur hexafluoride is measured using a gas chromatograph equipped with an electron capture detector. The sample is injected onto the GC column via a low dead volume gas sampling valve and a 1.0 cc gas sample loop. We employ a 3.17 mm × 1.83 m. Molecular Sieve 5A (40–60 mesh) column operated isothermally in the 40 to 50°C range. SF_6 elutes in about one minute and prior to the oxygen peak. In the chromatogram below (Fig. 9), three consecutive SF_6 (77 pptv, parts per trillion by volume) analyses are shown. The peaks at 1.75, 4.23 and 7.37 min are SF_6 and the other three are oxygen. Thus a triplicate analysis can be completed in less than ten minutes. Note that high precision for SF_6 is achieved by this procedure. The system is calibrated with a series of ppt level SF_6 standards ranging from about 30 to 1000 ppt SF_6. A concentration of about 100 ppt is appropriate for routine span checks.



a) Sample is run twice and the average area is 41.63.



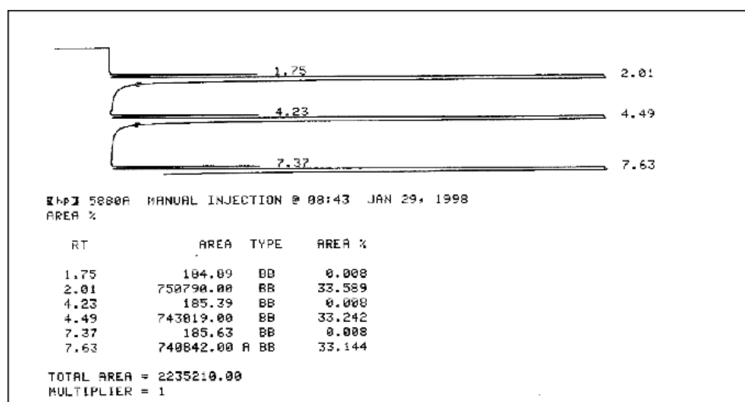
b)

Figure 8. Examples of CH_4 chromatographs showing a) CH_4 standard analysis, b) a duplicate run from a collection canister

3.2.1. Calculations

The tracer method utilizes SF_6 to account for dilution as gases exiting the cow's mouth mixed with ambient air. It is assumed that the SF_6 emission exactly simulates the CH_4 emission; thus, the dilution rates for SF_6 and CH_4 are identical. Mixing due to turbulent diffusion is much more important than molecular diffusion in the atmosphere. Similarly, gas transport from the rumen out of the mouth is dominated by forceful contractions and eructation so that molecular diffusion is unimportant in the emission process. The methane emission rate (QCH_4) can then be calculated from measured CH_4 and SF_6 concentrations and the known release rate of SF_6 (QSF_6) :

$$QCH_4 = QSF_6 \times [CH_4]/[SF_6]$$

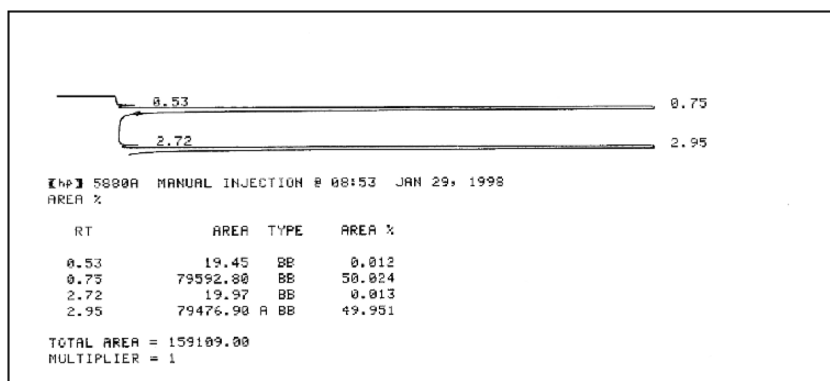


[hp] 5880A MANUAL INJECTION @ 08:43 JAN 29, 1998
AREA %

RT	AREA	TYPE	AREA %
1.75	184.89	BB	0.008
2.01	750790.00	BB	33.589
4.23	185.39	BB	0.008
4.49	743019.00	BB	33.242
7.37	185.63	BB	0.008
7.63	740842.00 A	BB	33.144

TOTAL AREA = 2235210.00
MULTIPLIER = 1

a) Notice that the small peak areas (<200 area units) are SF_6 while the later large peaks (~750 000 area units) are oxygen



[hp] 5880A MANUAL INJECTION @ 08:53 JAN 29, 1998
AREA %

RT	AREA	TYPE	AREA %
0.53	19.45	BB	0.012
0.75	79592.80	BB	50.024
2.72	19.97	BB	0.013
2.95	79476.90 A	BB	49.951

TOTAL AREA = 159109.00
MULTIPLIER = 1

b) The small peak is the SF_6 peak. Notice that the variation between runs is 2.6%

Figure 9. SF_6 chromatographs showing a) standard analysis and b) sample analysis a) Notice that the small peak areas (<200 area units) are SF_6 while the later large peaks (~750 000 area units) are oxygen. b) The small peak is the SF_6 peak. Notice that the variation between runs is 2.6%

Background concentrations of methane and sulfur hexafluoride should be subtracted from the concentration of these species measured in the collection yoke. The background SF_6 concentrations are normally very small compared to yoke concentrations and therefore can usually be neglected. However, background methane levels (−2 ppm; $[CH_4]_b$) should always be subtracted from the methane concentration measured in the yoke ($[CH_4]_y$):

$$QCH_4 = QSF_6 \times ([CH_4]_y - [CH_4]_b])/[SF_6]$$

3.2.2. Example Calculation

Yoke #: 19
Final pressure: 6.45 psi (atm = 13.5 psi)
Dilution pressure: 15.59 psi
Dilution factor: 2.42 = (15.59 psi / 6.45 psi)
Gas chromatograph results – Yoke #19 area counts: $CH_4 = 287$; $SF_6 = 929$
Gas chromatograph results – CH_4 standard area counts (3.87 ppm) = 94; SF_6 (321 ppt) = 1048
Methane concentration (ppm): [(Std conc/std area counts) × sample area count × dilution factor]
$3.87\,\text{ppm} / 94 \times 287 \times 2.42 = 28.6\,\text{ppm}$
Methane concentration** ($\mu g/m^3$): [CH_4 conc × 40.9 × 16]
$28.6\,\text{ppm} \times 40.9 \times 16 = 18716\,\mu g/m^3$
Methane background concentration ($\mu g/m^3$): 1112 $\mu g/m^3$
SF_6 concentration (ppt): [(Std conc/std area counts) × sample area count × dilution factor]
$321\,\text{ppt}/1048 \times 929 \times 2.42 = 687\,\text{ppt}$
SF_6 concentration ($\mu g/m^3$)**: [SF_6 conc × 40.9 × 146]
$0.000687\,\text{ppm} \times 40.9 \times 146 = 4.10\,\mu g/m^3$
SF_6 emission rate from permeation tube: 0.0000502 g/min
CH_4 emission rate: $QCH_4 = QSF_6 \times ([CH_4]_y - [CH_4]_b]) / [SF_6]$
$QCH_4 = 0.0000502\,g/min \times (18716\,\mu g/m^3 - 1112\,\mu g/m^3)/4.10\,\mu g/m^3 = 0.22\,g/min$
***Concentration units conversion (1 atm. 25°C):*
$\mu g/m^3 = \text{ppm} \times 1000/(R \times T) \times MW$
R = 0.0821 L atm/mole °K
T = 298°K
$\mu g/m^3 = \text{ppm} \times 40.9 \times MW$

3.3. Methods/Sampling Protocol

3.3.1. Pre-sampling considerations

Our first step is to gain approval to conduct research with animals from our Institutional Animal Care committee. Following approval we follow a sequence similar to that seen below.

Perhaps the greatest factor influencing the amount of methane a ruminant produces is the diet the animal is fed. The diet determines the balance of the microbes existing in the rumen and therefore the fermentation characteristics, including methane production. Thus, it is essential that the animal be fed the experimental diet at least 21 days prior to sampling. This time period allows the microbial population to change and stabilize.

During this time period, training of animals to be measured is recommended. Some cattle or sheep will not eat if they are wearing a halter and collection canister, others will never adjust to handling and are a danger to themselves as well as

to the people making collections. Therefore, it is recommended that the animals to be sampled be gentled, accustomed to a halter and to wearing a collection canister. We save any damaged canisters to be used during this training period. The number of animals to be measured is also an important consideration. There is variation in methane production from animal to animal on the same diet, so it is necessary to use several animals per treatment. Estimates of the variation in methane seen in similar circumstances from previous literature coupled with appropriate statistical formulas can result in fairly accurate estimates of the numbers of animals needed.

Preparation of permeation tubes is also necessary several months prior to making measurements. In most cases five to six weeks are needed to obtain accurate estimates of the release rate from a permeation tube prior to placement in the cow.

Halters should be prepared prior to sampling and capillary tubing checked to ensure the sampling interval is correct. We have found that there is variation among and within a lot of capillary tubing so we check each piece prior to use. We have also found it to be a good policy to have at least one completely fitted spare halter and several pieces of capillary tubing for prompt repairs. A 24 h capillary tube and collection canister are prepared for use in measuring ambient concentrations of CH_4 and SF_6. Canisters should be constructed or checked to insure they hold pressure, and GC's tested at this time as well.

3.3.2. Sampling

Permeation tubes with known stable release rates are placed in the ruminoreticulum using a balling gun or calf piller two days prior to initiation of sampling. The permeation tube will nearly always end up in the reticulum. This time period is to ensure that the rumen is equilibrated and to allow a day to check the level of SF_6. Prior to initiation of sampling, we always outfit the animal with a halter and canister for several hours to check that we have detectable levels of SF_6. This is particularly important if the tube has been in the animal from a previous collection period and is nearing the end of its functional lifetime. This pre-sampling check has the added advantage of ensuring the GC's and standards are available and working correctly.

The following is an example of a sampling sequence:

Day-3 Feed samples taken

Day-2: Permeation tubes are placed in the animals, feed samples taken

Day-1: Animals are checked to ensure SF_6 is detectable and everything is ready. Animals are weighed (for later description purposes). Feed samples taken.

Day 1: Sampling begins at 07:00h with the animals fitted with halters (with 12 h capillary tubes) and evacuated canisters. The halter should fit the animal so the filter is above the nose but far enough back on the nose that an animal can drink without filling the filter with water. The canisters have been checked to ensure they are evacuated using the pressure gauge. The line from the halter is connected into

the Quickconnect on the sampling canister and Velcro straps are placed around the canister legs and the halter on the sides and top to stabilize the can. The valve on the canister is then opened and the collection period begun. Another evacuated canister equipped with a 24 h capillary tube is placed in the area the animals will frequent to measure ambient concentrations of methane and SF_6 and collection is started here also. Time of collection initiation is recorded. Feed samples are collected and feed offered is weighed.

Note: Another possibility for measuring ambient concentrations of methane and SF_6 *when many animals are penned together is to outfit an animal with a sampling canister and modified halter. The halter is modified to have the capillary tube and filter on the back of the animal's head. This animal will travel with the rest of the herd and the canister will fill with ambient air instead of respired air.*

At 19:00h the canisters are changed and newly evacuated canisters placed on the animal. To change the canister, the valve on the used one is closed, the Velcro straps opened and the Quickconnect released. A new canister is placed on the animal, velcroed into position and connected with the Quickconnect. The halter position is checked and adjusted if necessary. The valve is then opened and the collection started. Once again the time of sampling, both final and initial, is recorded. The cans are numbered and care is taken to record the can number for each animal. Use of a tag or label on the canister can help the identification process. The used canisters are then connected to the dilution device and the final pressures recorded. This is especially important to do immediately because it is the first indication of a sampling problem. The pressure should be at one half atmosphere. The canisters are then pressurized to about 1.2 atm with pre-pure nitrogen. The final pressure is recorded and the canister is ready for analysis of CH_4 and SF_6. While we have found sample storage in the canisters for 10 days not to be a problem, we recommend immediate analysis if at all possible. This prevents loss of sample and also allows for examination of results. The information is then entered into a spreadsheet and methane production calculated.

Day 2 to Day 5: The same sequence is repeated as on Day 1. The ambient sample is collected and a new one started. The ambient canisters are treated exactly as the used sampling canisters. Feed samples or pasture clip samples are collected several times over the course of the measurement period and stored for future analysis. Feed intakes are recorded where possible and the feeding schedule is strictly adhered to.

Post-sampling: After a sampling period is completed, cleaning of the filters and capillary tubing by blowing nitrogen through them is recommended. Dust and very small particles may accumulate over time and eventually plug the filter or the tubing. Filters can be dissembled, washed and dried if needed.

A troubleshooting guide and comprehensive list of parts for establishing the SF_6 tracer technique, as well as potential suppliers are provided in Appendix D, Section 4.

3.4. Quality Assurance (QA) Plan

3.4.1. Introduction

Methane emissions from ruminant livestock are of interest to animal scientists and researchers trying to understand global climate change. The production of methane and release to the atmosphere represents an energy loss mechanism in cattle. Ruminant physiologists would like to find ways to rechannel the carbon loss associated with methane production into weight gain and/or milk production. Since methane is an important greenhouse gas, reduction in methane emissions from livestock may help to stem the constantly increasing levels of this gas in the atmosphere. A procedure for measuring the methane emission rate from free ranging livestock has been developed. This plan summarizes QA objectives associated with the sample collection and analysis.

3.4.2. Project Description

The rate at which cattle release methane is determined using a tracer technique. A permeation device that releases SF_6 at a known rate is placed in the cow's rumen. The animal is fitted with a sampling system such that ambient air from around the mouth and nostrils can be collected over an extended period of time. The air sample is then analysed for methane and sulfur hexafluoride and these concentrations along with the known release rate allow calculation of the methane emission rate. The components of the system include the permeation device, a halter that fits snugly around the cow's head, a PVC yoke that fits over the neck for sample collection, capillary tubing for air transfer, a gas dilution system, and gas chromatographs for methane and sulfur hexafluoride determination.

3.4.3. Project Organization and Responsibilities

The principal investigator (PI) for this work will be directly responsible for all aspects of the measurement program. A graduate student/technician versed in animal handling, welfare, etc. will be responsible for general animal care and all activities associated with sample collection. Experienced chromatographers will perform sample analyses. The PI will have primary responsibility for all quality assurance efforts with assistance from the entire team.

3.4.4. QA Objectives for Measurement Data

Table 1 lists the QA objectives for precision, accuracy and completeness for the methane-sampling program. All data will be collected in a manner that ensures it represents actual ambient or environmental conditions. Methane emission rates will be reported in units of grams/hour. The precision of gaseous concentrations from ambient samples will be determined from routine replicate analyses of the samples and span gas mixtures. The absolute accuracy of the methane and sulfur hexafluoride analyses is dependent upon the certified accuracy of the calibration gases. Whenever possible, standards will be intercompared with other laboratories.

The precision and accuracy results will be tabulated, as appropriate, in the analytical logbooks. The results will be compared to QA objectives following each calibration or span period as a means of checking system performance and to ensure that QA goals are being attained.

3.5. Sampling and Analytical Procedures

3.5.1. Sample collection

The sampling apparatus consists of a PVC yoke or canister that is formed to fit around a cow's neck. PVC pipe of 6.35 cm diameter and 200 psi pressure rating is satisfactory for yoke construction. PVC end-caps and an elbow are used to seal the sample container. The yoke is made to conform to the shape of the neck by heating in an oven and bending it while it is hot. Figure 5 provides a diagram of the sample collection yoke. The yoke's elbow is tapped with 0.25 pipe thread to allow attachment of a gas-sampling valve. A short piece (101 mm) of 6.35 mm Teflon tubing is attached to the valve via a Swagelok connection. The Teflon tube is fitted with a female 6.35 mm Quickconnect on the upstream end such that it can be attached to the capillary tubing that extends from the collection yoke to just above the animal's mouth and nostrils, 1.58 mm stainless steel tubing with an inside diameter of 0.127 mm serves as the flow restrictor and transfer line. The length of the restrictor tubing dictates the sample collection period. Short sample integration periods (e.g. 1 hour) may require only a short piece of the restrictor tubing. In this case, normal 1.58 mm stainless steel tubing is used to connect the remainder of the transfer line. A filter (50 micron) is placed on the upstream end of the capillary line to keep it from plugging. Immediately prior to sampling, the collection yoke is evacuated. Any vacuum pump capable of reducing pressure in the yoke to a few Torr is acceptable. The yoke is then placed over the cow's neck and connected to the capillary transfer line. To initiate sample collection, the valve on the yoke is opened. The evacuated collection device will fill at a constant rate until it reaches about 1/2 atm, at which time sample collection is stopped by closing the valve. Soon after collection and prior to analysing for methane and SF_6, the yoke is pressurized to approximately 1.2 atm. with nitrogen gas. The dilution system (Fig. 6) consists of a tank of compressed nitrogen, a valve for flow control and a pressure gauge that will provide readings above and below normal atmospheric pressure. When sampling is

Table 1. QA Data Objectives

Method	Parameter	Completeness %	Precision %	Accuracy %
FID/GC	CH_4 concentration	100	±2	±10
EC/GC	SF_6 concentration	100	±2	±10
Gravimetric	SF_6 release rate	100	±0.2	±1

complete the yoke should be at about 0.5 atm. pressure. Nitrogen is added slowly until the pressure is increased to about 1.2 atm. in the yoke. Pressure readings before and after addition of nitrogen are recorded in order to calculate the dilution factor. With the contents of the yoke under positive pressure, it is easy to transfer an aliquot of sample to the gas chromotography (GC) systems. The yoke can be attached directly to the gas-sampling valve on the GC via Quickconnect fittings. Simply opening the yoke valve allows sample transfer to the fixed volume loop on the GC (Fig. 7).

3.5.2. Methane analysis

Methane concentration in the collection yoke is determined by GC. The GC system consists of a 1.0 cc sample loop attached to a low dead volume gas sampling valve, a 3.17 mm× 1.22 m stainless steel column packed with Porapak N and a flame ionization detector. The GC oven is maintained at about 50°C for CH_4 analysis and then conditioned at 150°C for several hours (overnight) prior to the next analysis period. Each analysis can be completed in less than one minute. Duplicate or triplicate analyses should exhibit reproducibility of 2% or better. The GC calibration is based on a NIST methane standard reference material (SRM). A less costly certified standard available from several gas suppliers can be employed for routine span checks.

3.5.3. SF_6 *analysis*

Sulfur hexafluoride is measured using a gas chromatograph equipped with an electron capture detector. The system employs a 1.0 cc gas sample loop attached to a low dead volume gas sampling valve and a Molecular Sieve 5A column (3.17 mm × 1.83 m). With a column temperature of 50°C, SF_6 elutes in about one minute and prior to the oxygen peak. The system is calibrated with a series of ppt level SF_6 standards purchased from a specialty gas supplier. Standard concentrations should range from about 30 to 1000 ppt SF_6. A concentration of about 100 ppt is appropriate for routine span checks.

3.6. Calibration Procedures and Frequency

Calibration of the methane gas chromatograph is achieved by measuring instrument response to a known concentration of methane (3.87 ppm) in air (Scott Environmental Technology). This laboratory standard has been compared to a methane standard that has direct traceability to a NIST standard. Multiple span checks are performed during each analysis period. Sulfur hexafluoride calibration is accomplished using a series of certified standards purchased from a specialty gas supply house (e.g. Scott Marin). A SF_6 standard in the 100 ppt range is used for routine span checks. Calibration of the microbalance used to weigh the permeation tube is performed on a regular basis by a certified technician.

3.7. Data Reduction, Validation and Reporting

Output from the gas chromatographs is processed by the computerized data acquisition system associated with each chromatograph. This provides an integrated area under each individual peak in the chromatogram. Peak identities are assigned by comparison to a chromatogram obtained from standard runs with methane and SF_6. In both cases, data validity is based on a complete examination of each chromatogram. Any abnormalities will be flagged and a reason sought for the discrepancy. Decisions concerning the acceptance or rejection of data will be based on the recommendations of the principal investigator. The methane and SF_6 concentrations and pertinent sampling information will be manually compiled into a computer spreadsheet. Visual checks of all data entry will be performed. Any errors will be flagged and the source of error determined. Methane emission rates are calculated using the following relationship:

$$CH_4\ (g/h) = SF_6 \text{ release rate } (g/h) \times [CH_4\ (\mu g/m^3)]\ /\ [SF_6\ (\mu g/m^3)]$$

3.8. Internal Quality Control Checks

Field QA tests will be performed daily. This will involve checks for transfer line blockage and a careful examination of all parts of the collection system. Routine span checks of the chromatographs will be performed during every analysis day. Chromatograms will be examined for excessive baseline noise, unusual peaks, or artifacts. Pressure in the collection canister at the completion of the sampling period is the best indicator of sampling problems. The final pressure should be approximately 1/2 atm. A very low pressure indicates a blockage in the transfer line while a pressure approaching ambient means leakage has occurred in the system.

3.9. Specific Routine Procedures Used to Assess Data Precision, Accuracy and Completeness

Precision of the physical systems will be measured in terms of the standard deviation of replicate measurements obtained during lab calibrations. Precision of the gas chromatographic measurements will be determined by comparing the reproducibility of results from analyzing the laboratory standards. Since the methane and sulfur hexafluoride standards will be run each day, there will be ample data for a statistical (standard deviation) determination of precision. Interlaboratory comparisons and/or audits will be used to assess accuracy of the GC analyses.

Data completeness will be calculated as a percentage of valid data compared to the maximum amount of data obtainable during the study period.

3.10. Corrective Actions

Inspection of sampling systems and chromatographic span checks will be used to identify problems with specific systems. Decisions regarding replacement, repair, cleaning or re- calibration will be based upon the quality control checks and QA objectives outlined previously. Authority to initiate corrective action will reside with the principal investigator.

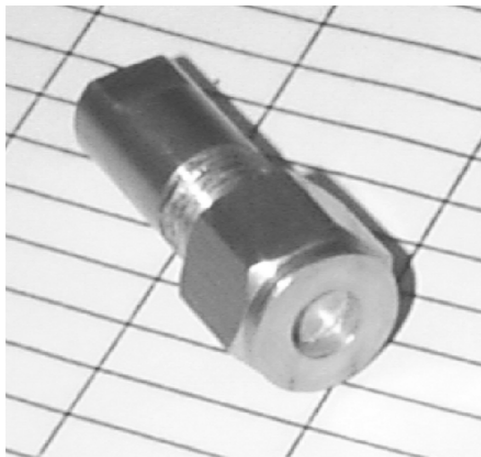
3.11. Quality Assurance Reports

The QA results will be summarized in terms of the calibration results, span and zero checks, and in terms of precision, accuracy and completeness along with the sampling data for the study period. These QA results will be incorporated into the published papers. Final QA data will be presented in a final QA report.

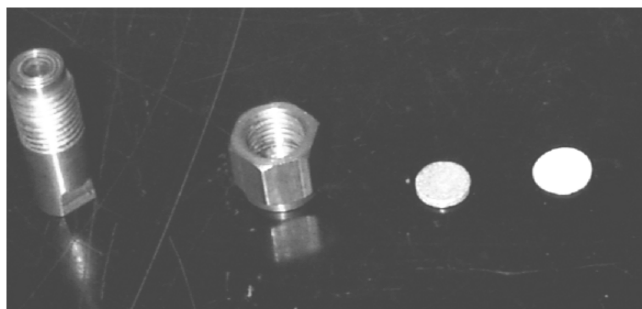
4. APPENDICES

Appendix A Illustration of steps for preparing and constructing an SF_6 permeation tube

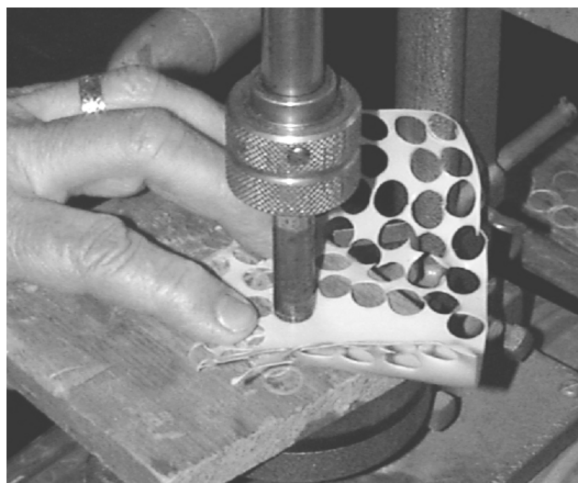
Parts required: Permeation tube body; Teflon window; Stainless steel frit (2 μ); 6.35 mm Swagelok nut; 14.3 mm wrench; Pure SF_6 gas (instrument grade); 2 – 60 cc syringes with wide bore needles (18G); Dewar; Cryogen; Styrofoam block to hold tube; Glass receptacle in 39°C water bath; Nitrogen gas; Locking pliers (vise grips).



SF_6 permeation tube



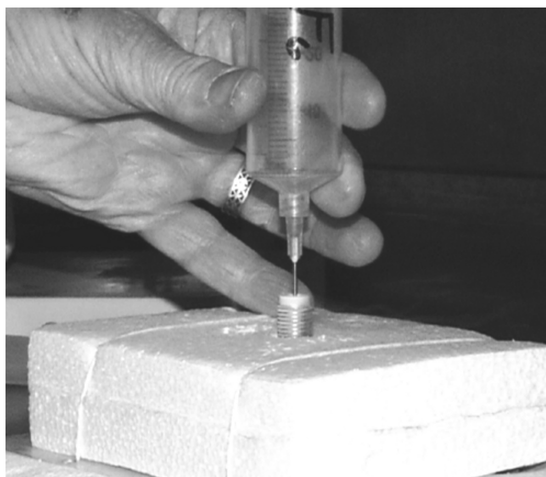
Unassembled SF_6 permeation tube. From left to right: permeation tube body, 6.35 mm Swagelok nut, stainless steel frit, and Teflon window



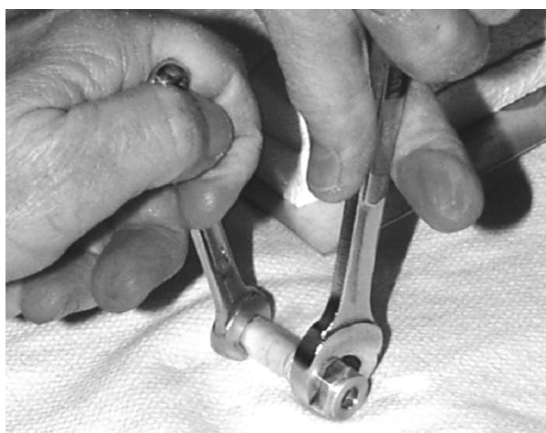
Step 1: Cut Teflon windows

Step 2: Stamp permeation tube body with identification. Weigh the empty body, frit, Teflon window and nut

Step 3: Cool permeation tube body in cryogen. Remove cryogen when tube reaches cryogen temperature

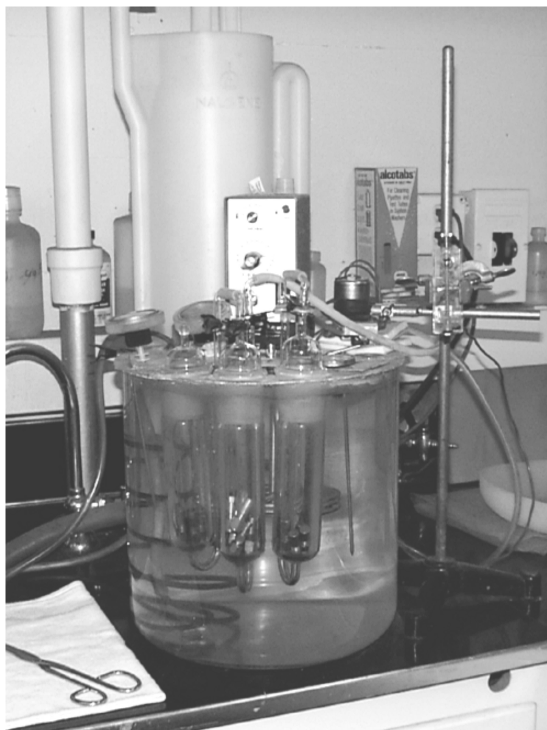


Step 4: Place cooled permeation tube body in a Styrofoam holder. Quickly fill the cavity with two 60 cc syringes full of pure SF_6



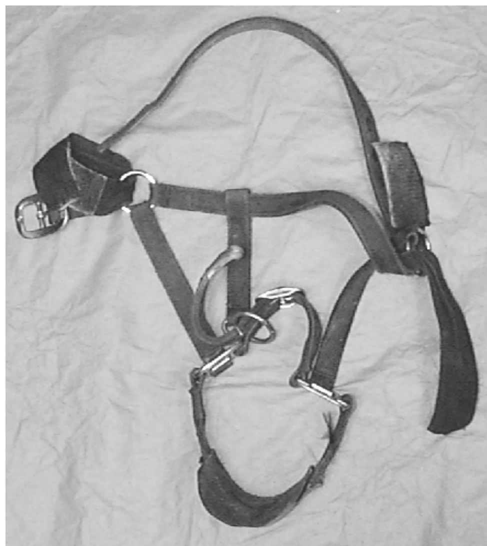
Step 5: Cap permeation tube body with a 6.35 mm Swagelok nut fitted with a stainless steel frit and Teflon window as soon as possible after filling with SF_6

Step 6: Record weight of filled permeation tube. Weights should be recorded weekly for at least 5–6 weeks for calculation of SF_6 release rate. Note: Empty permeation tube should also be weighed



Step 7: Place the permeation tube in a glass receptacle in a 39°C water bath. Nitrogen gas should run through the receptacle to purge SF_6

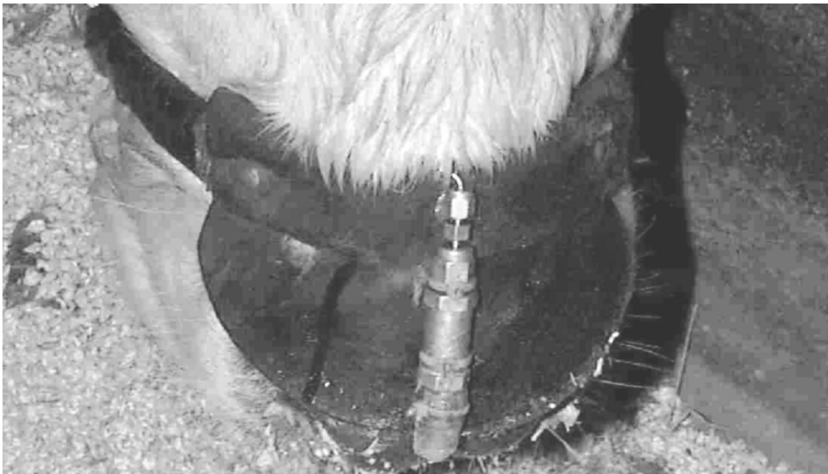
Appendix B Illustrations of the halter construction and attaching the capillary tubing



Step 1. Extra-large horse halter with adjustable chin strap. Extra holes are burned into the chin strap and crown piece for maximum adjustability. A leather piece is sewn or riveted to the noseband and 51 mm Velcro straps are held in place to the cheek pieces with strap ties

Step 2. Thread capillary tubing (without filter apparatus) through leather nosepiece

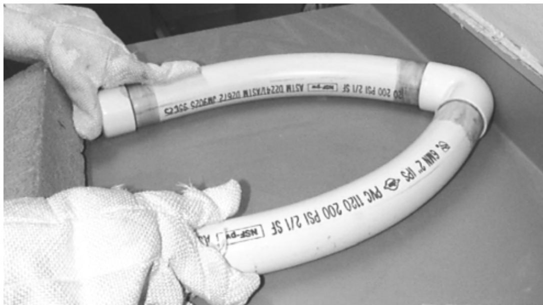
Step 3. Capillary tubing is secured to the halter with electrical (or other) tape



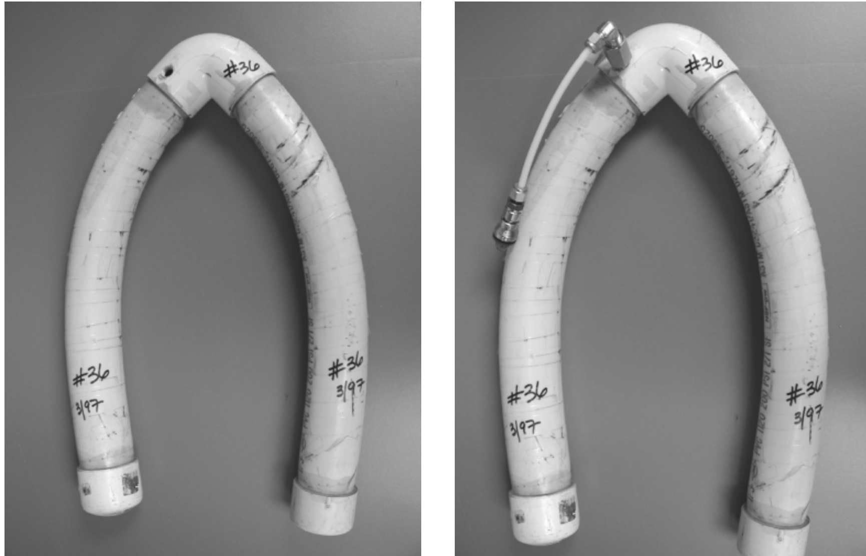
Step 4. After capillary tubing is secured to the halter with tape, the filter assembly is secured to the leather nosepiece with strap ties. Tygon tubing is placed over the end. Note: This photo shows correct location of the filter assembly on the animal's nose

Appendix C Illustrations of the steps involved in constructing the collection canister

Step 1. Primed pieces of PVC pipe and fittings ready to be glued



Step 2. After primed pieces are glued and dry, heat to ~120°C or until PVC pipe is pliable. Bend pipe by applying steady pressure to top and bottom of canister until legs bow. Set the form by cooling with water



Step 3. Completed PVC collection canister showing the correct location of the tap (left photo) and after addition of sampling valve (right photo). Legs of the collection canister are wrapped in packaging tape to protect against implosion or shattering

Appendix D

TROUBLE SHOOTING

Problem	Possible Solutions
Fill rate of the canister is too fast	1. Check for broken or cracked PVC canister. Pressurize the canister and submerge it to look for bubbles 2. Check fittings for tightness 3. Lengthen the capillary tubing
Fill rate of the canister is too slow	1. Check filter and capillary lines for blockages 2. Check fittings for possible occlusion 3. Check the valve on the canister to be sure it is full open 4. Shorten the capillary line
Pressure in the canister is atmospheric	1. Check all fittings for leaks 2. Check the canister for leaks 3. Capillary line is too short
No pressure change or low pressure in the canister	1. Was the valve on the canister open? 2. Check the filter and capillary line for blockages. Blow them out with compressed air.

TROUBLE SHOOTING *(continued)*

Problem	Possible Solutions
	3. Check all fittings for obstructions
No methane or SF_6 peaks on the GC	See GC manual

PARTS LIST

Part	Possible Source	Number
Vacuum Distillation Apparatus		
Vacuum pump	VWR	DD–22 P1298
Vacuum pump filter	VWR	P–1297
Pump oil	Local source	–
Portable Digital Absolute Gauge, 0–30 psig	Cole-Parmer	EW–68970–60
Dilution Apparatus		
3-Way valve	Swagelok Distributor	B–43x54
1 – 6.35 mm Bulkhead union	Swagelok Distributor	B–400–61
1 – 6.35 mm Union tee	Swagelok Distributor	B–400–3
1 – Male quickconnect	Swagelok Distributor	SS–QC4–S–400
8 – 6.35 mm Swagelok nuts	Swagelok Distributor	B–402–1
1 – Male elbow (6.35 mm Swagelock–3.17 mm NPT)	Swagelok Distributor	B–400–2–2
1 – 6.35 mm Swagelok ferrules	Swagelok Distributor	B–400 sets–100
1 – N_2 Regulator		CGA #580
1 – Male connector (6.35 mm Swagelok–3.17 mm NPT)	Swagelok Distributor	B–400–1–4
7.62 m – Copper tubing	Auto parts store	–
Sampling halter (# of parts/halter)		
1 – 51 mm piece 7.95 mm Tygon tubing (length should be adjusted based on size of cow)	Local source	
1 – 40.6 cm piece 3.17 mm PTFE tubing	Cole Parmer	G–06407–42
1 – Filter	Swagelok Distributor	B–2F–15
2 – 3.17 mm to 1.59 mm Reducing union	Swagelok Distributor	B–100–R–2
30 cm Capillary tubing (length must be adjusted according to lot & depends on duration of sampling)	Alltech Associates	30212
2 – 3.17 mm to 1.59 mm Reducing union	Swagelok Distributor	B–200–6–1
2 – 3.17 mm Swagelok nuts	Swagelok Distributor	B–202–1
1 – Male quickconnect	Swagelok Distributor	SS–QC4–S–200
2 sets 3.17 mm ferrules	Swagelok Distributor	B–200–set
1 – Halter (Extra large horse size works best)	Livestock supply	–
Leather nose flap + velcro straps	Local source	–
Electrical tape	Local source	–
Sampling can		
2 – 50.8 cm pieces of 51 mm ID 200 psi PVC pipe	Local source	–
1 – 25.4 mm 90° elbows	Local source	–

2 – 51 mm ID end caps	Local source	–
1 – Can of primer PVC	Local source	–
1 – Can PVC cement	Local source	–
1 – Valve (*Note: Special Order/Expensive*!!)	Swagelok Distributor	B–14DKM4–S4–A
Optional valve (Mini-Ball)	Parker Hannifin Distributor (Fluid Products)	MV608–4
Male elbow, 6.35 mm Swagelok to 6.35 mm female NPT thread (Must have if use the Mini-ball valve from Fluid Connector Products)	Swagelok Distributor	B–400–2–4
15 cm – 6.35 mm Teflon PTFE tubing (ID=3.17 mm, OD=6.35 mm, Wall=1.59 mm)	Fisher Scientific	14–169–15B (Chemfluor Fluoropolymer tubing)
2 – 6.35 mm Swagelok nuts	Swagelok Distributor	B–402–1
2 – 6.35 mm Swagelok ferules	Swagelok Distributor	B–400–set
1 – Female quickconnect	Swagelok Distributor	SS–QC4–B–400
Miscellaneous Supplies		
Tools – wrenches, etc.	Local sources	–
Fittings – nuts, ferrules, valves, etc.	Swagelok Distributor	–
Supplies – tape, tubing, etc.	Local source	–
Permeation tube		
Teflon film, 0.38 mm thick	McMaster-Carr	8569K18 8569K41 8569K63
Stainless steel frits, 9.5 mm OD X 1.59 mm thick	Alltech Associates	718225

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Fisher Scientific PO Box 1490 Houston, TX 77251–1490 Tel: 800–766–7000	Parker Hannifin Distributor (Fluid Connectors, Brass Products) See: http://www.parker.com/distloc/english/search_2V4.asp?menu_gid=46&menu_divid=

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CHAPTER 4

ESTIMATION OF RUMINAL METHANE PRODUCTION FROM MEASUREMENT OF VOLATILE FATTY ACID PRODUCTION

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INTRODUCTION

Reduction of carbon dioxide to methane is one of the terminal steps in electron transfer within the network of fermentation reactions conducted by rumen micro-organisms. Clearing the rumen of hydrogen gas by methanogenesis allows ongoing regeneration of NAD^+ and FAD^+ and the continuous conversion of dietary carbohydrate and protein into microbial cells and volatile fatty acids (VFA). Given this mechanistic link between fermentation and methanogenesis, it is not surprising that stoichiometric associations based on hydrogen recovery have been considered to be the basis for linking methane production and VFA production data (8, 16, 26). The association is quite strong when fermentation occurs in a normal manner but is uncoupled when methane inhibitors are included, presumably because alternative hydrogen pathways are induced.

This chapter describes in three sections the technical procedures associated with infusing or injecting ^{14}C-labelled VFA into ruminants, the mathematical procedures for calculating VFA production rates and the stoichiometric relationships that allow methane production rate to be predicted from VFA production rates. A critique of this approach is also included.

1. MEASURING VOLATILE FATTY ACID PRODUCTION RATE IN THE RUMEN BY ISOTOPE DILUTION

1.1. Principle of Method

VFA labelled with isotopes can be used as tracers to estimate the rate of VFA production in the rumen. A known quantity of VFA tracer is injected or infused

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into the rumen and the dilution over time of the labelled VFA (tracer) by unlabelled VFA (tracee) arising from fermentation is determined (this method is referred to as the tracer dilution method). The most widely used tracer methods involve the continuous infusion of ^{14}C- or ^{3}H-labelled VFA either singly or as mixtures of VFA, with samples of rumen fluid being taken at intervals over a period of several hours (19, 12). The *net flux* of VFA (mol/min) through the compartment into which a tracer is infused is calculated by dividing the rate of infusion of radioactive isotope (MBq/min) by the concentration of radioactivity in the VFA (MBq/mol) isolated from the rumen fluid samples. A more complete understanding of the rates of production of individual VFA and their interconversions can be obtained by solving multi-compartment models (e.g. 22, 23).

A general three-compartment model showing the total fluxes of acetate, propionate and butyrate through their respective compartments from internal and external sources and the interconversions of tracee is shown in Figure 1.

The terminology used to describe the flows of VFA in a model such as the one shown in Figure 1 can be a cause of confusion. It is important to focus on the *tracer* being used. If a ^{14}C- or ^{13}C-labelled VFA is used as the tracer, then the material being traced (or *tracee*) is carbon. If ^{3}H or deuterium is the label, then flow rates refer primarily to the flows of hydrogen. The estimated flow rates are fluxes of the tracee through the kinetic *compartments* that comprise the model, e.g. acetate, propionate and butyrate in rumen contents. For the acetate compartment, the rate of *total flux* of tracee is given by the sum of all inflows into the acetate compartment which, if the rumen VFA system is in *steady state* will be the same as the sum of all outflow rates. This value is greater than the rate of *net flux* of acetate-C because, during a continuous infusion of labelled acetate, some of the acetate tracer will leave the acetate compartment and be converted to other materials (e.g. butyrate) and then subsequently be returned to acetate, i.e. *recycled.* Net flux rate has been referred to as *irreversible loss rate* by some workers. The

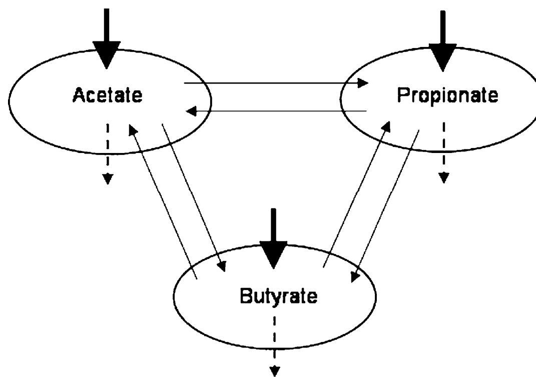


Figure 1. An open, general, three-compartment model describing all possible carbon fluxes into, from and between each of the three major volatile fatty acids in the rumen

net flux rate (g C/d), which excludes this recycled tracer, is calculated by dividing the ^{14}C-acetate infusion rate (MBq/d) by the plateau specific radioactivity (SR) in acetate (MBq/g C). The compartment size, total flux rate and recycling rate are not readily determined by means of a *continuous infusion* of labelled VFA, but can be determined if a *single dose* of tracer is administered at one time and the SR versus time curve thereafter is analysed. Despite this apparent advantage, the single dose method has other limitations that will be discussed later.

The above general three-compartment model (Fig. 1) can be solved if three separate experiments, each involving the continuous infusion of one of the three acids containing labelled carbon, are performed. The plateau SR values for each compartment are determined at known rates of tracer infusion in each experiment, giving 9 plateau SR values and 3 infusion rates. These values enable all the flow rates in the model to be quantified. Several more terms can be defined. Each compartment has a flow of material into it from outside the model, and a flow from that compartment to the outside. The first represents the rate of incorporation of tracee (new or *de novo* production of VFA; bold solid arrows in Fig. 1) and the second represents tracee that is permanently removed from the model system (*final disposal rate*; bold dashed arrows in Figure 1 which reflects VFA available for absorption from the gut).

There is no ideal isotopic tracer for estimating the rates of VFA production. Tracer VFA can be labelled using radioactive or stable isotopes. Rates of net flux of the major VFA (acetate, propionate and butyrate) can be estimated individually using separate tracers for each VFA. Alternatively, one labelled VFA can be used and a good estimate of the combined rates of net flux of all VFA obtained (34, 35). Production rates of the individual VFA can be predicted reasonably accurately from their molar concentrations in rumen fluid (20).

Prediction depends on the linear relationship that exists between net production of an individual VFA in the rumen, expressed as a fraction of total VFA production, and its concentration, expressed as a fraction of the total VFA concentration (19, 20). However, Weston and Hogan (35) and Rowe (27) suggest that a single VFA infusion gives unreliable estimates of the ‘effective production rate’ of individual VFA because factors affecting absorption rate are not well understood and there are variable rates of interconversion of carbon between the individual VFA.

As little propionate is metabolized to either acetate or butyrate in the rumen, ^{14}C-labelled sodium propionate is nearest to being an ideal tracer in experiments using only a single labelled VFA, but it is also the most expensive of the three ^{14}C-labelled VFA. As a less expensive alternative, 1-^{14}C, 2-^{14}C or uniformly labelled (U-^{14}C) acetate can be used to determine acetate (or total VFA) net production rate. Acetic acid is the predominant VFA produced by ruminal fermentation and is closely associated with ruminal hydrogen production. Acetate interconverts with butyric acid and this interconversion can cause underestimates of acetate production by 6% if not allowed for (15). This error can be overcome by making separate injections or infusions of labelled acetate and butyrate on separate days to quantify the interconversion. These complexities are explained in Section 2.

Procedures for estimating VFA production in ruminants *in-vivo* are described in detail below. They apply mainly to experiments to be made in rumen-cannulated sheep or goats but additional notes for cattle studies are provided where needed. Considerable planning is required before an infusion experiment is undertaken but, with good planning, one scientist can comfortably conduct injections or infusions and take samples from 6 sheep simultaneously.

While there is no maximum time for infusions, a 9 h infusion, with sample collection over the final 6 h can give a good estimate of daily production if animals are given their daily ration in equal hourly portions. The 6 h period of sampling is long enough to allow averaging of small variations in VFA production rate that may occur as the substrate supply for the rumen microorganisms varies between meals.

1.2. Preparation of Animals

1.2.1. Materials

- Rumen cannulated sheep or cattle
- Automatic feeders delivering feed at regular intervals
- Peristaltic infusion pump and flow-rated pump tubes
- Infusion tubing (Silastic; 1.5 mm i.d.)
- Ruminal probe with infusion and sampling tubes

1.2.2. Procedure

Animals should have been fitted with cannulas at least 4 weeks prior to VFA production being measured and should have been held in the experimental housing for at least two weeks to allow them to adjust to their experimental diet and surroundings. Animals should be housed in metabolism cages or tethered so that they are not able to rotate in the pen, which can cause infusion lines to twist and fail. The area around the animals should be well ventilated, especially if radioactive tracers are to be used, to prevent the accumulation of hazardous concentrations of radioactive end-products in the area. The diet should be provided in equal portions at regular frequent intervals in the 7 days leading up to, and throughout, the infusion period. Ideally, the diet should be provided in equal hourly portions by an automatic feed-dispensing machine fitted with a 24 h timer. Animals should also be accustomed to their attendants and the procedures for taking fluid samples. Unaccustomed animals may become stressed and refuse to eat during the period of VFA measurements, which will compromise the results obtained.

1.3. Preparation of Isotope Stock and Infusion Solutions

1.3.1. Materials

- Isotope source (supplied in glass ampoule or screw-top bottle)
- Tray (20 cm × 30 cm) with sides and absorbent liner to contain isotope during transfer

- Pasteur pipettes
- Latex gloves
- Tissues
- Water
- 10 M NaOH (< 10 mL)
- 20 mL McCartney bottles and lids
- Radioactive waste disposal bag
- 10 mg of non-radioactive sodium salt of the VFA

1.3.2. Procedure

Volatile fatty acids are normally purchased as the sodium salt (> 99% chemical purity) and isotopically labelled on one or more carbons. Deuterium label (at least in acetic acid) exhibits exchange with other hydrogen atoms in the rumen so is not used (18) although tritiated butyrate is used (19). During preparation of the isotope stock solution, the operator should wear disposable latex gloves to prevent contact with the highly radioactive β–emitting ^{14}C isotope. The ampule containing the dried down VFA salt can be dissolved in water and transferred into a thick walled glass bottle (20 mL) as follows (Fig. 2).

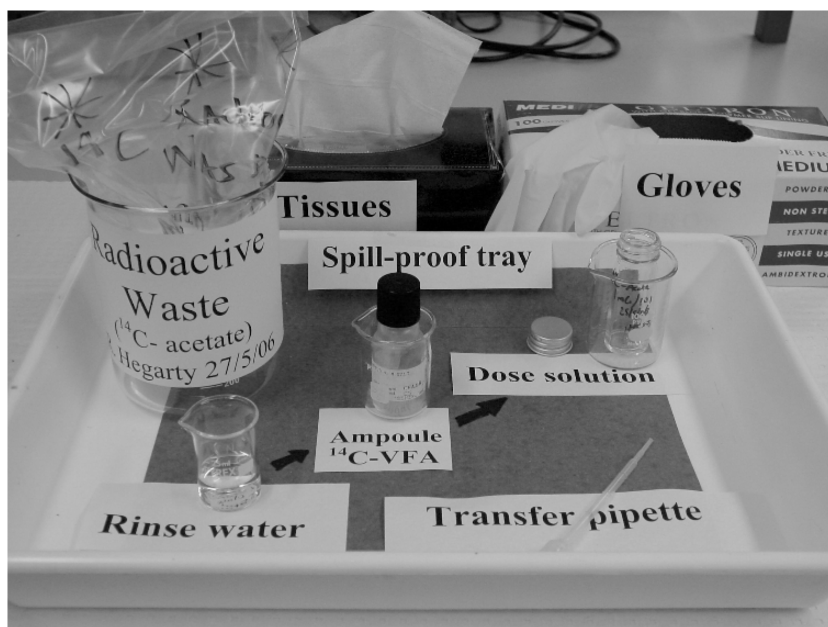


Figure 2. Tray for preparation of radioisotope stock solution. The tray has raised edges to prevent spillage, is lined with absorbent paper and has a waste disposal bag. The vial containing the isotope to be opened, together with transfer equipment (pipette, wash solution) and a labelled bottle to receive the isotope solution are present. Tissues are present in case of spillage and to help safely snap the ampule when tracers come as powders in ampules

- Line a waterproof tray having raised edges with absorbent paper.
- Place in tray:
  - Ampule, sitting upright in 50 mL glass beaker
  - File/scratcher to score ampule
  - Pasteur pipette with functional rubber bulb
  - Tissue to place around scored ampule to contain glass when snapping scored neck of ampule
  - Open-topped bottle (scintillation vial) containing 10 mL of distilled water into which 10 mg of the sodium salt of the VFA had been dissolved (this non-radioactive 'carrier' is added to prevent the radioactive acid binding extensively to glass and infusion lines)
  - Empty 20 mL McCartney bottle. This bottle should be labelled 'STOCK' or 'DOSE SOLUTION' and have all details of the isotope solution it will contain written onto it with a permanent marker. The sticker adhering to the ampule or its packaging can also be removed and stuck to this dose bottle.
  - 1 L glass beaker lined with plastic bag for waste. This should be labelled with your name, ^{14}C waste, a date and 'Caution radioactive waste' written in large visible letters.
- Using the file, score the neck of the ampule at the narrowest place, then wrap the scored section in tissue before snapping the ampule at the score mark. Dispose of tissue into waste bag.
- Draw approximately 1 mL of water into the pasteur pipette and dispense into the open ampule. After swirling the ampule gently, all the VFA salt should dissolve. The solution can then be drawn back into the pasture pipette and dispensed into the labelled McCartney bottle.
- A further 9 × 1 mL washes of water can be transferred into the ampule to wash it and then drawn back out and placed in the McCartney bottle being used for storage.
- The final step in preparation is to add 0.1 mL of 10 M NaOH to make the stock solution alkaline, screw the lid on the stock solution and place in freezer until needed. NOTE: To prevent cracking during freezing, the bottle should not be more than ¾ full and should be allowed to cool and freeze slowly.
- The empty ampoule, the tissue/s, the small bottle that originally held water and the pasture pipette and the paper lining the tray can all be placed in the radioactive waste bag. This bag should be disposed of according to nationally accepted procedures.

From this stock solution of isotope, working solutions for intraruminal injection or infusion can be prepared and utilized as described below.

1.4. Intra-Ruminal Infusion of a ^{14}C-Labelled VFA

The amount of activity infused (MBq/min) is the product of the activity in the infusion solution (MBq/mL) and the rate of infusion (mL/min). In sheep, infusions are typically made at a rate of 0.6–0.8 mL/min and for this rate, the infusion solution

should be prepared by dilution of the dose solution to contain 11.1×10^3 Bq/ml (0.3 μ Ci/mL). For cattle the infusion rate is higher (10 mL/min) and the radioactivity content (Bq/mL) in the infusion solution similar. Alkali (0.7 mL of 10 M NaOH per litre of infusion) should be included to prevent microbial growth and volatilization of VFA. Infusion solutions should only be prepared on the day prior to infusion and held under refrigeration prior to use.

1.4.1. Materials

- Dual inlet rumen probes (Fig. 3)
- Silastic tubing (1.5 mm i.d.). This must be at least 1 m longer than the distance between pump and sheep
- Short (60 cm) lengths of silastic tubing to conduct infusion solution from infusate bottles to pump
- Woollen thread
- Scales accurate to 1 mg
- Clock/timer
- Bottles or Erlenmeyer flasks containing the infusion solution. One bottle per sheep plus one bottle of 'spare' infusate per pump used. These must be numbered with the number of the animal/pen to which the infusion is being made (Fig. 4)
- Peristaltic infusion pump fitted with correct flow-rate pump tubing
- Tray lined with absorbent paper to house pump
- Connectors to join pump to inlet and outlet lines and join infusion line to rumen probe

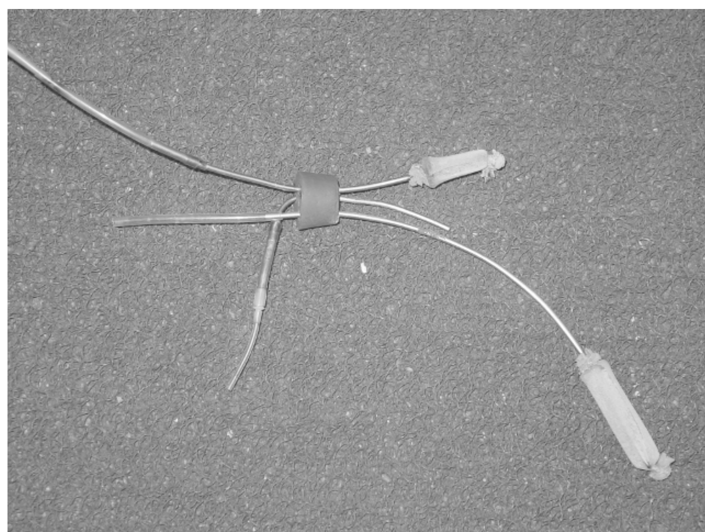


Figure 3. Rumen probe showing positioning of isotope infusion probe (middle) relative to sampling probes for rumen fluid (lower) and headspace gas (upper)

Figure 4. Infusion pump set up for intraruminal infusion. The plastic tray contains the peristaltic pump, an infusion bottled labelled with animal number, a spare bottle of infusate. Scales to determine the weight of infusion bottle, a clock and a data book to record weights, times and comments are nearby

- Infusion solution
- Rumen fluid collection bottles (stock solution) labelled with animal and sample number. Approximately 8–10 bottles will be required per animal. Each bottle should be prepared with 0.3 mL of 18 M H_2SO_4 as a preservative before the rumen fluid is added.

1.4.2. Procedure

Assuming animals have been eating as required, have recovered from cannulation and are tethered or caged so they cannot turn in circles, a 9-h ^{14}C-VFA infusion can be conducted as follows:

- Place peristaltic pump in tray on a table at a close but safe distance from the animal.
- Connect short (60 cm) inlet line to the inflow side of pump and long infusion line from pump outlet reaching to the sheep. This tube will need to have flexibility to permit the animal to stand or lie down and move in the metabolism crate. This can be set up some days before the infusion is to happen.
- The infusion line should preferably be suspended from above and behind the sheep (to avoid it being chewed), and be tied to tufts of fleece above the tail and again near the rumen cannula (Fig. 5). This prevents the infusion line pulling

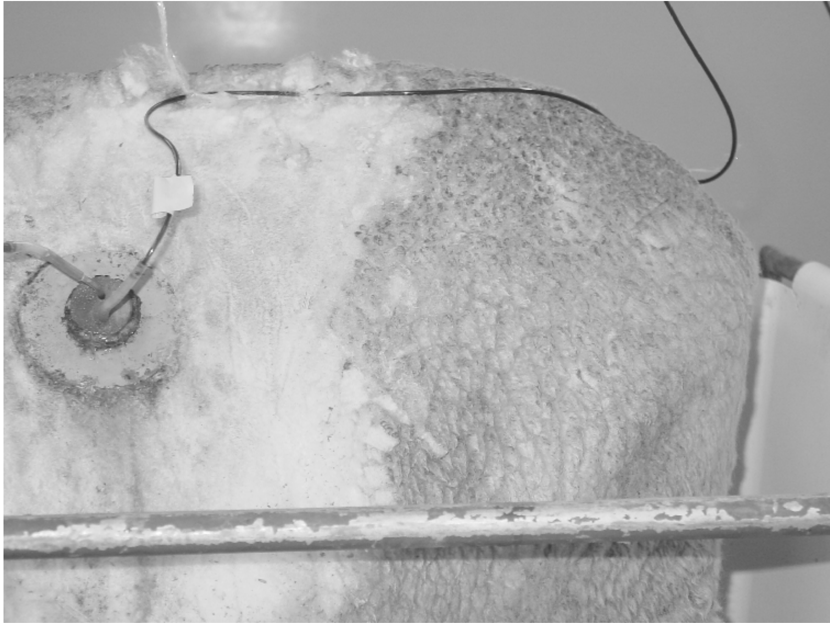


Figure 5. Infusion of ^{14}C-VFA and CrEDTA in progress. The infusion line is tied to the wool near the cannula and near the tail to prevent the infusion line being pulled loose. The infusion line is hung from the ceiling using a chain of linked rubber bands to allow the animal to stand up or lie down without the line going slack and being damaged

from the cannula when the animal lies down. A system for keeping the tube taut above the animal as it moves in the cage should be devised (e.g. suspending the infusion tubing from the ceiling using a length of elastic).

- On the day of the infusion, the rumen probe should be inserted in the rumen and a background sample(s) of rumen fluid collected before tracer is administered. It is important that the inlet probe should reach deep into the rumen contents, while the sampling probe should have its intake at least 15 cm from the infusion outlet. This is essential as it must be assumed that the sample taken is representative of the whole rumen contents at that time and the infusate has mixed completely in the rumen. The rate of mixing of rumen contents is a major infusion issue as discussed subsequently.
- Subsamples of the infusion solution MUST be taken into acid-free clean, fully labelled bottles (McCartney) at least before and at completion of the infusion. These should be stored frozen with all samples collected. No sample is more important than the sample of infusion solution!
- The rate of infusion is determined from the loss in weight of infusate over the period of infusion. Record the full (starting) weight of each animal's infusion bottle immediately before commencement and record in data sheet (worked example in Table 1).

Table 1. Data Recording Sheet to Monitor Rate of Infusion (g/Min) by Change in Weight of Infusate Reservoir During Intraruminal Infusion of Labelled VFA. A Separate Sheet Should also be Prepared to Record the Time at Which Each Sample from Each Animal is Collected

Date	16/02/2005			
Infusion	1–^{14}C acetate (Na salt) approx 0.25 μCi/mL			
Diet	500 g lucerne + 100 g barley			
Real time infusion started	07:52 h			
Sheep	1	2	3	4
Bottle weight start (g)	1526.21	1725.91	1550.16	Not eating
Bottle weight end (g)	1350.94	1553.22	1390.88	Not done
Weight change (g)	*175.27*	*172.69*	*159.28*	X
				X
Time start (by digital clock)	0012	0012	0013	X
Time end	0220	0220	0222	X
Duration (min)	208	208	209	X
Infusion rate (g/min)	***0.8426***	***0.8302***	***0.7621***	
Weight and time cells for repeat measures below				

- Commence the tracer infusion by connecting the infusion line to the rumen probe and then turning on the infusion pumps and recording the "start time" from the clock onto the data sheet.
- No samples will be collected during the first 3 h of infusion.
- After 3, 6 and 9 h of infusion, the bottles of infusion should be weighed and the time and weight recorded on the data sheet. While the bottles are being weighed, the intake lines leading to each pump can be transferred to the spare bottle of infusate to ensure the infusion into animals is not interrupted. When the bottles have been weighed, the intake lines can be quickly swapped back into the infusion bottle for each sheep. These measurements allow three three-hourly measures of infusion rate (g/min) to be obtained for each animal and help identify pump failure if it occurs.

1.4.3. Sample collection and storage

- After 3 h of infusion, samples of rumen fluid can be collected at approximately 50 min intervals.
- Rumen fluid samples should be taken after drawing fluid into the syringe (25 mL) and either discarding or re-injecting this fluid into the rumen at least three times. This ensures the sample is fresh fluid from the rumen digesta. Some frothing of sample may occur due to the H_2SO_4 already in the sample bottle so the 15 mL of sample should be dispensed slowly into the sample bottle.
- Samples should be sealed and frozen on collection.

All tracer dilution methods are based on assumptions about kinetic compartments. By definition, a compartment is a biological entity within which the movement of materials is relatively rapid when compared with movement to and from areas outside the compartment. This means that the boundaries of the compartment effectively restrict the movement of the material of interest.

For accuracy of estimates of VFA flux rates from infusion (or single injection) studies there must be a rapid and uniform mixing of labelled VFA throughout the entire reticulo-rumen. This requirement is seldom ideally met. Variations in VFA concentrations can occur in different sites within the reticulo-rumen indicating that the rumen contents are not always homogenous.

Slow or incomplete mixing of tracer can be a major source of error when tracer techniques are used to estimate VFA production rates. In recognition of the importance of mixing in the compartment into which tracer is administered, researchers have tried many options for improving the rate of tracer mixing. For example, Sutherland *et al.* (31) and Blake *et al.* (3) devised pumping systems that artificially circulated rumen contents to achieve more rapid mixing of tracer and tracee. To assist the rate of distribution of tracer through the rumen contents, the rate of infusion of solution containing the tracer should be as high as possible – with a balance being struck between the volume added and the extent of perturbation of normal water kinetics in the rumen. To improve tracer and tracee mixing in the rumen ammonia compartment during a continuous intraruminal infusion experiment using labelled ammonium chloride, Oldham *et al.* (25) placed a stainless steel frame in the rumen on which there were two tracer infusion sites approximately 20 cm apart, sited near the centre of the rumen, and three separate sampling tubes. The frame was designed to improve the distribution of tracer in the rumen contents and also to shorten the time taken for tracers to become evenly distributed throughout the rumen contents, and to keep a discrete distance between the points of infusion and the points of sampling.

To help workers to recognize and allow for problems of less-than-ideal mixing of tracer, we recommend that a liquid marker (such as Cr-EDTA or Co-EDTA) is included in the infusate containing the labelled VFA. The Co or Cr kinetics indicate how quickly and uniformly the infusion solution mixes throughout the rumen contents.

1.5. Single Ruminal Dose (Injection) of ^{14}C Labelled VFA

An alternative to infusion is to make a single injection of a more concentrated ^{14}C-VFA into the rumen. Sheppard *et al.* (29) and Gray *et al.* (12) used single injections of ^{14}C-labelled VFA to estimate their production rates in the rumen of sheep. An instantaneous injection offers several advantages over infusion, *viz.*

- problems associated with infusion pumps and broken infusion lines are avoided,
- the injection can be made at multiple sites within the rumen assisting the more rapid and even distribution of the tracer in the rumen contents, and
- the isotope dilution curve allows compartment size of the VFA and total flux rate to be determined, not just net flux rate.

A disadvantage of single injection experiments is that the production rate estimates apply to the few hours after injection which may not give a good estimate

of daily VFA production rate. The method also requires that samples are taken more frequently during this critical time.

The conducting of an intraruminal ^{14}C-VFA injection should be as follows:

- Dose prepared of 50 mL × 56 kBq/mL and containing 25 mL of Cr-EDTA solution (1).
- Sub-samples of injection solution taken into labelled empty McCartney bottle and stored frozen (do not fill > 3/4 full).
- Dose dispensed into 50 mL syringes and full-syringe weighed immediately prior to injection.
- Dose introduced via 40 cm (× 3 mm i.d.) curved steel tube and rapidly dispensed into 10 rumen sites with approximately 5 mL/site.
- Dose syringe removed and new syringe with 100 mL of water used to wash residual dose from steel tube into the rumen.
- Final (empty) weight of injection syringe MUST be determined to enable the weight of dose injected to be determined.
- Time of dose introduction and time of each individual sample must be recorded precisely.
- Samples taken 20, 40, 60, 90, 120, 150, 180, 240, 300, 360, 420 min post-injection and stored in acidified McCartney bottles and frozen.
- If more than one ^{14}C-VFA infusion or injection is to be conducted, the preferred dosing order is butyrate, propionate then acetate and if possible, a day should be allowed between each infusion to allow residual radioactivity to be diluted out.

1.5.1. Sample Analysis

The fundamental analytical requirement for this or more complex analyses is to establish the specific radioactivity (SR) of the infused VFA in the rumen, *i.e.* radioactivity (MBq per mole of VFA). This requires the VFA of interest be isolated, quantified and its radioactivity determined.

In early studies, Gray *et al.* (12) simply steam distilled rumen fluid to release all VFA and determined the concentration of radioactivity in the distillate after determining the total VFA present by titration. This method is still used by some workers (30). Weller *et al.* (34) subsequently showed that the SR of a single labelled VFA could be used to give satisfactory estimate of the net flux of all three major VFA. There are a number of means by which radioactivity in rumen contents can be obtained including:

- Quantification and isolation of labelled VFA by HPLC and determination of radioactivity by liquid scintillation counting (e.g. 13).
- Quantification and isolation of labelled VFA by liquid chromatography and determination of radioactivity by liquid scintillation counting (e.g. 19).
- Gas chromatographic quantification of component VFA and on-line determination of radioactivity by means of a proportional gas flow through counter. Czerkawski and Breckenridge (1975) utilized this radioactivity sensor system but it has not been widely used. The simplicity of the system and low cost compared to liquid chromatography and liquid scintillation could justify its renewed investigation for application in developing countries.

2. CALCULATION OF VFA PRODUCTION FROM SAMPLE SPECIFIC RADIOACTIVITY

Several calculation options are presented. The analysis to be used will depend on whether the results are derived from single dose experiments, or single or multiple infusion experiments. In all cases, VFA samples taken before tracers were administered should be assayed for background radioactivity and the results subtracted from VFA samples taken after the administration of tracer. Most analyses of the data involve certain assumptions, and the analyses in common use often do not use all of the information implicit in the SR versus time curves resulting from tracer dilution.

2.1. Single Dose Experiments

These are studies in which a single bolus of ^{14}C-VFA is injected into the rumen at a single time and the dilution of added radioactivity followed over 6–9 h. One limitation is that instantaneous mixing of tracer and tracee is assumed. Also VFA production in the period soon after injection contributes more to the flux estimate than production later in the sampling period.

- If a liquid marker is introduced with the ^{14}C-VFA tracer, the concentration of marker in all samples (mg Cr-EDTA/L) should be analysed. If the decline in marker concentration with time is well fitted by a single exponential function, this indicates there was rapid mixing of the labelled solutions and a steady state of fluid turnover in the rumen existed. If early data points vary from the line of best fit, the implication is that the injection solution was slowly distributed throughout the rumen contents.
- The rumen fluid volume is given by dividing the dose of Cr-EDTA injected (g) by the intercept of the curve at time zero (g Cr-EDTA/L rumen fluid). This information, together with values for the molar concentrations of individual VFA, can be used to estimate the VFA compartment sizes (g C) in rumen contents.
- Assuming mixing of the dose solution was deemed satisfactory, the SR versus time curve should be fitted using single or multi-exponential functions (Eqn. 1), depending on the tracers used (22).

$$(1) \qquad SR_t = \sum A_i \exp(-{}^{k}i^{t})$$

where SR_t is the SR at time t, A is the intercept of the curve at time zero and k_i is the rate constant of the ith exponent.

- If propionate is infused, the propionate flux rate can be closely approximated from a single exponential decay whereas if acetate or butyrate were infused, where interconversions and recycling of ^{14}C will have occurred, then a multi-compartment model must be assumed and two or more components must be fitted to the curve as described above (Eqn. 1).

At time zero (the moment of injection) the multi-exponential equation simplifies to

(2) $SR_0 = \text{Sum } (A_i)$

The exponents (A_1, $A_{2...}$ A_i) are the intercepts of the component vectors that can be summed to give the SR at the moment of injection. Dividing the dose (Bq) by this SR (Bq/g C) gives an estimate of the size of the VFA compartment (g C).

The total flux of VFA carbon (TF, g C/d) and net flux of VFA (NF, g C/d) are given by

(3) $TF = \text{Compartment size (g C)} \times \text{(tangent to curve at time zero)}$

(4) $NF = \text{Dose injected (Bq)/(area under curve (Bq/g C.d)}$

(see Nolan and Leng (22)).

2.2. Continuous Infusion Experiments

- Again the background radioactivity in the pre-infusion samples of VFA should be subtracted from the radioactivity in all samples taken after the start of the tracer infusion.
- The SR (Bq/g C) versus time curve should be plotted to demonstrate that a constant SR had been reached after 3 h.
- A plateau SR can either be calculated as the mean of measured SRs from, say, 3–9 h after the start of infusion (assuming there is no trend in SR), or can be calculated after fitting an appropriate model to the data and predicting the SR at plateau.
- The net flux of the VFA through the primary compartment is calculated as follows:

(5) $$\text{Net flux (g C/d)} = \frac{\text{Infusion rate (Bq/d)}}{\text{Plateau SR (Bq/g C)}}$$

The use of the net flux equation (Eqn. 5.) is a standard method for deriving the net flux of a tracee when only one acid is infused. However, various different ways of analysing results from one or more ^{14}C-VFA infusion have been used, producing different measures of VFA production. These options and their implications for estimates of methane production are discussed below. Differences in estimates arise depending on whether cycling of VFA carbon (between acetate and butyrate) is ignored or allowed for, and whether radioactivity is determined in individual VFA (e.g. SR of acetate-C) or in the bulked VFA (radioactivity in total VFA carbon).

The most detailed analysis of VFA production will be a complete analysis of the flows depicted in the general three-compartment shown in Figure 1. The model can

be solved by matrix algebra using Microsoft Excel™or other software (Excel version available on request). If several continuous infusions of tracer have been used, the data required are plateau SR values for acetate, propionate or butyrate during individual infusions of each acid, and the tracer infusion rates. It is assumed that the same conditions (animal diet and steady state etc) applied to each experiment.

To illustrate the range of possible combinations of ^{14}C-VFA tracer application in the animal, and laboratory analysis procedures, three approaches to analysing and interpreting VFA production studies are considered with respect to their effect on estimated methane production. Data of Bergman *et al.* (2) are used as they represent a comprehensive database that can be drawn on selectively to evaluate all data options. These workers infused labelled acetate, propionate and butyrate separately into sheep offered grass cubes continuously from a moving belt feeder (900 g/d). The rumen contents were artificially mixed with a pump. The total VFA concentration in rumen fluid was 105 mmol/L, acetate, propionate and butyrate concentrations expressed in molar percentages were 68:19:13 and compartment sizes of 395, 110 and 75.6 mmol or 9.49, 3.98 and 3.63 g C, respectively.

All production parameters for each VFA were derived from complete solution of the 3 pool model (total, net flux, *de novo* synthesis and VFA disposal) and stoichiometry used to predict the anticipated net hydrogen synthesis and methane (Approach 1). Data were then re-analysed to provide stoichiometric estimates of methane production had only the SR of a single infused VFA been known (acetate or propionate) (Approach 2) and from the gross SRs of the combined VFA together being measured after steam distillation (Approach 3).

Approach 1. *Infusion of radioactively labelled acetate in one experiment, propionate in another experiment and butyrate in a third experiment in sheep maintained under similar conditions throughout the three experiments*

This most costly and lengthy procedure, requires separate infusions of three individual ^{14}C-VFA tracers and determination of the SR of all three VFA during each experiment (e.g. 2, 17). This 3 compartment model gives estimates of total flux, net flux and *de novo* production rates for each compartment and requires as data inputs, VFA infusion rate in each experiment (mBq/d) and the SR at plateau (Bq/g C) for each of the VFA during each experiment. The infusion rates in each experiment (adjusted to 100 μCi/h) and the corresponding plateau SR values for Bergman *et al.* (2) are given in Table 2.

The general three-compartment model can be solved by solving 12 simultaneous equations describing the flows of tracer and tracee, or by a matrix algebra procedure. The model solution with all fluxes (g C/d) is given in Figure 6. The measures of VFA flux and methane production are summarized in Table 3.

The difference between total flux and net flux rate for each VFA compartment is an estimate of the rate of carbon recycling to the compartment. From Figure 6 and Table 3 it can be noted that:

- Use of total flux gives the highest estimate of methane production. This is because total flux includes carbon of the specific VFA that has been changed to some

Table 2. Plateau Specific Radioactivity of Rumen Acetate, Propionate and Butyrate in Sheep During Infusions of Labelled Acetate, Propionate and Butyrate in Separate Experiments. Results are Means From 3 Separate Infusions of Each of Three VFA Tracers and are SRs are Adjusted to Infusion Rates of 100 μCi/d Bergman *et al.*, (1965)

Tracer	Infusion rate (adjusted)	Acetate SR μCi/g C	Propionate SR μCi/g C	Butyrate SR μCi/g C
[1–^{14}C]-acetate	100 μCi/h	21.1	2.42	9.42
[2–^{14}C]-propionate	100 μCi/h	3.00	54.3	2.17
[1–^{14}C]-butyrate	100 μCi/h	12.8	2.58	47.5

other compound and back to that VFA during the experiment. Total flux should not be used to estimate methane production because hydrogen production or consumption need not be associated with remaking the VFA.

- The rate of net flux (also referred to as irreversible loss rate) from each VFA compartment is intermediate between the total flux and the rate of *de novo* synthesis (bold arrows) or final disposal (dashed arrows). This is because irreversible loss of tracee occurs not only by direct outflow from the compartment but also from other compartments after interconversion between acids has occurred.
- The sum of the rates of *de novo* incorporation of carbon into each VFA (bold arrows) is equal to the sum of the rates of final disposal of VFA carbon (dashed arrows), but usually differ for each compartment as a result of the VFA interconversions.
- For each VFA, disposal rate should most closely reflect the quantity of VFA available for absorption from the rumen.

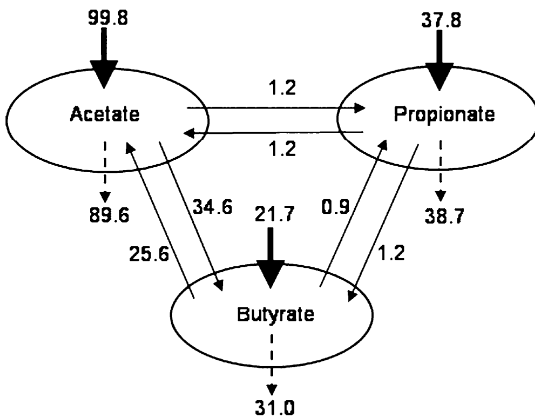


Figure 6. Solution to general three-compartment model of acetate, propionate and butyrate kinetics (g C/d) in the rumen of sheep based on data of Bergman *et al.* (2). Bold arrows = *de novo* synthesis; dashed arrows = final disposal rate

Table 3. Estimates of Methane Production Rate Derived from a Range of Flux Parameters from ^{14}C-VFA Infusions in Sheep. Methane Stoichiometry Explained in Section 3

Model	Flux Parameter	Flux			
		Acet. g C/d	Prop. g C/d	Butyr. g C/d	Methane moles/d
3 pool model *(3 VFA infused)*	Total flux	129.9	44.5	57.4	3.00
	Net flux	113.8	44.2	50.5	2.59
	De novo	99.8	37.8	21.7	2.04
	Disposal	89.6	38.7	31.0	1.92
	Net effect of including VFA interconversions	–	–	–	0.21
Acetate infusion, SR of acetate determined	Net flux	113.8	47.7	43.5	2.50
Propionate infusion, SR of propionate determined	Net flux	105.4	44.2	40.3	2.31
Acetate infusion, SR of bulk VFA determined	Net flux	92.8	38.9	35.5	2.03

*: Stoichiometry described in section 3

Approach 2. *Infusion of a single ^{14}C-labelled VFA and determination of its unique specific radioactivity*

A single infusion of one ^{14}C-VFA and determination of its plateau SR allows VFA production to be determined and stoichiometric estimates of methane production. The net flux of the VFA (net flux equation; Eqn. 5.) is obtained but no data on recycling is generated. Assuming the molar proportion of the component VFA in rumen fluid reflect their net flux, the net flux of each VFA is derived from the measured flux of the one infused VFA. The data of Bergman *et al.* (2) indicate that if only ^{14}C acetate had been infused, the estimates of acetate, butyrate and propionate net flux would have been 113.8, 47.7 and 43.5 g/d, respectively (Table 3). In contrast, the infusion of ^{14}C-propionate and determination of the SR of propionate would lead to slightly lower estimates of VFA production. This is a consequence of a lesser proportion of propionate carbon recycling into the propionate pool than occurs for acetate, but no measure of recycling can be obtained from any single acid infusion (Table 3).

Approach 3. *Infusion of a single ^{14}C-labelled VFA and determination of the radioactivity in bulked VFA*

A less complex and less expensive procedure that can still give useful information, especially for hydrogen and methane production, was described by Weller *et al.* (34).

A single VFA (e.g. ^{14}C-labelled acetate) is infused and rumen fluid samples are collected after the plateau SR is reached. All VFA are steam distilled simultaneously and the total VFA concentration (mmol acid/mL of distillate) and total radioactivity (DPM/mL of distillate) are determined so that the mean SR of VFA (DPM/mmole VFA) can be calculated. The DPM/mg total VFA-carbon during an intraruminal ^{14}C-acetate infusion was calculated from the experiments of Bergman *et al.* (2) Allowance was made for the proportion of VFA carbon in the total VFA contributed by acetate, propionate and butyrate to give the SR of all three VFA that would have been found in a single distillate (i.e. 16.9 μCi/g C, cf. row 1 of Table 2). The net flux of combined VFA was estimated using the equation $SR_0 = \text{Sum}(A_i)$ (5.40 mol/d) and net fluxes of the individual VFA were then determined by multiplying combined net flux rate by the molar proportions of each acid.

This gave flux rates of 3.87, 1.08 and 0.74 mol/d or 92.8, 38.9 and 35.5 g C/d for acetate, propionate and butyrate, respectively (Table 3). These net flux estimates are fairly similar to the estimates of de novo incorporation for each VFA as determined by separate infusions of all three VFA, with the greatest error being associated with butyrate. This is on account of butyrate having a different absorption: ruminal concentration relationship to other VFA and because of the large amount of butyrate that is produced from acetate. Weller *et al.* (34) have undertaken a similar analysis and reached similar conclusions; however their estimates of net production of combined VFA (mol/d) differed depending on which VFA tracer was used, viz. 5.7 with propionate, 5.4 with acetate and 5.2 with butyrate compared with 5.4 when all three VFA tracers were used.

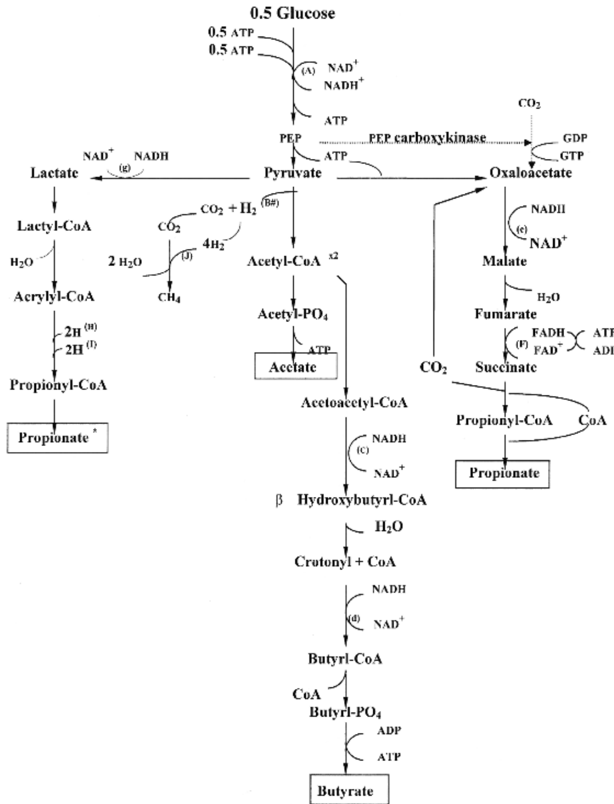
3. STOICHIOMETRIC ESTIMATION OF METHANE PRODUCTION FROM VFA PRODUCTION DATA

As indicated in the introduction, methane production in the rumen helps to limit hydrogen accumulation in anaerobic rumen contents. This in turn, prevents ‘feedback inhibition’ of reactions in the fermentation pathways, namely glycolysis, acetate production and butyrate production, which would reduce the rate of organic matter digestion. The hydrogen is removed from reducing agents such as NADH produced within these pathways and the majority is used to reduce CO_2 to methane gas that is then removed from the animal with other expired gases.

If the fermentable organic matter (cellulose, starch etc.) entering the rumen is considered to be degraded via glucose, the pathways of VFA formation can be represented according to the scheme in Figure 7. (The scheme is a simplification because degradation of other organic materials such as proteins is not explicitly represented.)

The individual pathways present in most micro-organisms are depicted in Figure 7, which also summarizes the hydrogen inputs and outputs associated with production of each VFA.

The pathways in Figure 7 show that NADH is produced in glycolysis and also when acetate and butyrate are formed from the resulting pyruvate. NAD^+ is



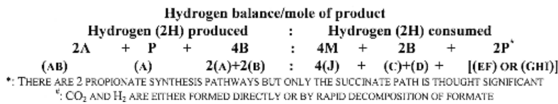
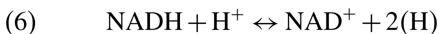


Figure 7. Pathways leading to VFA production in rumen. Hydrogen (2H) utilized and consumed in the synthesis of acetate, propionate and butyrate are summarized. After Gottschalk (10)

regenerated when propionate (and valerate – not shown in Fig. 7) are formed, or when the NADH dissociates to form H_2 gas. High rates of fermentation of feed carbohydrates result in increases in intracellular NADH concentrations that may tend to increase further if microbial growth is impaired for any reason, and fermentation and growth are uncoupled. NADH is in reversible equilibrium with H_2 gas, represented here as 2(H) in the rumen.



$$(6) \qquad NADH + H^+ \leftrightarrow NAD^+ + 2(H)$$

Regeneration of NAD^+, promoted by the removal of 2(H), is necessary to enable fermentation reactions that generate NADH (glycolysis, and acetate and butyrate

formation) to continue. Thus, synthesis of acetate and propionate are complementary processes and often occur simultaneously in anaerobic microbes.

When NADH concentrations increase, the normally low rumen H_2 concentration (about 10^{-4} atmospheres; Wolin (36)) moves the equilibrium to the right, promoting release of more gaseous H_2. (H_2 is also released from formate derived from pyruvate by some methanogenic species.) Gaseous H_2 seldom accumulates in the rumen because it is apparently transferred from hydrogen-producing microbes to methanogenic organisms that obtain ATP by using it to reduce CO_2 to CH_4, according to the following equation

$$(7) \qquad CO_2 + 8(H) \quad \rightarrow \quad CH_4 + 2H_2O$$

If all 2(H) produced during the production of these VFA ('A' is acetate etc) were used in methane synthesis, then potential methane production would be given by

$$(8) \qquad 0.5A + 0.5B - 0.25P - 0.25V$$

Predicting methane production from the above equation would be valid only if:

- these VFA were the only fermentation end-products (i.e. no hydrogen is used in cell polymer production)
- no free H_2 escaped from the rumen
- the microbial digestion processes were strictly anaerobic (i.e. no H_2 is used to reduce O_2 to H_2O)
- H_2 is not used in other reactions, e.g. the reduction of sulphates to sulphides or of double bonds in fatty acids.

In real life, production of methane will be lower than the equation predicts because these assumptions are not totally correct. We know, for example, that some NADH, or 2(H), is oxidized to provide energy for synthesis of cell polymers (e.g. lipids, amino acids and nucleic acids) during growth of cells, and in various other redox reactions (6).

In summary, if the net disposal rates of individual VFA are known or can be estimated, the net NADH and hydrogen generation can calculated and methane production predicted. If ATP production during hexose fermentation is also calculated, estimates can be made of potential cell synthesis and of the 2(H) removed by this synthesis. This will allow a better prediction of methane production.

One means of making the relevant calculations is to use a model similar to that proposed by Czerkawski (6). This model has been implemented as software by Nolan (24).

At this point it is also worth noting that many workers have relied on molar concentrations of the VFA, expressed as molar percentages, to develop equations for predicting methane production, rather than molar *de novo* synthesis rates. This introduces another error that is often not explicitly stated. This error will arise if it is assumed that molar concentrations of individual VFA (expressed as molar percentages of total VFA) will correctly represent individual VFA disposal rates. Even though quite good relationships between VFA concentrations in rumen fluid and net production rates have been demonstrated (19, 15, 33), interconversions of

VFA are not fully accounted for, and factors such as changes in rumen pH (11, 28) and osmotic pressure (21) alter these relationships to some extent and will affect some VFA more than others (7).

3.1. General Discussion

Since VFA are the principal energy source for ruminant tissues, the techniques of measuring VFA production have made major contributions to understanding of ruminant physiology and energetics. There are however, a number of possible experimental procedures, and a number of descriptions of 'VFA production' that can be derived from any one *in vivo* procedure used to measure VFA flux. Selecting the appropriate measure of VFA flux to use in taking a stoichiometric approach to predicting methane production is somewhat subjective and it is easy to have circular arguments about which flux should be used.

The comparison summarized in Table 3 identifies that the differences in estimated methane production arising from use of different VFA flux measures may be up to 25% and this variation due to flux measurement will magnify the already considerable experimental errors associated with making ruminal measurements over 6 h to 3 d. Using the data from studies by Bergman *et al.* (2), Leng and Leonard (18) and Leng and Brett (19), Rowe (27) attempted to calculate errors associated with the procedures mentioned above and concluded that (a) combined production rate of acetate, propionate and butyrate was always overestimated by the single VFA infusion technique (3.5% ± SE 1.1, n=6), (b) accuracy of estimates of 'effective production rate' of individual VFA was variable, the difference between predicted values and those estimated by using individual infusions of each VFA ranging from −36% to +44% of the mean, which is more than our estimates in Table 3.

On the basis of modelling conducted to create Table 3, it can be recommended that for most (grain and roughage) diets, the single acid infusion procedure (preferably using propionate) should be used, with isolation of that acid from the VFA and determination of its SR. This approach is intermediate in terms of complexity and also in its ability to avoid calculating the interconversion of the VFA. Consequently, the estimation of daily methane production by this method (2.49 moles/d) was 89% of that estimated if the net flux of each VFA had been measured separately, but 14% higher than if the SR of bulked VFA had been relied on. The error in estimating methane production from ignoring the interconversion of VFA (acetate : butyrate, butyrate : propionate) of 0.21 moles was less than 10% of methane production.

On diets that have high proportions of butyrate (such as molasses based diets), this procedure can be expected to generate greater error. This is because acetate: butyate interconversion and because the relationship between butyrate proportion and absorption differs from that of acetate and propionate (7). Only a 'three-acid' infusion or injection protocol will suffice for such diets.

From *in vitro* studies, where rises in concentration of VFA and methane are subject to far less experimental error than occurs in whole animal studies, stoichiometry does typically produce good hydrogen recoveries although again, recoveries fall when

methanogenesis is interrupted. *In vivo*, however, stoichiometry should not be viewed as an accurate way of estimating methane production. Clearly, the most appropriate way to determine methane production in a ruminant is to measure the amount produced by direct methods (as described in other chapters of this IAEA text) rather than by using predictions based on stoichiometric relationships.

If the animal is housed in a chamber or an enclosed room the mixed gases released (both expired and flatus) can be analysed for methane concentration. If the rates of flow of gases from the chamber or room are determined, then a direct estimate of methane release from all segments of the gut of the animal can be determined directly. Estimates can also be made in enclosed chambers by determining the small increase in methane concentration over short periods.

Probably the next most appropriate means of determining methane production is to estimate it by tracer dilution methods. Methane labelled with ^{14}C, ^{3}H or deuterium can be used and the SR of methane determined over time (14). The precision of these estimates will depend on how effectively the tracer and tracee are mixed in the rumen, and on the analytical techniques. The method based on constant release of SF_6 in the rumen is another potential method, and the possible errors have been discussed (32, 4, 9).

Finally, VFA production can be determined *in vivo* as described above and predictions of methane production can be made using stoichiometric equations. This is probably the least appropriate measurement option because the predictions based on VFA production will theoretically result in an overestimation of the true methane production due to assimilation of hydrogen during the synthesis of microbial polymers and in other reactions are not been allowed for. The importance of these unspecified reactions is difficult to gauge and may depend on the mix of species of bacteria and other microbes present. Their effects may be greater when inhibitors of methane production have been included in the animal's diet.

While determinations of ruminal VFA production *in vivo* are helpful for digestive and energetic studies of diet utilization and the animal, they should not be looked upon as the most appropriate method to quantify enteric methane production. Leng (7) envisaged measurement of methane production as a simple way of estimating VFA production rather than the reverse. This is because (a) the current requirement for cannulated animals, (b) the complex laboratory procedures associated with isolating individual VFA and (c) the errors associated with assuming rumen conditions are constant over the 3–4 d of experimentation. Consequently, for laboratories seeking a single direct means of measuring only enteric methane production, procedures utilizing direct methane tracers or respiratory hoods or chambers are recommended.

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CHAPTER 5

MEASUREMENT OF METHANE PRODUCTION RATE IN THE RUMEN USING ISOTOPIC TRACERS

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INTRODUCTION

Methane production by ruminants has been estimated by isotope dilution (10) using both ^{3}H- and ^{14}C-labelled CH_4. As with any technique of this type, its accuracy depends on the efficient mixing of the labelled material (tracer) within the pool that is to be measured (tracee), in this case CH_4 in the rumen. The solubility of CH_4 is low and its diffusibility is high, so these properties cause difficulties not encountered with non-gaseous solutes. However, with care these problems can be minimized, and the continuous infusion of $^{14}CH_4$ into the rumen can yield valuable measurements of methane production. The technique can be used to estimate production in the rumen by sampling the gas phase in the dorsal rumen, or alternatively total tract CH_4 production (less a small correction for methane lost from the anus) if total expired gases are collected.

1. PRINCIPLE OF THE METHOD

The principle of using an isotopic tracer infusion to measure methane production (10) is similar to that applied when SF_6 is used as a tracer (6). The isotope is introduced into the rumen continuously at a constant rate (MBq/d) leading to a rise in the specific radioactivity (SR) of ruminal methane to a constant 'plateau' value (mBq/g methane carbon). Arithmetic division of the infusion rate by the specific radioactivity of methane on plateau provides a measure of the flux rate of methane (g methane carbon/d).

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2. METHODS AND MATERIALS

2.1. Experimental Animals

Cattle or sheep need to be fitted with gas-tight rumen cannulas to enable labelled methane to be introduced (10). Metal infusion lines must be used to deliver the ^{14}C-methane solution into the ventral rumen. These infusion lines are typically pliable 1/4 inch diameter copper tubing of the type used to carry gas to a gas chromatograph. A stainless steel tube passing directly through the rumen cannula into the gas phase in the dorsal rumen can be used to withdraw gas samples (Fig. 1), or a face mask can be used to collect samples of expired air. Since intraruminal permeation devices have been used to deliver other gases (e.g. SF_6), it may also now be possible to create permeation devices to deliver labelled isotopes of methane into the rumen, and in combination with breath sampling, thereby remove the need for rumen cannulas in the future (refer to Delivery of isotopic CH_4 by intraruminal permeation devices on Page 100).

2.2. Reagents and Equipment

Reagents

- $^{14}CH_4$ (3.7 MBq/sheep)
- Distilled water
- 1M NaOH (CO_2 absorbent)
- 5% (w/v) NH_4Cl (25 mL/animal)

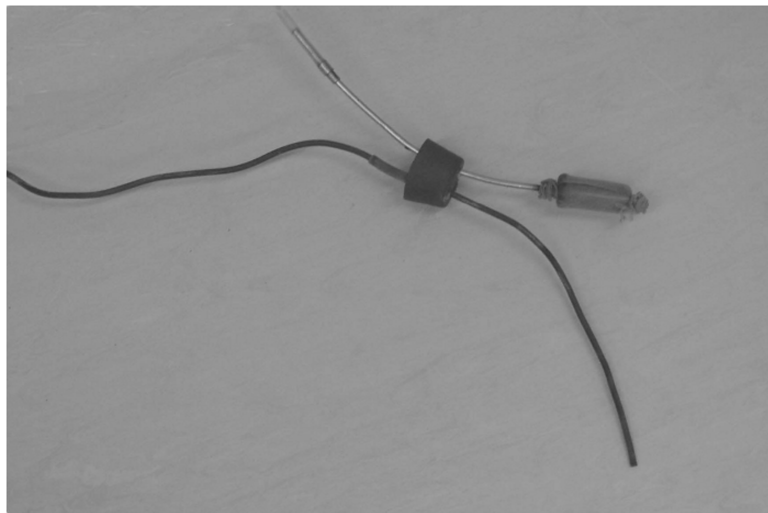

Figure 1. Plug from rumen cannula showing copper infusion line penetrating 15 cm below plug and smaller gas sampling probe directed upwards

- 20% (w/v) $BaCl_2$ (15 mL/animal)
- Acetone (technical grade; 200 mL/animal)
- Scintillation gell (suited for supporting suspended powders; 3.5% cab-o-sil thixotropic powder, 0.4% PPO and 0.2% POPOP (w/v) in xylene has been used)
- Cylinder of non-radioactive pure methane (1L STP/8 animals)

Equipment

- Bunsen burner to boil water (5 L)
- Erlenmeyer flask to prepare infusate (Fig. 2)
- Steel infusion syringes to store and pump infusate
- Piston or Palmer pumps for infusion syringes
- 5m/animal of 1/4 inch copper tubing
- Peristaltic pump for water infusion
- Rumen infusion and sampling probe (Fig. 1)
- 100 mL glass syringe with 3 way tap for rumen gas collection
- 10 × 25 mL Terumo™ brand syringes for gas collection/animal
- 10 × blind (sealed) needles to seal collection syringes
- 10 × 5 mL syringes for trapping CO_2 in NaOH per animal (Fig. 4)
- Tube furnace (Fig. 3)
- Filter disks (2.5 cm; Whatman No. 1)
- Planchette and suction system for 2.5 cm diameter filter papers
- Drying oven (95°C)
- Scintillation vials (12/animal)
- Glass beads (20/animal; 2 mm dia)
- Vortex mixer

2.3. Preparation of Infusion Solution

For this description of methodology it will be assumed that $^{14}CH_4$ methane is to be infused via a ruminal cannula. Methane has a very low solubility, which creates technical challenges to infusing it, and the possibility of losses during handling due to diffusion and leakage. Thus at all stages during the preparation of an infusion solution, strict precautions must be taken to avoid loss of gas. Achieving the correct solution for infusion is the most difficult part of the whole procedure, and most errors arise from the production of the infusion solution or its transfer to the rumen.

The apparatus for preparing ^{14}C-methane solution is made of glass and metal, with butyl rubber bungs and seals (Fig. 2) except for a flexible plastic bottle whose purpose is to enable the system to be flushed with water and made gas-tight. Distilled water is de-gassed by boiling for 20–30 min and introduced into the conical flask and flexible plastic bottle. The vial breaker with a 1 mL vial of ^{14}C-methane (37 MBq or 1.0 mCi; 131 KBq/mmol) is then introduced into the flask while the water is hot, and the flask is closed and *all* pockets of air or bubbles are expelled by squeezing the collapsable container. The outlet line is then closed and the whole assembly is allowed to cool. The plastic bottle collapses as the volume contracts.

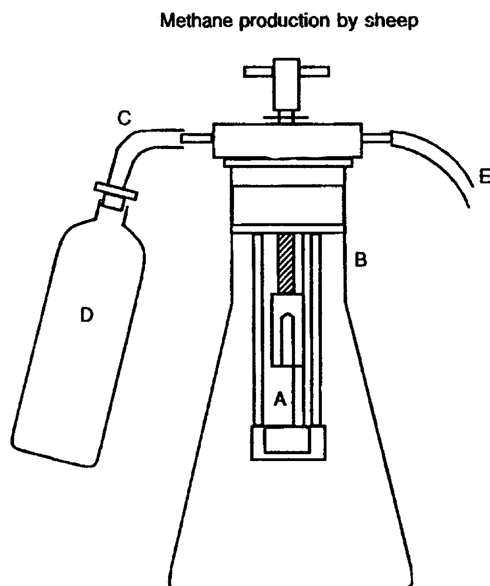

Figure 2. Apparatus used to transfer radioactive methane gas to the infusion solution. A: vial breaker; B: sealed 2 L flask; C: outlet tube; D: collapsible 250 mL plastic bottle; E: outlet tub. After the methane ampule has been broken and the methane gas dissolved (6–48 h) into the refrigerated water, the solution is rapidly transferred into stainless steel gas infusion syringes (300 mL each) or other sealed storage vessel

The glass vial containing the stock ^{14}C-methane gas is then crushed by turning the screw, and the methane is allowed to dissolve for 3–6 h. Cooling improves the rate at which methane dissolves in water but it may take several days for the bubble of methane to fully dissolve. This ^{14}C-methane solution can be used as prepared, or alternatively can be transferred to air-tight glass bottles and stored in a cold room. This transfer of solution containing dissolved $^{14}CH_4$ to storage bottles (or preferably to brass infusion syringes) is made as quickly as possible while the solution is still cold, minimizing the time the solution is open to air.

2.4. Infusion Procedure

When rumen gas is to be sampled via the cannula rather than as diluted in exhaled air, a lower ^{14}C dose can be used than the 18 to 54 kBq/min (or 0.5–1.5 μCi/min) used by Murray *et al.* (10). A dose rate 3.7 MBq/sheep of $^{14}CH_4$ dissolved in 60 mL of infusion solution pumped at a rate of 0.1 mL/min for 9 h is used for intraruminal infusion. This solution is blended with a larger flow of clean water pumped by a peristaltic pump (1.2 mL/min) via a ‘T’ piece as it exits from the syringe. Blending water with the infusate before it goes into the copper rumen-delivery pipe means that only a small volume of stock ^{14}C infusate needs to be dispensed. This makes

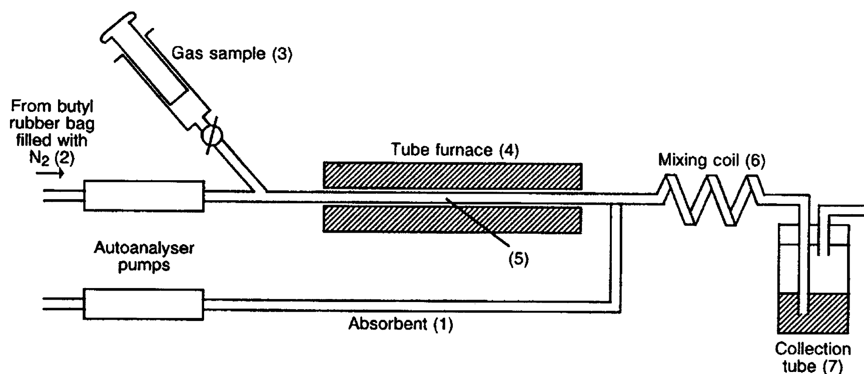

Figure 3. Apparatus used for oxidation of CH_4 to CO_2 in determining specific radioactivity of CH_4 in rumen gas samples

1. Absorbent: 1M $C0_2$-free NaOH is stored in a reservoir equipped with a carbasorb CO_2 trap. The flow rate is 2 mL/min.
2. Carrier gas: Nitrogen is stored in an impermeable butyl rubber bag and pumped through the system at a rate of 9 mL/min.
3. Sample entry: The sample is injected from a syringe via a rubber septum into the carrier gas stream and into the column or via a 3-way tap.
4. Oven: The column is heated by an electric element to 780–790°C.
5. Combustion: A quartz glass tube containing a catalyst of copper oxide, 150 g (wire from 0.6 mm BDH Chemicals or Merck). Alternative catalysts include nickel oxide or cobalt oxide.
6. Mixing coil: An autoanalyser type coil (1 m in length) is used to mix gas and absorbent streams and the latter is collected.
7. Collection tube: A quick-fit test tube is used to collect the sample and the $BaC0_3$ is precipitated with 2.0 mL 5% (w/v) NH_4 Cl and 0.8 mL 20% (w/v) $BaCl_2$.

storage and infusion of the necessary dose easier than if a large dilute volume of infusion solution is prepared in the first instance. In the original method published by Murray *et al.* (10), the radioactive infusion solution was delivered by a Palmer slow injection pump (C.F. Palmer Ltd, London, U.K.). Modern piston pumps would also be suitable, but a peristaltic pump will lead to the loss of gas by diffusion through the peristaltic pump-tubing even if Viton™ pump tubes are used. The rate of infusion (g/min) is calculated by weighing the infusion syringe at the start and end of the infusion and dividing the weight loss (g) by the duration of infusion (min).

The infusion is typically run for 9 h with rumen gas samples being collected at 50 min intervals over the last 6 h. These gas samples are withdrawn into glass 100 mL gas-tight syringes through a sampling probe directed dorsally into the rumen gas space (Fig. 1). Any rumen fluid collected with the gas is voided from the collection syringe when inverted and approximately 20 mL of gas sample is transferred via a metal 3-way tap into a 25 mL Terumo® disposable syringe for storage. As this storage syringe is being disconnected from the collection syringe, it is immediately reconnected to a 21 gauge needle sealed into a rubber bung to prevent gas loss from the syringe.

2.5. Determination of Specific Radioactivity

Various methods can be used for the determination of the SR (MBq/mg carbon) of rumen methane. Proportional radioactivity counters for gases are available for the measurement of radioactivity in CH_4 after it has been isolated and its concentration determined by gas chromatography. Alternatively, the CH_4 can be burned to CO_2 and the radioactivity of the resultant CO_2 can be subsequently determined in apparatus similar to that shown in Figure 3. Rumen gas samples contain approxi-

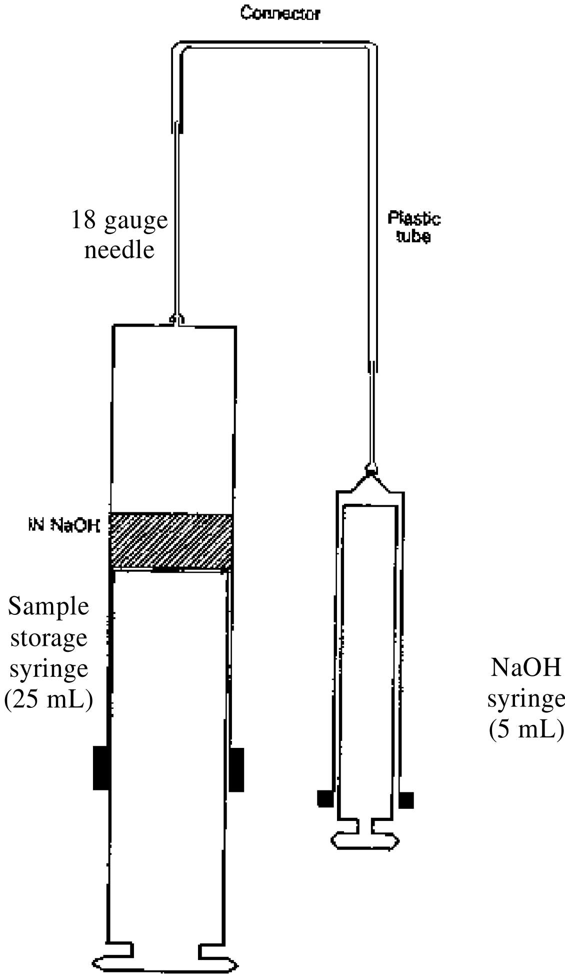

Figure 4. A simple apparatus for removal of CO_2 from samples of rumen gas. A 5 mL plastic syringe containing 3 mL 1 M NaoH is connected by a plastic tube to the storage syringe. The NaoH solution is transferred to the storage syringe to absorb CO_2. Although absorption is rapid, the connection is left overnight to ensure complete CO_2 absorption.

mately 70% CO_2 and this CO_2 in the sample must first be removed by adding 3 mL of 1 M NaOH to the 20 mL of gas sample held in the storage syringe. This is done by connecting the storage syringe to a 5 mL syringe containing the 3 mL of 1 M NaOH and pushing the NaOH into the gas storage syringe (Fig. 4) and shaking the interconnected syringes. The NaOH solution is then voided from the syringe when inverted and the remaining gas injected into the oxidation apparatus via a 3-way tap (Figure 3). Nitrogen is used as the carrier gas to take methane through the oxidizing tube furnace. The gas passes through copper oxide or a similar catalyst at 780 – 790°C where the CH_4 is oxidized to CO_2 (Fig. 3). The resultant CO_2 is then stripped from the gas mixture by blending the effluent gas stream with a CO_2 absorbent stream pumped by a peristaltic pump (1 M NaOH; 2 mL/min) in a glass mixing coil. Radioactivity in this solution could be counted directly using a scintillation cocktail but both the quantity of carbon and the radioactivity must be determined. Normally the effluent stream from the mixing coil is collected in a 20 mL bottle to which NH_4Cl (2 mL, 5%) and $BaCl_2$ (0.8 mL, 20%) have been placed. The absorbed CO_2 is immediately precipitated in this solution as $BaCO_3$.

The precipitate can be washed twice with water and once with acetone on a filter paper disk under mild (water) suction to remove contaminating salts then oven dried on the filter paper overnight. A subsample of $BaCO_3$ (20 mg) is qualitatively transferred into an empty weighed tared scintillation vial which is then over-dried overnight re-weighed. The SR of the $BaCO_3$ is determined from the weight of oven dry precipitate in the vial and the radioactivity it contains. Radioactivity is determined after addition of 2×2 mm glass beads and agitation on a vortex mixer to reduce the $BaCO_3$ precipitate to a fine powder. The powdered $BaCO_3$ is then stirred into a gell-type scintillation cocktail prior to scintillation counting. Any radioactivity determined in the pre-infusion (background) rumen gas sample is deducted from radioactivity present in plateau samples collected from hours 6–9 of infusion.

The same principle is applied to determine the radioactivity infused (which is normally determined at least in triplicate). Approximately 5 mL of non-radioactive methane gas ($> 99\%$ purity) is drawn into a 25 mL syringe that has been pre-weighed and pre-calibrated using water so that the volume of gas at a specific mark on the syringe is known exactly. The temperature and pressure of the laboratory are measured when the syringe is filled to enable the exact volume of methane in the syringe at STP to be calculated. The syringe containing this known amount of pure methane is sealed by attaching a blind needle. The methane filled syringe is then connected to a brass infusion syringe used in the experiment and approximately 2 mL of infusion solution is drawn into the methane-filled syringe. The weight of this syringe must be determined exactly before and after adding infusion solution so that the weight of infusion solution (approximately 2 g) is known. ^{14}C-methane from the infusion solution mixes with the non-radioactive methane in the syringe and the contents of the syringe are injected into the sample oxidizer exactly as for samples. From knowledge of the amount of methane-carbon in the syringe and the SR of the carbon in the $BaCO_3$ produced, the total radioactivity that must have been present in the infusion solution drawn in from the infusion syringe (approx. 2 g)

can be calculated. Consequently, the infusion rate (MBq/min) can be calculated as the product of the change in weight of the infusion syringe during the experiment (g/min), multiplied by the radioactivity in the infusion solution (MBq/g).

3. CALCULATION AND INTERPRETATION

Specific radioactivity determinations are made on 6–7 rumen fluid samples taken per sheep over 6–9 h of infusion, or by bulking these gas samples, storing and performing duplicate analyses. The rate of CH_4 production (g C/min) is calculated from the rate of labelled methane infusion (MBq/min) and the SR of methane carbon in the sample (MBq/g C) using the equation below:

$$CH_4 \text{ production} = \text{Infusion rate/mean SR of all samples.}$$

For animals fed once or twice daily, the rate of CH_4 production will fluctuate. In this situation the analysis of hourly samples then gives the most reliable picture of the overall pattern of production.

A laboratory setting up the $^{14}CH_4$ infusion procedures for the first time may have challenges preparing the $^{14}CH_4$ infusion solution and introducing this into the rumen without the $^{14}CH_4$ coming out of solution in an air-pocket or in transfer and being lost. Several alternative possible ways of administering labelled CH_4 into the rumen are presented below.

4. POSSIBLE ALTERNATIVE DELIVERY MECHANISMS FOR $^{14}CH_4$

4.1. Delivery of Isotopic CH_4 by Intraruminal Permeation Devices

There is potential for the use of permeation capsules to deliver labelled methane into the rumen contents of animals without the need for animals to be fitted with rumen cannulas. Intraruminal permeation devices like those used for SF_6 (6) have been prepared by Hegarty (5) to and tested with unlabelled methane (Fig. 5). Perhaps because methane has a lower boiling point (−161°C) and higher vapour pressure at 39°C (46 Bar) than does SF_6 (37.6 Bar), the release rate of methane is not constant but shows a linear decline over time (Fig. 5).

4.2. Introduction of Radio-Labelled Methane Precursors

Other potential ways of introducing labelled methane into the rumen have been suggested but appear to have serious theoretical objections. Ruminal methane is produced by the reduction of CO_2 or formate by hydrogen gas dissolved in the rumen contents (4). Protons in hydrogen gas exchange readily with hydrogen atoms in water (2) so it cannot be assumed that tritium gas or deuterium gas would be quantitatively metabolized to methane. Therefore, infusion of labelled H_2 as a

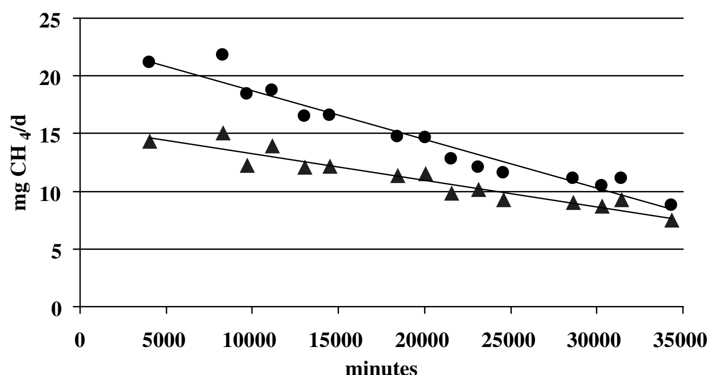

Figure 5. Release (mg/d) of methane from brass permeation tubes (tube 1 •; tube 2 ▲) containing approximately 1500 mL of methane (STP) and fitted with dual 125 μm thick teflon permeation windows

methane precursor is unlikely to give estimates of methane production that match those obtained by using infusing labelled methane itself.

While bicarbonate or formate (which is readily degraded to release carbon dioxide) can be infused into the rumen without risk of volatilization, they will also not be uniquely and quantitatively reduced to methane. Consequently, the 'dose' of ^{14}C label entering the methane pool from these tracers cannot be known. Similarly, ^{14}C-choline is thought to be largely metabolized to methane, but some is incorporated directly into rumen protozoa so the rate of flow of ^{14}C into methane from ^{14}C-choline cannot be easily established from the rate of labelled choline infusion.

5. USE OF NON-RADIOACTIVE TRACERS OF METHANE

5.1. Stable Isotopes

The fact that tritiated methane gives the same estimate of ruminal methane production as does $^{14}CH_4$ (10), indicates that methane hydrogen atoms do not readily exchange with hydrogens in water or other rumen compounds. Consequently, deuterium (CD1 – CD4) may offer a more user-friendly (but still ideal) tracer of ruminal methane than does $^{14}CH_4$. Studies are currently underway in Armidale to establish technologies for the delivery and analysis of deuterated methane in ruminal studies.

The $^{12}C/^{13}C$ signature of collected methane is routinely used to discriminate between sources of methane (9) and substrates used by methanogens (1). Harvey *et al.* (3) have described the variation in the carbon signature of exhaled methane from sheep and the between-animal variation that occurs. For isotopic analysis of atmospheric methane, large volumes of air are usually available and methane can be cryogenically concentrated. For animal studies, however, the same techniques cannot be used as sample size is normally 1–2 L at most. This significantly reduces the capacity to quantify the low enrichment of carbon that can be

anticipated in exhaled methane. It is apparent that some concentrating of collected methane, either at the point of collection or in the laboratory afterwards, will be required to use ruminally delivered $^{13}CH_4$ or deuterated methane as tracers for enteric methane production. Since the cost of high-enrichment deuterated methane is approximately half of the cost of high-enrichment ^{13}C labelled methane, cost of tracer as well as cost of analysis may favour deuterium as the preferred stable isotopic tracer.

5.2. Other Marker Gases

SF_6 is the principal tracer currently used for enteric methane studies, being chosen because of its inertness, its suitability for delivery by permeation devices and its ability to be measured at extremely low concentrations (parts per trillion) in expired air. While SF_6 has been routinely used as a non-metabolizable marker gas to 'trace' the behaviour of methane, the fact that SF_6 is considerably more dense than air and has a relatively higher solubility in water than does methane (41 v 26 mg/L) while only minute amounts are released from permeation devices (1–10 mg/d), does suggest it is not ideal for tracing enteric methane production.

Moate *et al.* (8) has used intraruminal injections of ethane into the rumen headspace gas to determine head-space methane production over short (20 min) periods but this technique would be of limited application for longer term assessment of ruminal methane production. Ethane has also been tested in high flow permeation devices (7) with a view to its continuous release in the rumen. The low (ppb) concentrations of ethane that are likely to be present in samples of expired air could however, make analysis difficult.

6. CONCLUSIONS

Isotope labelled forms of methane provide the ideal tracer for ruminal methane studies as the user can be confident their behaviour will mimic that of naturally occurring enteric methane. A method for introducing $^{14}CH_4$ in solution into the rumen and measurement of rumen methane is presented and can provide direct and accurate measures of ruminal methane production. To use this approach for field studies or to apply it on a broader scale, alternative means of tracer delivery to the rumen need to be developed. Options for this are being developed at present.

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CHAPTER 6

MEASUREMENT OF METHANE FROM GRAZING ANIMALS – THE TUNNEL METHOD

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INTRODUCTION / BACKGROUND

Because of the significance of CH_4 release from ruminants, both in terms of the utilization of metabolizable energy and its importance in greenhouse gas inventories its production has been measured directly or estimated through an understanding of energy balances (3, 4, 7). In all cases, these studies used animals housed under controlled conditions, receiving defined diets with the aim of assessing the dietary quality and intake on the release of CH_4 and energy loss.

Data from this type of study have formed the basis of current estimates of national and global CH_4 budgets. However, most ruminant production systems involve some element of grazing, where interactions between soil, plant and animal may influence the net CH_4 emission. Thus, not only will there be direct effects of grazing patterns and dietary quality on CH_4 production, but soil (as a potential source or sink) and excreta (as a source) may exert significant effects. Estimates of some of these individual effects have been made (2) but there have, until recently, been few attempts to provide integrated measurements of net CH_4 release from a grazing system (6, 9). In order to provide the necessary information, a tunnel system for measuring CH_4 release from grazing systems was developed at the Institute of Grassland and Environmental Research, UK (6).

1. METHODS

1.1. Tunnels

The original system used to measure CH_4 production from grazing sheep has been fully described in an earlier paper (6). Briefly, it consisted of (i) a large polythene

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tunnel, (ii) two small wind-tunnels used to blow air into, and draw air from, the larger tunnel, (iii) apparatus to measure and record the concentration of CH_4 in air entering and leaving the tunnel and (iv) apparatus to monitor and record airspeeds and temperatures.

The tunnel was a commercial, polythene-clad greenhouse modified to make the entire structure portable; it measured 4.27 m × 9.91 m, with a height at the ridge of 2.06 m and an approximate volume of 66 m^3. Its framework was covered with white polythene sheeting drawn down at both ends of the tunnel to form funnel-like connections to each of the small wind-tunnels.

The design and operation of the small wind-tunnels have been described by Lockyer (5); each consisted of a steel duct, 1.5 m long and 0.4 m i.d., housing a variable speed co-axial fan and a vane anemometer. Air flow through a tunnel could be controlled at rates of up to 0.9 $m^3 s^{-1}$. The output from each anemometer was fed to a data logger to provide an integrated measurement of airspeed from which the volume flow of air through the tunnel could be calculated. Subsequent studies found that the small wind-tunnel at the inlet was not required.

Further modifications were made to the system, essentially to enhance the portability and robustness. The large tunnel was renewed and a purpose built tunnel constructed from nylon reinforced plastic tubes that were inflated to give the tunnel shape (similar in principle to a child's 'bouncy castle' see http://www.southerninflatables.net/ for examples, (Fig. 1). When required the tunnel could be deflated, rolled up and transported. The dimensions of the tunnel were 4 m wide, 10 m long with a maximum height of 2.1 m, giving an approximate volume of 80 m^3. The rear wall of the tunnel was designed with a collar to fit around one of the smaller wind-tunnels.

Figure 1. Apparatus used to measure methane production

1.2. Methane Measurements

In order to determine the net CH_4 release from the grazing system it is necessary to take at least two air samples, one from the outlet wind-tunnel and one ambient sample taken up wind of the tunnel. Depending on the required accuracy of the data samples are taken as often as necessary. In the work of Lockyer and Jarvis (5) and Murray *et al.* (9) the CH_4 concentrations in air entering and leaving the polythene tunnel were measured through an automatic sampling and injection system connected to a gas chromatograph (GC) fitted with a Flame Ionisation Detector (FID). Samples were taken every 2 min, with alternate samples being drawn from the inlet and outlet of the tunnel, and injected onto the GC column; the output from the FID was scanned continuously by a data logger, which was programmed to detect, integrate and record each CH_4 peak.

Methane detectors such as GC systems or photoacoustic infra-red spectrometer trace gas analysers (10) can be housed in mobile laboratories and taken out to the field (1). Alternatively, it is possible to take a 'low-tech' approach and manually sample the outlet and ambient air by taking a syringe of the air and injecting it into a previously evacuated container. This sample can then be transported back to the laboratory and analysed using a GC.

It is also essential to know the airflow rate through the tunnel. This is usually measured on the anemometer in the small wind-tunnel and set at around $0.25\ m^3\ s^{-1}$ and logged on the data logger at the same interval as the CH_4 measurements are recorded. Alternatively, the air speed can be determined using a hand-held anemometer and checked at regular intervals (we suggest twice daily).

1.3. Recovery of Added Methane

Tests of the ability of the measurement system to make a quantitative recovery of added CH_4 should be made regularly. This is done by bleeding CH_4 into the tunnel through a precision gas flow meter for a known length of time and with a defined flow-rate.

1.4. Operation

The tunnel is placed on the grass sward and the small wind tunnel is attached to the rear of the tunnel. A set of hurdles or electric fencing is set up within the tunnel to stop the animals damaging the tunnel walls. Animals are provided with a source of drinking water and air temperature and humidity should be monitored to maintain animal welfare. In excessive temperatures animals must be removed from the tunnel.

An estimate of the amount of herbage on offer inside the tunnel is made by cutting two $0.5 \times 0.5\ m^2$ quadrats cut to ground level. The herbage is then dried at 80°C and weighed. The amount of herbage is monitored and the whole tunnel moved to a fresh area of grass before the animals eat all the available herbage.

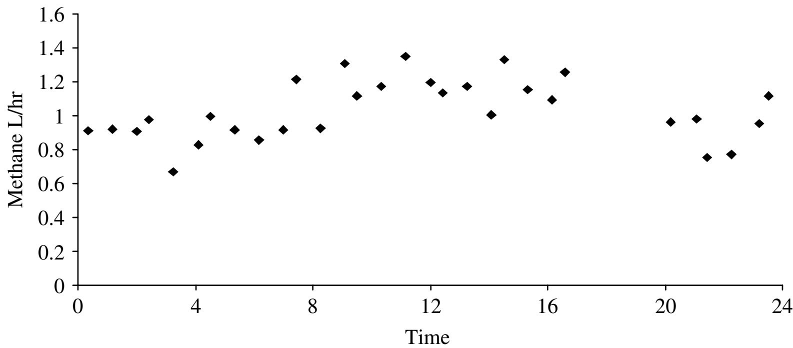

Figure 2. Methane production over 24 h in sheep fed an *ad lib* concentrate diet

Emission measurements should be conducted over an extended period of time, since previous work has demonstrated that emission rates can vary considerably throughout the day (8), depending on animal behaviour, e.g. whether animals are grazing, ruminating, sleeping or drinking. Clearly depending on the diet methane emissions will peak following a meal (8), and even in continuously fed animals methane emissions tend to be lower during the hours of darkness (Fig. 2). For this reason we would recommend measurements continuing for a minimum of 24 hours, in order to take into account the variability in emission rates throughout the day, typically we measure methane production over a 4 day period. Clearly automated systems will allow regular and repeated sampling of inlet and outlet methane concentrations. However, if using manual sampling, consideration will have to be given to sampling frequency, with our recommendation being that hourly sampling be considered a minimum requirement. Livestock should remain undisturbed, as far as possible, during the measurement period and consideration of sampling protocols will need to take this into account when using manual sampling.

2. COMPARISON WITH OTHER METHODS

Murray *et al.* (8) compared methane production by sheep fed diets of cutgrass or high temperature dried grass using both open-circuit respiration chambers and the tunnel system described above. Methane production tended to be higher in the open circuit chambers compared to the tunnels (31.7 $\pm$ 0.35 v 26.9 $\pm$ 0.46 L/kg dry matter intake, respectively). However, recovery of added methane in both systems was high (95.5–97.9% vs 89.2–96.7% for the tunnels and chamber respectively) and it was suggested that differences in animal behaviour between the two systems, the animal in tunnels are less restrained than is required in the chambers, may have accounted for the differences in methane production.

3. CONCLUSION

The tunnel system as described above offers the opportunity to measure methane production by ruminants under grazing conditions; the tunnel can be constructed using locally available resources and can be portable or fixed. The greatest precision will be achieved using regular analysis of inlet and outlet gases with an automated system but given the availability of manpower comparative measurements should be possible with little additional capital outlay using manual sampling.

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CHAPTER 7

A PROTOCOL FOR THE OPERATION OF OPEN-CIRCUIT CHAMBERS FOR MEASURING METHANE OUTPUT IN SHEEP

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INTRODUCTION

Methane is the leading man-made contributor to climate change after carbon dioxide (1, 7) and ruminant livestock are the largest source of methane. For example, sheep and dairy cattle produce approximately 5.0–11.0 kg and 56–118 kg of methane, respectively, per head, per year (6). Methane is formed in the rumen when hydrogen, released by other microbes during fermentation of forage, is used by methane-producing bacteria-like organisms (i.e. methanogenic Archaea) to reduce carbon dioxide. It has been estimated that as much as 94% of the enteric methane emitted by ruminants is belched out and expired via the lungs (5). The loss of energy for ruminant animals has been estimated to represent between 2 and 14% of the animal's gross energy intake (2, 4).

For many years researchers have tried to manipulate the numbers or activity of methane-producing microorganisms in order to improve the efficiency of ruminant production in an ecologically sustainable way. Assessing the effectiveness of mitigation strategies depends on the accuracy of methane measurements. Although measurement of methane using a chamber system restricts studies to a small number of housed animals fed under controlled conditions, it has been shown to be the most reliable and accurate methodology available. The following chapter describes the use of this methodology for sheep, but it could also be applied to other animals, or measurement of different gases.

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Figure 1. Design of the low-cost open-circuit methane chamber cover. Size (L × W × H): 1.73 × 0.9 × 1.56 m; Frame: 25 × 25 mm square aluminium; Covering: 1.0 mm UV resistant clear flexible PVC sheeting; Chamber air inlet (i.e. front-end): 40 mm PVC flange fitted centrally 50 cm above the floor; Chamber air outlet (i.e. rear-end): 40 mm PVC flange fitted centrally 125 cm above the floor

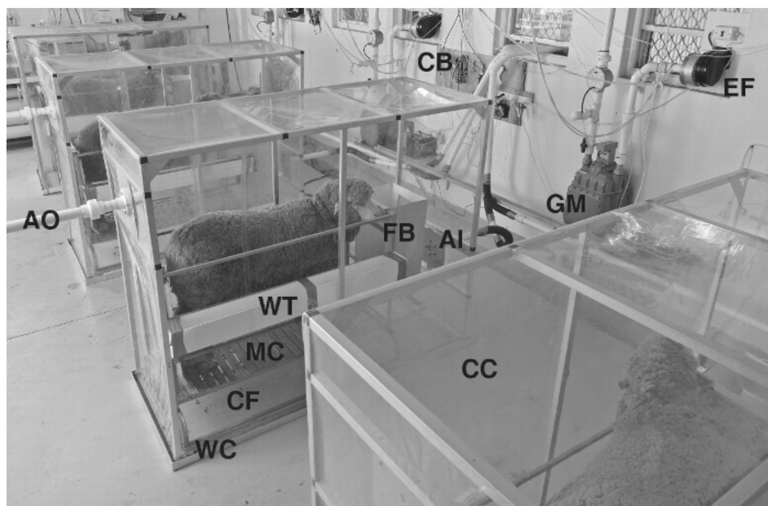

Figure 2. Methane chambers under full operation. **AI**, air inlet through 40 mm PVC pipe; **AO**, air outlet through 40 mm PVC pipe; **CB**, control/circuit board; **CC**, 1.0 mm thick UV resistant clear PVC chamber covering; **CF**, chamber floor (1.77 × 0.94 m), made of 6 mm thick high density PVC sheet, with a 50 × 50 × 50 mm water channel around the floor perimeter; **EF**, high speed (2,500 rpm) extractor air fan; **FB**, feed bin; **GM**, air flow gas meter; **MC**, metabolism crate (1.21 L × 0.52 W × 0.73 m H) on a 20 cm stand; **WC**, water filled channel (50 × 50 × 50 mm); **WT**, water trough (17 L). Photo from Klein and Wright (3)

1. DESIGN AND CONSTRUCTION OF CHAMBERS

A detailed description of the design and construction of low-cost open-circuit methane chambers has been described by Klein and Wright (3). Briefly, the methane chambers are constructed from square aluminium tubing and covered by 1.0 mm thick UV resistant clear flexible polyvinyl chloride (PVC) sheeting (Fig. 1). An air tight seal is achieved when the chamber frame is lowered into a water-filled channel that forms part of the chamber floor.

Inside the chamber is a crate, on a 20 cm high steel stand, to confine the sheep (Fig. 2). Attached to the sides of the crate are a water trough and a fibreglass feed bin (Fig. 2). A high speed (2,500 rpm) air fan extractor motor (Fig. 2) is used to draw dehumidified air through the chamber system at 300 L/min. The volume of total air flow through the chamber is calculated from the final and initial gas meter readings from an aluminium case diaphragm dry gas meter (American Meter Company, Horsham, PA) (Fig. 2).

2. CONSIDERATIONS PRIOR TO SAMPLING

2.1. Training Sheep to the Chambers

Acclimatizing sheep to being in the chambers is important and this can be time-consuming, especially if there are many experimental animals. The more trained the sheep are to being in the chambers the more consistent your data collection will be. However, the amount of training time required varies for individual sheep depending on temperament and their previous experience to handling and people. Therefore, allow plenty of time for this aspect of animal preparation.

Sheep should be moved to the chambers in a quiet manner. Use of a trolley to wheel them from their housing to the chambers is an acceptable method and maybe less stressful particularly if the flooring in the chamber room does not provide appropriate footing for hoofed animals (e.g. sealed smooth concrete). Animals that are quiet, used to handling, and with suitable flooring may be led to the chambers.

Initially, the animals should be accustomized to being confined in the crates, without the chamber covers in place, gradually progressing to confinement with the chamber covers over the sheep. In our experience, the sheep did not panic as the chamber covers were clear so vision and sound were not restricted. It is important for the sheep to get used to people coming in and out of the room, walking around them, peering into feed bins, and adjusting the equipment. Be aware that some animals may try, and even succeed, to jump out when the chambers are in place if they are startled.

Always provide feed when animals are placed into the chambers because they then associate being in the chambers with receiving food. Because they associate the chambers with feeding, and they are not distressed by being in a confined space. Several 6–8 h sessions in the chambers are usually necessary for each animal.

Most animals will turn around in the crates facing away from their feed. This is not a problem provided they can also turn back to eat their feed. The main

issue with turning around is that they can contaminate their feed with faeces. Some animals can be manoeuvred in the crates to turn back around to face their feed. However, more stubborn or larger animals may need to be removed from the crate and put back facing the right direction. For animals that continuously turn around, foul their feed and don't eat, a piece of wood can be attached to the long side of the crate to prevent them from turning by constricting the width of the crate.

It is recommended that animals that do not settle or refuse to eat when in the chambers, are not used for the experiment.

2.2. Standard Gas Mixes and Gases for Gas Chromatograph (GC) Analysis

Cylinders of mixed gases are used as standards for GC analysis and may have to be specially made-up by gas suppliers which could take several weeks to arrive. Ensure there is ample supply of these prior to beginning sampling. Also, ensure that there are enough gases available for continuous running of the GC for your sampling days.

2.3. Chamber Recovery Tests

Chamber recovery tests should be performed before and after an experimental sampling period to ensure that the chamber system is functioning correctly, particularly to determine that there is no loss of methane from the system. The basis of the recovery test is that a known amount of methane is added to the chamber at a rate similar to what is produced by the sheep. The amount of methane in the chamber over time is then measured by the GC. The amount measured by the GC should be very similar (i.e. $\pm$ 0.7 g of methane (3)) to the known amount added to the chambers.

Steps for preparing the recovery tests:

1. Setup the chamber system with all air flows, sample pumps, and data loggers running.
2. Begin the GC analysis.
3. Record the weight of the standard methane (99.95% purity) gas cylinder and insert the cylinder outlet line from the regulator into the chambers air intake pipe.
4. If you have more than one chamber, use a separate cylinder for each chamber or test one chamber each time.
5. Record the time and the initial gas flow meter reading at the start using cylinder weight loss.
6. Turn on the methane gas cylinder and set regulator dial to bleed in around 1.0–1.5 L standard methane/h. If a greater rate than this is used you may not get peak separation on the GC output due to the concentration exceeding the sensitivity of the GC.
7. Measure the chamber gas standards on the GC as usual.

8. Allow the test to run for at least 12 h.
9. After 12 h, turn off methane gas and immediately record the weight of methane cylinder.
10. After about 5 min record the time and final gas flow meter reading.
11. Leave the GC's analysing for an additional 30 min. Once all the methane has been collected and only background levels are present on the GC traces, then stop the GC instruments.
12. Repeat the process for the other chambers, if necessary.
13. Determine the chamber methane output (g) as you would for sheep using GC results, standards, airflow and temperature and pressure data.
14. Determine the weight (g) of methane released into the chamber from cylinder (= initial weight – final weight).

$$\text{Recovery (\%)} = \text{g chamber output} / \text{g methane released} \times 100$$

2.3.1. Chamber system airflow

The chamber and gas chromatography systems that we use to measure methane output of sheep have been described by Klein and Wright (3) and are schematically illustrated in Figure 3.

Briefly, air pressure in the meter side of the filter and barometric pressure are measured by differential (Model 1 INCH-G-4V-REF; All Sensors Corp, Morgan

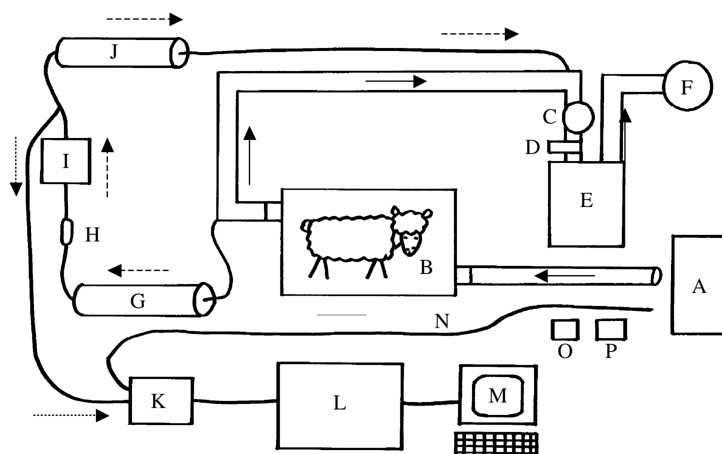

Figure 3. Schematic diagram of open system methane chambers. Solid arrows indicate flow of air through chamber system. A, dehumidifier; B, methane chamber; C, chamber pipe differential pressure sensor and gas meter air filter; D, chamber air temperature logger; E, gas meter; F, extractor fan. Dashed lines indicate flow loop for air sampled from chamber; G, homogenizer 1; H, flow meter; I, diaphragm pump; J, homogenizer 2. Dotted lines indicate flow of homogenized and subsampled chamber air to gas chromatograph for methane analysis; K, peristaltic pump; L, gas chromatograph; M, computer; N, ambient air sampling line; O, Ambient air temperature logger; P, atmospheric pressure sensor and data logger

Hill, CA) and absolute (Model HD 9408T BARO; Delta Ohm) electronic pressure sensors, respectively. Readings from the pressure sensors are recorded at 5 min intervals by a CR10X data logger (Campbell Scientific, Kirwan, Qld). The temperature and relative humidity meter (Watchdog Model 150; Spectrum Technologies Inc., Plainfield, Illinois) are housed in a 40 mm sealable T-piece, fitted in line with the 40 mm main air pipe before entering the gas meter. Temperature and relative humidity of the air in the pipe are recorded at 5 min intervals.

2.3.2. Sampling chamber air

A "Rietschle Thomas" inline diaphragm pump (Model SK107/2107; Seven Hills, NSW) is used to continuously draw part of the exhaust air from the 40 mm main air pipe just after the chamber exit through a homogenizing loop (Fig. 3). The homogenizing loop consists of a 70 L homogenizer, the inline diaphragm pump, and a second small 10 L homogenizer. The homogenized exhaust air from the homogenizing loop is returned back into the 40 mm main air pipe just before the filter (Fig. 3). The homogenizers are made out of 150 mm PVC pipe and end caps fitted with 6 mm barbed air fittings at each end. The smaller homogenizer acts as a silencer blocking the diaphragm pump's chatter from reaching the chamber. The homogenizers and diaphragm pump are connected by 6 mm internal diameter (ID) flexible PVC tubing and 6 mm brass barb fitting and clamps.

A small sub-sample of the homogenized exhaust air is drawn out continuously through 2.5 mm infusion tubing by a Technicon III peristatic pump (Labequip, Markham, ON) at approximately 3.9 mL/min from a section of the 6 mm PVC flexible tubing that connects the diaphragm pump to the second homogenizer in the homogenizing loop.

2.3.3. Methane concentration

Methane concentration in sub-samples is determined by GC at 5 min intervals. A Shimadzu GC17A gas chromatography is equipped with two flame ionization detectors (FID) and two 1/8" × 10 ft stainless steel columns packed with molecular sieve 5A, 80/100 mesh (custom made by Alltech Associates Pty Ltd, Baulkham Hill, NSW). Two 10-port Valco valves (Model P36-220) (VICI-Valco Instruments, Houston, TX) (Fig. 4), fitted with 1.0 mL sample loops, are configured to inject two different samples into the same column. Nitrogen is used as the carrier gas at 400 kPa head pressure and the oven and detector temperatures are set isothermally at 150°C and 300°C, respectively. Ambient methane (Fig. 3) concentration is sampled directly from the dehumidifier outlet to the sample loops of a Valco valve of a second GC.

2.4. Methane Collection and Measurement

The following is an example of the sequence of events to follow when using these open-circuit chambers to measure methane output:

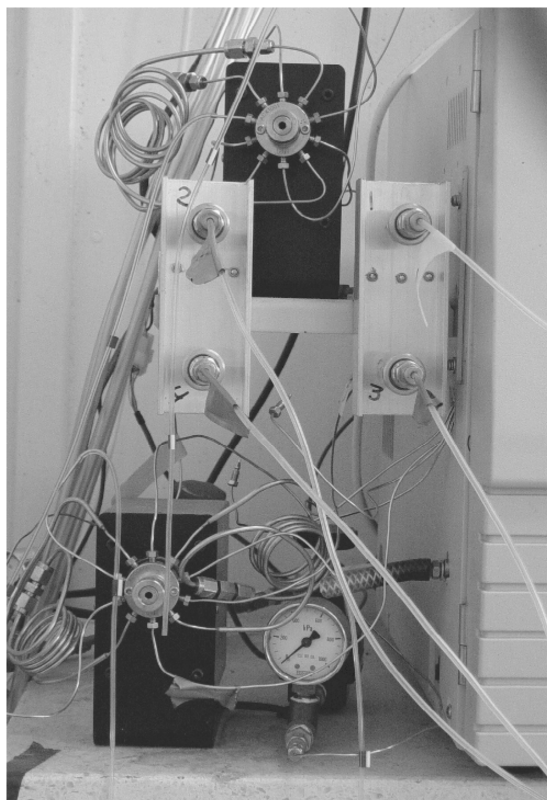

Figure 4. Two 10-port Valco valves with 1.0 ml sample loops, configured to inject two different samples to the same column. At time zero, valves 1 and 2 inject samples from chambers 1 and 3 onto columns 1 and 2, respectively. At 2 min, valves 1 and 2 inject samples from chambers 2 and 4 onto columns 1 and 2, respectively. The sample lines are purged of sample for 30 s prior to each injection. Separation of the two methane peaks on each column is achieved within 5 min and the cycle repeated every 5 min over the measurement period

2.4.1. Day before sampling

1. Switch on the GC's and allow them to heat and stabilize with the required programme/method running. We leave them on overnight, prior to measurement the next morning.

2.4.2. Morning of sampling

At 08:00 h

1. Turn on the air conditioning system for the room (if installed).
2. Turn on the dehumidifier and extractor fans that draw air through the chambers.
3. Turn on the sample pumps that draw a subsample of air exiting the chambers.

4. Turn on the peristaltic pump that continuously feeds a homogenized sample of chamber air from the subsample line to the GC.
5. Locate the ambient air sampling lines at the air intake for the chambers.
6. Initiate the data logger that records the atmospheric and pipe pressure sensor outputs.
7. Initiate the data loggers that record the temperature of the chamber air and ambient air.
8. Set up the chamber floor pans and crates, ready for sheep to be placed in them, that includes filling the water troughs on each crate and the floor pans sealing channels.
9. Ensure GC's are functional with ample gas supply.

At 09:00 h

1. Start GC analysis.
2. Put sheep in each chamber crate. Tape the backing panels of the crates closed so that sheep cannot dislodge or open them whilst in the chambers.
3. Put feed ration into feed bin, put the chamber cover on, attach the air flow pipes at both ends and then immediately record the time of day and gas meter (air flow) reading.
4. Start methane collection, one sheep at a time.
5. Run a first round of GC standards.

At 16:00 h

1. Run a second round of GC standards.
2. Sheep will need to be checked hourly while in the chambers unless some form of alarm system has been fitted that alerts the user to reductions in or a loss of air flow through the chambers (3) (Fig. 5).

2.4.3. The following morning

At 08:00 h

1. The third round of GC standards are run.
2. Methane collection is now finished.
3. Record the time and the gas meter reading for the chamber, then immediately lift off the chamber cover.
4. All the fans and pumps are left running and the GCs are left analysing for another 20–30 min, until the last bit of methane comes through.
5. Sheep are returned to their housing and any feed residues are recorded.
6. Chamber floor and metabolic crates are cleaned out and set up, and feed bins and water troughs are filled for the next round of measurements.

Circa 08:30 h

1. GC analysis is stopped and all the data are downloaded and backed-up.
2. Data from data loggers are also downloaded if necessary.
3. GC gas supply is replaced, if required.

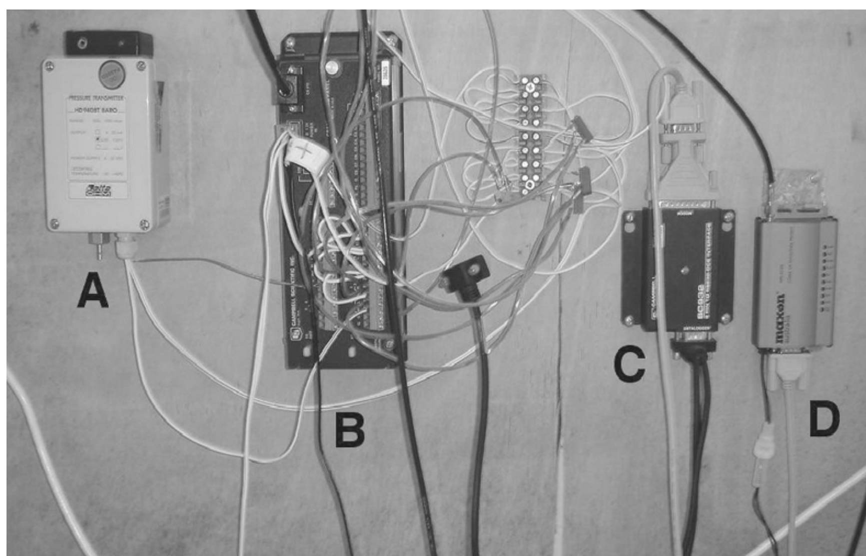

Figure 5. Chamber pressure monitoring and alarm setup. A, barometric pressure sensor; B, datalogger that logs outputs from the barometric sensor and the individual chamber pipe differential pressure sensors; C, data logger/modem interface, when connected to the data logger allows the modem to be activated (i.e. sends an SMS text message warning to the investigator's mobile phone) when chamber pressures sensor voltages fall below a designated threshold; D, voice/data/SMS modem

4. Peristaltic pump tubing is replaced, if necessary.
5. GC analysis is then recommenced if required and the next group of sheep put in the chambers.

2.5. Calculating Methane Output

The amount of methane produced by the animal in the chamber is calculated as a product of the amount of air drawn through the chambers, corrected to standard temperature and pressure (STP), and the concentration of methane in the air leaving the chamber (corrected for the concentration of methane in the ambient air).

2.5.1. To calculate the volume of air drawn through the chambers

Air volume through chambers (m^3) = final gas reading- initial reading

If your gas meter does not measure in m^3 then you will have to convert the air volume to these units.

2.5.2. To correct this air volume to STP

- Convert all temperature data to absolute temperature:

$$\text{Absolute temperature (K)} = 273.15 + \text{temp in °C}$$

- Determine air pressure in chamber pipes: The calculations required here will depend on the type of pressure sensors used. Absolute sensors will record the actual pressure in the pipe while differential sensors will record the difference in pressure between the inside of the pipe and the outside air pressure. Convert all pressures to millibars.
- Apply pressure and temperature corrections to the air flow volume:

STP air volume through chamber (m^3) =
Pressure in pipe × air volume through chamber/absolute temperature of air through chamber/1013.25 × 273.15

2.5.3. To determine the methane concentration of the ambient air and exited chamber air

- Calculate the peak areas for methane in all the air samples taken from each chamber and from the ambient sampling lines using the software for the GC. We use a batch integration option that allows all the GC files from a channel to be analysed at once rather than manually integrating each one. With more than 270 files created per channel each day (and we have 3 channels) this would be rather time consuming otherwise.
- Calculate standard correction coefficients for each chamber and ambient line from a standard curve. Plot the peak areas obtained from the methane standards against their known concentration (ppm). Include a (0,0) point and fit a straight line through the data. The slope of the line is the standard correction coefficient. This is done separately for each sampling.

For example: As a minimum, only 100 ppm methane in nitrogen standard is released through a chamber's GC sampling lines on three occasions during the measurement period. The peak areas of this standard gas are determined to be 8703251, 8841581, and 9122168. A standard curve is then constructed and the coefficient in the regression equation (e.g. 1.1246) (Fig. 6) is the standard coefficient used to correct the methane peak areas from that chamber on that day to concentrations of methane (ppm). To be more precise, prepare the standard curve by injecting different levels of methane.

- Convert methane peak areas from all sampling lines to concentrations of methane using the appropriate standard correction coefficient:

Methane concentration (ppm) = peak area / 100,000 × standard correction coefficient

- Correct chamber air concentrations for ambient air methane.

Corrected chamber methane (ppm) = chamber methane concentration − ambient air methane concentration

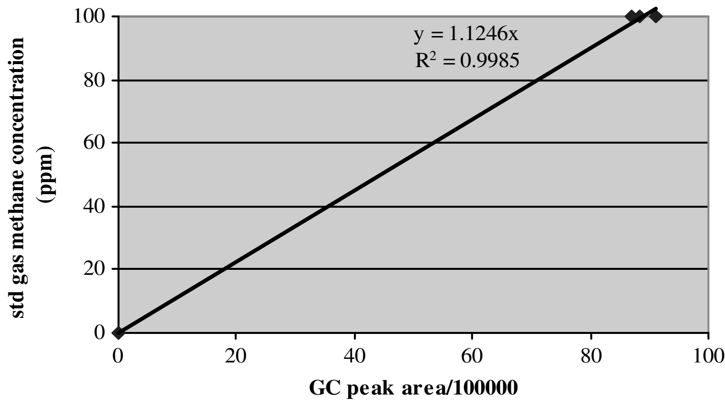

Figure 6. A standard (std) curve used to calculate the standard coefficient to correct methane peaks from the GC to concentrations of methane (ppm)

For example: If the methane concentration from chamber 2 is 89.4 ppm and the ambient air concentration is 1.9 ppm then the contribution to the chamber air output by the animal is 89.4 – 1.9 = 87.5 ppm.

2.5.4. To calculate methane output

$$\text{Methane output (L)} = \text{STP chamber air vol}/1,000 \times \text{corrected chamber methane}$$

Methane output (g) = methane output (L) / 22.4 × 16

2.5.5. Methane output can also be expressed on a feed intake basis

Methane output = Methane output / kg dry matter intake

Methane output can be calculated as an average for the whole period by averaging all the peak areas, temperatures and pressure measurements made in a period and obtaining a single output value for that period. Alternatively, it can be calculated over shorter periods of time (e.g. hourly) by assuming that the rate of air flow through the chambers is constant over the whole measurement period. In this case air volume drawn through in, for example, 22 h is divided by 22 to get the hourly volume drawn through. Peak areas, temperatures and pressure measurements are then averaged for each hour and the above calculations are performed for each set of hourly data. This method of data presentation allows daily patterns of methane output to be observed.

2.5.6. *Correcting to a standard time frame*

If desired, the measurement of air volume through the chambers can be standardized to a particular time frame so that all results are calculated for the same time frame. This assumes that the rate of flow through the gas meter was constant over time.

$$\text{Air volume in corrected time frame} = \text{recorded volume}/\text{actual time frame} \times \text{desired time frame}$$

For example: If a volume of 400 m^3 was recorded to go through the chambers in 22.5 h (1,350 min), this can be corrected back to 22 h using the above equation. Thus, air volume in 22 h = 400 / 22.5 $\times$ 22 = 391 m^3.

The time corrected volume is then converted to STP in the usual manner. These volumes are then used with the methane concentrations to determine methane output as above. This process is useful where the deviation of measurements carried out vary slightly among animals – e.g. up to 15 min.

It is not recommended that the standardized time frame is extrapolated too far from the actual measurement period as methane output by animals over time is not consistent and depends on feeding patterns and intake amounts.

For example: Using a 20 h methane concentration average to extrapolate the output after 24 h will overestimate if the animals outputs were declining with time in that 4 h extrapolation period, or will underestimate it if the outputs were increasing in 4 h due to an event outside the recording period.

3. CONCLUSIONS

The low-cost open-circuit methane chambers permit uninterrupted, real-time measurements. The design and use of the heavy-duty transparent chamber covering allows the sheep in the chambers to see each other and the researchers. This means the sheep are less stressed, so they behave naturally, and their feed consumption is not affected once they are acclimatized to the system.

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CHAPTER 8

CONSTRUCTION AND OPERATION OF VENTILATED HOOD-TYPE RESPIRATION CALORIMETERS FOR *IN VIVO* MEASUREMENT OF METHANE PRODUCTION AND ENERGY PARTITION IN RUMINANTS

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INTRODUCTION

Indirect calorimetry refers to the methods for quantifying heat production from quantitative measurements of materials consumed and produced during metabolism. Most of these measurements involve respiratory gas exchange including enteric methane production. Calorimeters are typically classified according to the specific design of the apparatus (5). The three main types of indirect calorimeters are (i) confinement-type, (ii) closed-circuit and (iii) open-circuit systems. In confinement-type systems, animals are housed in completely sealed chambers and the changes in gas concentrations in the chamber measured. In closed-circuit systems the animals are similarly housed in completely sealed chambers. Water vapour and carbon dioxide produced by the animal is collected and measured using absorbers. Oxygen consumption is measured by the amount of oxygen required to maintain constant oxygen concentrations in the sealed system.

In open-circuit systems animals are housed so that they breathe into a one-way stream of air passing across the face or body. Airflow is typically measured at the

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outlet side of the animal cage and a sub-sample of air collected for analysis using an on-line gas analysis system. The ventilated hood-type calorimeter presented in this chapter is a type of open-circuit calorimeter where the animals head, rather than its whole body, is positioned within the head cage of the calorimeter.

The details reported in this chapter refer to the respiration calorimeter that was constructed as part of a JIRCAS (Japan International Research Centre for Agricultural Sciences) project at Khon Kaen Animal Nutrition Research and Development Centre (Department of Livestock Development, Thailand Government) in north-east Thailand. This calorimeter system replaced a former 'face mask'-based measurement system that formed the basis for the present 'head cage' calorimeter (2). The advantage of a ventilated hood system over whole body chamber calorimeters is that there are fewer components, which makes the system less complicated to construct and operate in a developing country where the availability of various components and expertise is often more difficult to access.

1. CALORIMETER DESIGN AND ITS COMPONENTS

The ventilated hood-type respiration calorimeter system constructed at Khon Kaen Animal Nutrition Research and Development Centre consists of five components, including (a) the digestion trial pen, (b) head cage, (c) gas sampling and analysis, (d) behaviour monitoring and (e) data acquisition and processing. These components are discussed in more detail below. A schematic diagram of the ventilated hood-type calorimeter discussed in this chapter is shown in Figure 1.

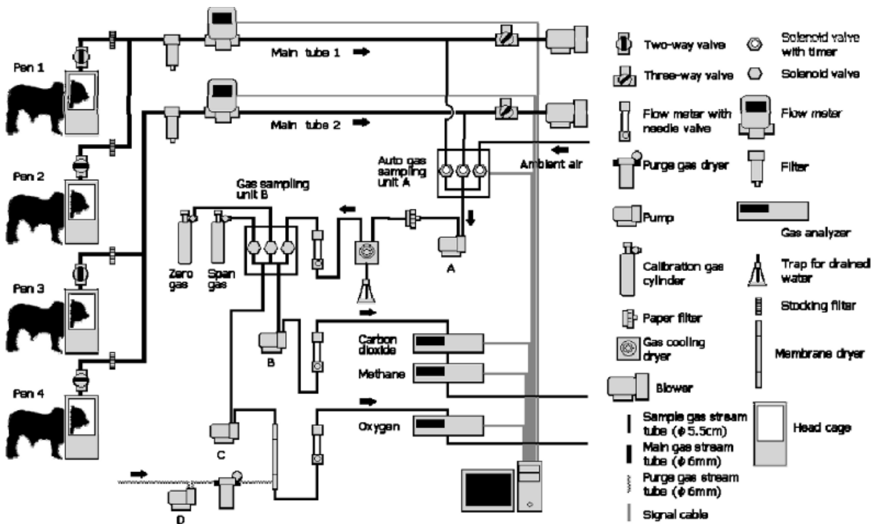


Figure 1. Schematic diagram of the ventilated hood-type respiration chamber system. The black arrows indicate the direction of air flow through the system

1.1. Digestion Trial Pen

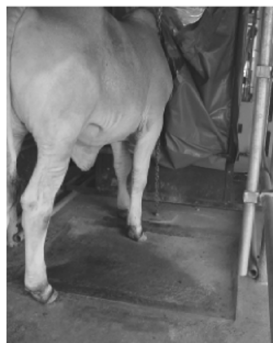
The digestion trial pen is designed to house animals to enable accurate measurement of feed intake and excreta output to be made. The experimental apparatus is typical of other apparatus used in many animal nutrition laboratories around the world where classical feed digestibility studies are conducted. The pen also functions to position the animals head inside the head cage. Some photos of the digestion trial pen and head cage are shown in Figure 2.

1.2. Head Cage

The head cage is installed at the front of the digestion trial pen. A technical drawing of the head cage used at Khon Kaen Animal Nutrition Research and Development Centre

(a)

(b)



(c)

(d)

(e)

Figure 2. Photos of the digestion trial pen and head cage at Khon Kaen Animal Nutrition Research and Development Centre

(a) Digestion trial pen and head cage;
(b) Faecal and urine collection from a cow;
(c) Urine collection pan on floor for male cattle;
(d) Urine tube, bottle and box for faecal collection from male cattle;
(e) Collection of feed refusals from head cage through door

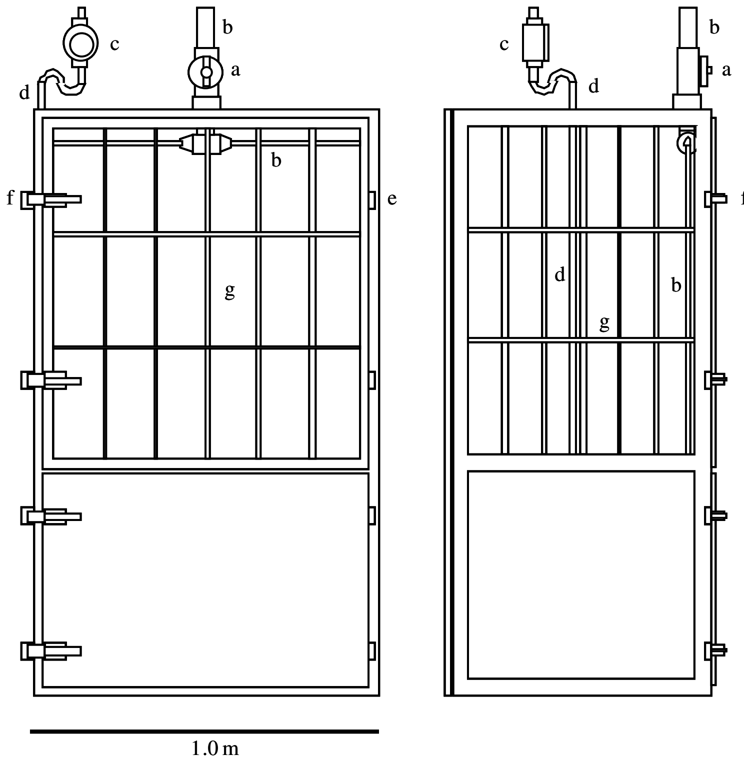


Figure 3. Diagram showing the front (left) and side (right) view of the head cage. (a) two-way valve; (b) gas outlet pipe; (c) water gauge; (d) water pipe; (e) hinge; (f) lock; (g) acrylic panel

is shown in Figure 3. This head cage is designed to be air-tight, with the exception of the air inlet, which is an adjustable 'loose-fitting' collar. A rear view of the collar can be seen in Figure 2. The position of the head cage and yoke are adjustable in order to permit animals of various sizes to be housed in the digestion trial pen.

The head cage incorporates the following components: an air-tight outer wall constructed with acrylic plastic, a water supply for the animal, a feed bin and outlet to main stream of air. The front of the cage has a door that permits easy access for feeding and collecting feed residues. The amount of water consumption can be measured with an in-line water gauge fitted to the mains water supply. The main air streams from two head cages are combined to one air stream so that only two head boxes of the four head cages can be measured at any time (Fig. 1). These air streams are changed manually with a ball valve positioned close to the head cage.

1.3. Gas Sampling and Analysis

1.3.1. Flow meters

Flow meters are an essential component of the calorimeter and underpin the accuracy of the whole measurement system. Thermal mass flow meters are the simplest type

of flow meter available, and are calibrated in the factory to output measurements of flow rates at standard temperature and pressure. In recent times, thermal flow meters have included a 'hot-wire'-type air mass flow sensor.

1.3.2. Blower

The purpose of the blower is to move the main air stream though the calorimeter system, from the inlet point at the loose fitting collar of the ventilated hood through to the exhaust point. Industrial blowers are typically used, and positioned at the exhaust point. It is important to position the exhaust point of the blower outside the barn in order not to contaminate the inlet air. Air flow rate through this main air stream can be adjusted using a 3-way valve in the main line immediately preceding the blower. The flow rate through the main air stream is set at a value determined by animal size, feed intake and production level. In the respiration calorimeter used at Khon Kaen Animal Nutrition Research and Development Centre, a flow rate of 450 L/min is used for a 300–400 kg liveweight American Brahman steer fed at 1–2 times maintenance energy requirement.

1.3.3. Air filter and dryers

Air filters and dryers are installed in the system to remove dust particles and moisture before gas samples are sent to the gas analysis system. In the main air stream both coarse 'stocking' filters and fine filters (filtration rating 5 μm) are positioned near the outlet of the ventilated hood preceding the flow meter. A paper filter and a dehumidifier are used in the gas stream being sampled to remove any dust particles and moisture. Dust and moisture in air sampled for gas analysis leads to unstable measurements of gas concentration, and may damage the analytical equipment permanently.

1.3.4. Gas analysers

One set of gas analysers is used to measure sampled gas from three positions (i.e. background air and two different head boxes). An automated system involving solenoids was established to permit the gas sampling point to alternate between the three positions. In this automated system the gas sampling point is switched at 90 second intervals between the three positions (Fig. 4). The first 60 seconds is used to allow stable gas concentrations to stabilize before measurement, while the final 30 seconds are for data acquisition.

There is another separate gas sampling line that connects the calibration gases (span and zero calibration) directly to the gas analysers. During calibration of the gas analysers the flow rate to the gas analysers is controlled with a needle valve. Infrared gas analysers are used for measurement of carbon dioxide and methane gas concentrations. The oxygen analyser used in this system is a paramagnetic analyser. The response of the oxygen analyser is slower than infrared analysers and hence is the primary limit to the responsiveness of the whole calorimeter system.

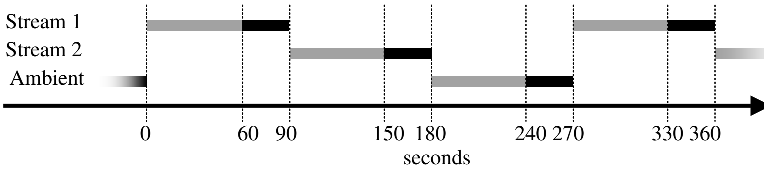


Figure 4. Order and timing of selection of sampling gas by auto gas analysis unit (■) and accumulation of gas concentration data by the data logging system (■)

1.4. Monitoring Animal Behaviour

Various other components can be installed in the system to monitor animal behaviour, which can assist greatly later on when trying to interpret the gas production data. The system in Khon Kaen includes a device for monitoring chewing activity of the animal and a video camera surveillance that is logged onto a separate computer that can be used for later reference. The halter with the mechanical switch positioned under the animals jaw for counting chewing activity and the web camera positioned in front of the head cage for recording cattle activity are shown in Figure 5 (a and b).

1.5. Data Acquisition and Processing

It is essential to have a system for collecting accurate data for accurate calculations. An automated system should be established to enable 24 hour data collection. Commercial systems are available (e.g. Testpoint, CEC, Bedford, NH, USA) or you can develop your own set of hardware and software. The essential components of the system include electronic devices (i.e. flow meters, gas analysers, selection

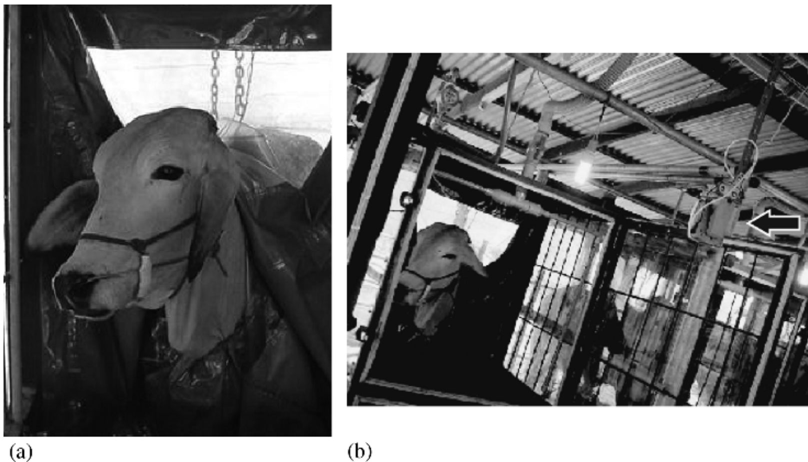


Figure 5. Photos of animal behaviour monitoring equipment. (a) is the device used to monitor chewing activity and (b) is the web camera positioned in front of the head cage for recording cattle activity

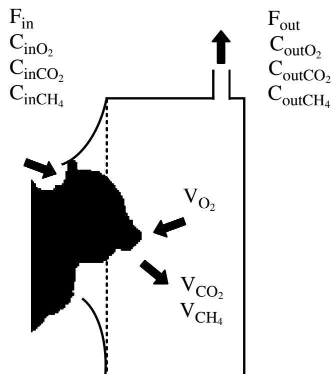


Figure 6. Schematic diagram illustrating the calculation of methane and carbon dioxide gas production and oxygen consumption. F_{in}, flow rate of inlet gas; C_{inO_2,CO_2,CH_4}, concentration of O_2, and CO_2 in inlet gas (ambient gas); F_{out}, flow rate of outlet gas; C_{outO_2,CO_2,CH_4} concentration of O_2, CO_2 and CH_4 in outlet gas; V_{O_2}, volume of O_2 consumed; V_{CO_2} and $_{CH_4}$, volume of CO_2 and CH_4 produced

of gas sampling points) termed A/D converters that are used to convert analog signal outputs from various electronic devices to digital signals that are sent to the computer and recorded. The software has functions of data accumulation and post experiment calculations.

A diagram showing the parameters required to calculate methane production and respiratory gas exchange from an animal is presented in Figure 6. The measurement parameters required include air flow out of the calorimeter, along with concentrations of methane, carbon dioxide and oxygen and the inlet and outlet point of the calorimeter.

It should also be noted that air flow into and out of an open circuit respiration calorimeter are not equal, because the total volume of gases (methane and carbon dioxide) produced is not equal to the volume of oxygen expenditure. In situations where you want to determine the difference between inflow and outflow of the system, you should compare calculated nitrogen gas in the ambient air and the head cage. Because nitrogen is not consumed or produced by cattle, the amount of nitrogen introduced into and coming out from the calorimeter is the same and, therefore, the flow rate into the calorimeter can be corrected by an adjustment of the measured outflow rate. This is particularly important in whole-body open circuit calorimeters where the volume of the animal cage is high, whereas the effects are negligible for the head cage calorimeter reported here. The procedures required for the measurements and calculations mentioned above are described in full detail by (4).

2. OPERATION OF THE SYSTEM

2.1. Instrumentation

The accuracy of the whole calorimeter system is only as accurate as the component instruments being used. It is therefore advisable to match the accuracy of the components at the time of purchase.

2.2. Recovery Tests

Recovery tests are the final validation of the system. The procedures used are described in full detail elsewhere (5). A newly constructed calorimeter system should achieve 95–105% recovery values before any measurements are made with the system. In essence, recovery tests are required to confirm that there are no leaks in the system and that the measurement system is accurately measuring emissions from animal. In the Khon Kaen laboratory, a pure gas source (i.e. carbon dioxide or nitrogen) is introduced into the head box from a gas cylinder positioned on a gravimetric balance. The injection of pure (99.99%) carbon dioxide gas during a recovery test is shown in Figure 7. The injection rate is measured gravimetrically. The test gas is then measured using the gas analysis system and the measurements compared to the gas introduction rate. An example of gas concentration measurements from a recovery test is shown in Figure 7.

2.3. Measurement of Net Volume of the System

It is important to consider the effect of differences in gas concentration in the chamber throughout periods of gas measurement. To calculate this effect, it is essential to know the real volume of the respiration calorimeter system. Such a calculation is more important for whole-body respiration calorimeters than the hood-type system that we are discussing here, because the volume of the whole-body calorimeter is greater. Hence, the reaction time of the measurement system to changes in gas production by the animal is slower. The measurement of net volume of the system is used to consider the accuracy of the whole measurement system.

Values for recovery rate and net volume of the hood-type respiration calorimeters in Khon Kaen are provided in Table 1.

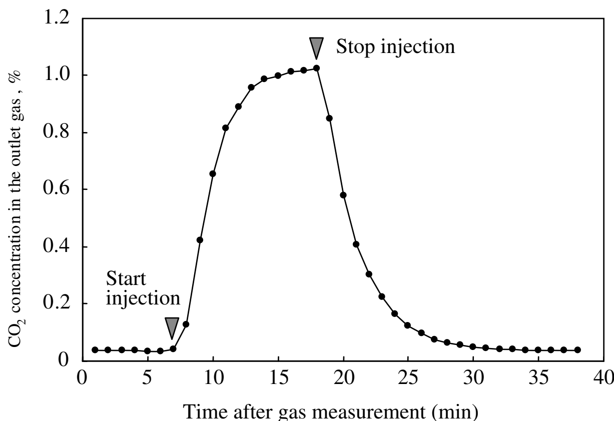


Figure 7. An example of the results obtained from an experiment where carbon dioxide concentration is measured in the outlet air during and after a period of carbon dioxide introduction into the calorimeter head cage

Table 1. Carbon Dioxide Recovery Rate and Net Volume of the Ventilated Hood-Type Respiration Calorimeter at Khon Kaen Animal Nutrition Research and Development Centre

	Recovery rate %	Net volume $\times 10^3$L
Head cage No.		
1	96.5 ± 1.0*	1.74 ± 0.12
2	97.0 ± 2.7	1.63 ± 0.09
3	95.7 ± 1.9	1.64 ± 0.10
4	101.8 ± 0.8	1.73 ± 0.07
Mean	97.8	1.70

* mean ± SD

2.4. Calibration of Gas Analysers with Certified Standard Gases

Calibrating the gas analysers with certified standard gases is an essential part of quality control for the measurement system and should be conducted at least on a daily basis during measurement periods. If measurement conditions are unstable then more frequent calibration is required. In the unit in Khon Kaen calibration is done with certified gases sourced from Japan.

2.5. Training Animals for Measurement and Experimental Design

It is important to train the animals to be used in the experiment and consider the experimental design carefully before making measurements with a respiration calorimeter. The respiration calorimeter is an artificial environment so all attempts should be made to ensure that behaviour of the animal when housed in the calorimeter, including feed intake, is representative of normal behaviour. At Khon Kaen Animal Nutrition Research and Development Centre, the training protocol for animals with no experience in experimental apparatus is two weeks housing in animal house pens, followed by one week housing in respiration calorimeter pens, then introduction to the head cage when the animal is sufficiently familiar. The most common difficulty is with individual animals that use their head and/or horns to damage the head cage.

Careful consideration needs to be made in the experimental design to make sure that the results can be analysed statistically using valid statistical procedures. An example of the order and timing of gas measurement and animal behaviour during a six day collection period is shown in Figure 8.

2.6. Calculating Energy Partition

A diagram of the partition of feed energy is provided in Figure 9. The full set of calculations for energy partition of ruminants is outlined elsewhere (3). In brief, energy of feed, feed refusal, faeces and urine are measured directly by bomb

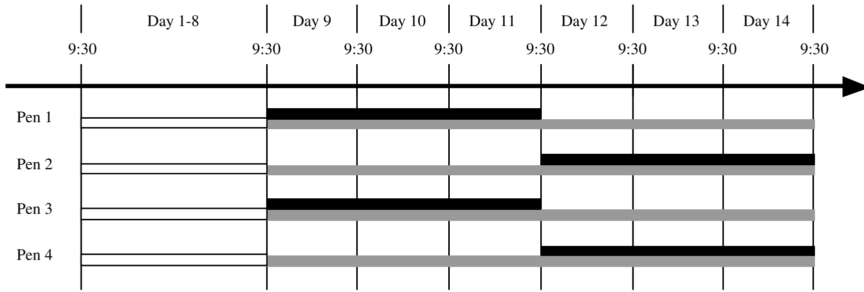


Figure 8. An example of the order and timing of gas measurement and collection of animal behaviour data (■) during a 6 day collection period (■)

calorimetry, and energy of methane and heat production are determined using the following relationships:

$$\text{Energy of methane (kJ)} = 39.5 \times V_{CH_4}$$

$$\text{Heat production (kJ)} = 16.18 \times V_{O_2} + 5.02 \times V_{CO_2} - 2.17 \times V_{CH_4} - 5.99 \times N$$

Where V_{O_2} is the volume of consumed oxygen, V_{CO_2} and V_{CH_4} are the volumes of carbon dioxide and methane produced in litres and N is the amount of nitrogen excreted in urine (g).

2.7. An Example of Typical Output Measurements

An example of typical results of a measurement run is presented in Figure 10, where methane and carbon dioxide production, oxygen consumption and chewing activity over a 24 hour period have been recorded for an American Brahman steer.

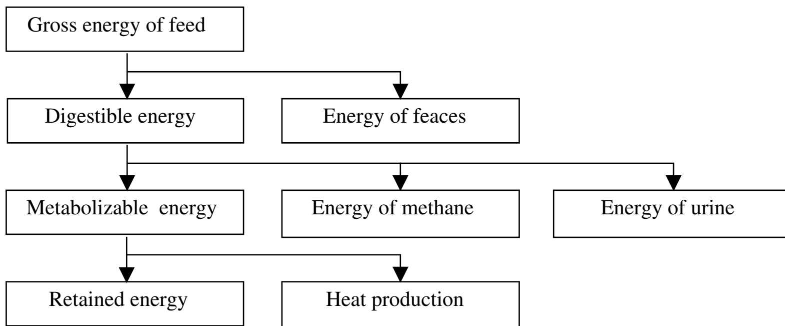


Figure 9. Schematic diagram energy partition of feed

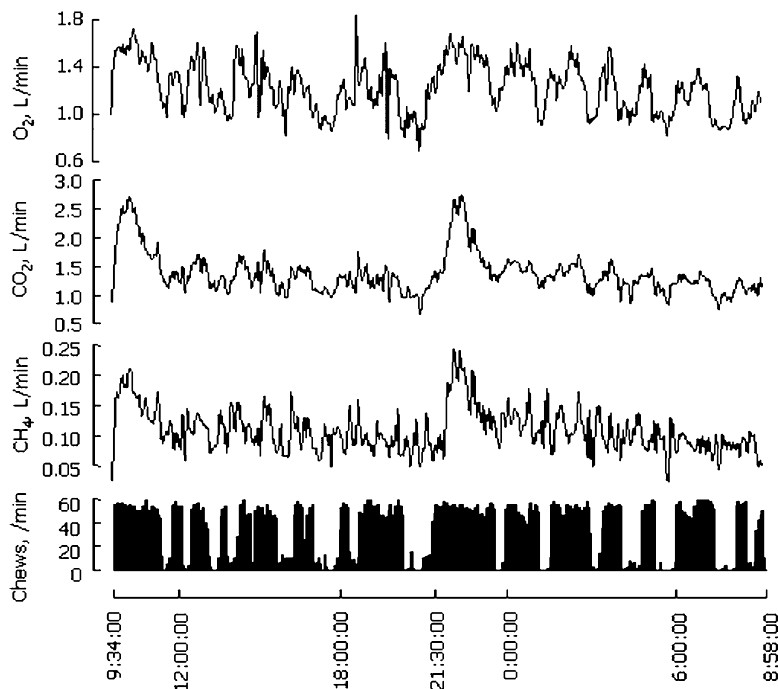


Figure 10. An example of methane and carbon dioxide production, oxygen consumption and chewing activity over a 24 hour period for an individual American Brahman steer

3. CONCLUSION

The ventilated hood-type respiration calorimeter is useful for *in vivo* measurement of methane production and energy partition in ruminants. Such a system can be established in animal research laboratories in developing countries. The need for trained expertise during construction and the initial stages of operation is considered essential.

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