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Development of proxy indicators of methane output by sheep using rapid-throughput field and laboratory technologies

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Supervisor: Dr Jon Moorby

CONTENTS

1. Abstract

Methane production by ruminants is a significant contributor to agricultural greenhouse gas emissions (Webb *et al.*, 2013). However, current values used to estimate methane output by sheep are default values and do not take into account animal and dietary factors that may affect methane output (Bernstein *et al.*, 2007). Strategies to reduce ruminant methane output are the focus of a large body of research (Iqbal *et al.*, 2008) and, in order to implement these strategies fully, a greater understanding of factors that influence ruminant methane emissions is necessary.

The "gold standard" method for measuring methane output by sheep is the use of respiratory chambers (Blaxter and Clapperton, 1965). However, this method is expensive, time-consuming and labour intensive, making it unsuitable for use in an on-farm situation. The work presented in this thesis explores the potential of three proxies to estimate methane output by sheep, which could be used or adapted to be used as a practical means of estimating methane emissions from sheep on a large scale.

The proxies investigated here are a Laser Methane Detector (LMD), used to take measurements of methane concentration from air expired by sheep, *in vitro* gas production analysis of feeds offered to sheep, and Fourier-transform infrared spectroscopy (FTIR) analysis of feeds offered to sheep. Predictions of methane output obtained from each of the proxies are validated using respiratory chamber measurements taken from sheep offered a variety of feeds during different experiments.

With further development and validation, all three proxies presented in this thesis demonstrate potential to be used to successfully estimate or predict methane output by sheep as measured in respiratory chambers. A novel and very successful approach to the method for use of the LMD and calculation of daily methane emissions from LMD data is presented in this thesis. However, the methods used were relatively labour intensive and time-consuming. Further work should, therefore, focus on simplifying these methods as much as possible. To my knowledge, the results presented for *in vitro* gas production and FTIR spectroscopy are also novel, although these are established methods. Both of these methods are rapid-throughput techniques and, therefore, have real potential to be used on a large scale. Further work using larger data sets may provide a more comprehensive idea of the aspects of feeds that affect their methane potentials.

2. Introduction

Methane production by ruminants is a significant contributor to agricultural greenhouse gas emissions (Webb *et al.*, 2013). However, current values used to estimate methane output by sheep are default values and do not take into account animal and dietary factors that may affect methane output (Bernstein *et al.*, 2007). Strategies to reduce ruminant methane output are the focus of a large body of research (Iqbal *et al.*, 2008) and, in order to implement these strategies fully, a greater understanding of factors that influence ruminant methane emissions is necessary.

The hypothesis upon which this project is based is that proxy indicators can be used to provide simple and accurate estimates of methane emissions by sheep that could be used at a large on-farm scale. The work presented in this thesis explores the potential of three proxies to estimate methane output by sheep, which could be used or adapted to be used as a practical means of estimating methane emissions from sheep on a large scale.

The overall aim of the project was to develop proxy indicators, which could be used to quickly, simply and accurately estimate methane output by sheep at a large on-farm scale. The objectives used to achieve this aim were:

1. To review the literature and identify field and laboratory techniques that could be used as proxy indicators for methane output by sheep.
2. To develop the identified techniques and to establish methods for their use for estimating methane output by sheep.
3. To validate the methods developed by comparing the estimates of methane output by sheep obtained from proxy data, with those obtained using methane output data from respiratory chambers, the "gold standard" measure for methane production by ruminants.

Based on a review of literature, three techniques were identified as having the potential to provide quick and simple measurements of methane output by sheep at a large on-farm scale. These were the Laser Methane Detector (LMD, Tokyo Gas Engineering Ltd.), the *in vitro* gas production technique, and Fourier-transform infrared (FTIR) spectroscopy. The LMD was a field technique, which could be used in conjunction with respiratory chamber methane measurements from sheep. The *in vitro* gas production technique and FTIR spectroscopy were laboratory techniques, which were used to evaluate the methane potentials of ruminant feeds.

Much of the methane data collected from sheep in this way was generated as part Defra project AC0115: “Improvements to the national inventory – methane.” Some of this data was collected with the help of IBERS technicians, but I was directly involved in most of the experimental work.

2.1. Laser Methane detector

The concept of using the LMD to measure methane emissions from ruminants is relatively new, and there was, therefore, considerable scope for developing methods for its use. The nature of

using the LMD is simple and non-invasive, providing a potential means to measure methane emissions with minimal disturbance to the animal. Using the LMD to measure methane is a method that requires development: the measurements taken using the LMD are measures of concentration of methane. In order for the LMD to provide useful information regarding methane output by ruminant livestock, methods must be developed to estimate daily methane emissions from the methane concentration data, which are simple enough to use at a large, on-farm scale. The experiments carried out during this project were focused on obtaining estimates of daily methane output by individual sheep, assessing the potential of the LMD to determine methane emissions from animals and exploring methodologies to achieve methane emissions estimates.

2.2. *In vitro* gas production

In vitro gas production is a simple laboratory technique (Theodorou *et al.*, 1994), requiring relatively little feed material, which could provide measurements for methane potential of plants when incubated with rumen fluid, simulating a rumen environment. Coupled with DM intake information, this method could be used to predict methane emissions by animals on certain feeds or feed mixtures. During this project, methane production profiles for a variety of upland plants and sheep feeds were created using *in vitro* gas production analysis. The data obtained were then used to predict methane emissions by sheep, based on the feed given and the DM intake of sheep in methane chambers.

2.3. FTIR spectroscopy

Fourier-transform infrared spectroscopy is a rapid-throughput non-destructive spectroscopy method, requiring very little sample material. It could provide useful information about the components of feed material and whether any particular components affect methane potentials of feeds *in vivo* and *in vitro*. The experiments conducted in this project aimed to use the FTIR spectra of feeds to predict daily methane emissions by sheep as measured in methane chambers and *in vitro* gas production analysis of feeds.

3. Materials and methods

3.1. Respiratory chambers for the validation of proxy methods

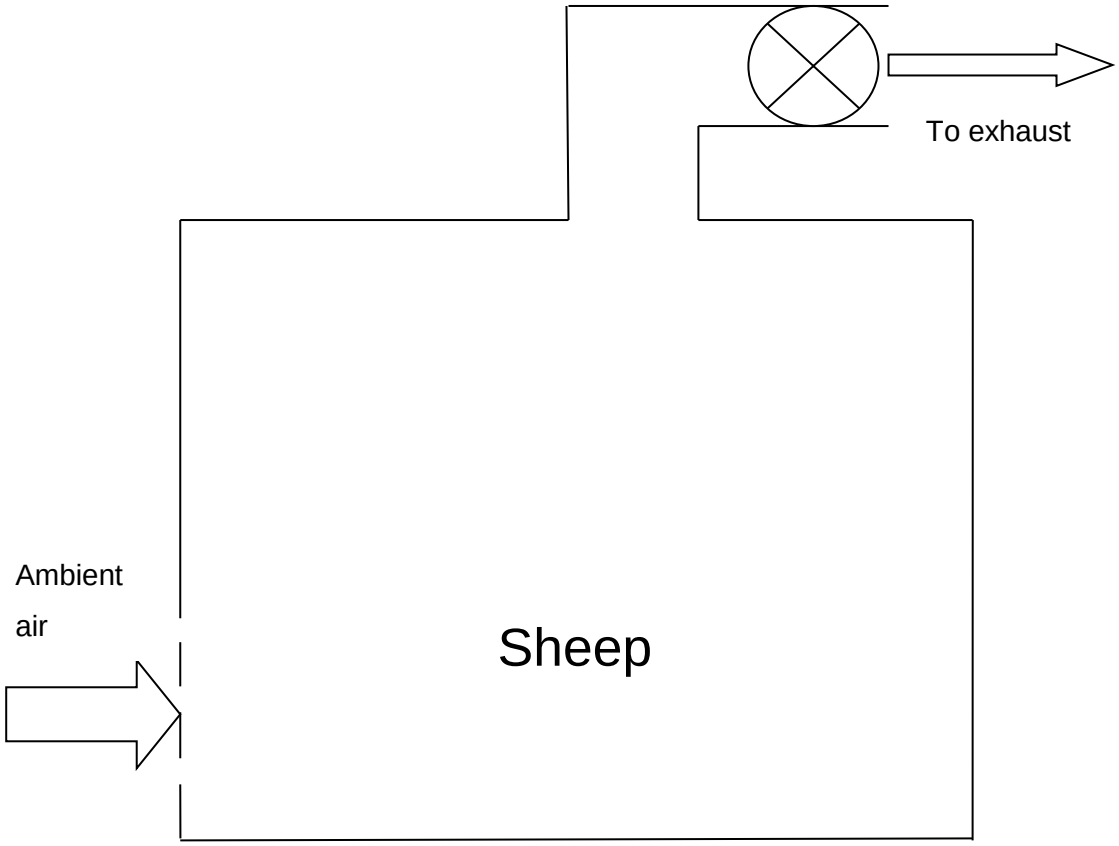
The use of open-circuit respiration chambers, or "methane chambers", to measure methane emissions by sheep is considered to be a 'gold standard' and, although subject to errors and inaccuracies, is the most accurate form of methane measurement from individual sheep currently available. Throughout the project, any methane measurements taken using potential proxies were validated against methane chamber measurements that were taken either from the same animals or diets as described in the relevant experimental methods sections.

The aim of using the respiratory chambers was to obtain reliable and repeatable measurements of methane output by sheep, which could be used for comparison with methane output estimates from proxy indicators.

3.1.1. Chamber principles

The principle of an open circuit respiration chamber (“methane chamber”) is that fresh air enters the chamber via an inlet, and air mixed with gases released by the animal exits the chamber via an outlet. The methane concentrations (ppm) sampled from the inlet and outlet gases are measured, along with the airflow through the chamber, which enables the calculation of daily methane emissions from animals inside the chambers. The general design of methane chambers is shown in Figure 1.

Figure 1: diagrammatic representation of general design of methane chambers



3.1.2. Chamber structure

The methane chambers at IBERS (Gogerddan, Aberystwyth University) were 1.8 m x 1.8 m x 1.5 m boxes (width x depth x height) constructed using a 25 mm x 25mm square tubing soft steel frame covered in clear polycarbonate sheets fixed with standard self-drilling and self-tapping screws. A 75 mm x 75 mm weld-mesh on the inside of the metal frame stopped animals damaging the polycarbonate from the inside, while the clear sheets allowed animals to see each other in neighbouring chambers. The bottom 300 mm of each side and back wall of the chambers was constructed of galvanised steel sheet to allow easy cleaning. The front of each chamber comprised two large hinged doors that had an unsealed area 300 mm high, with the same weld-mesh grid, at their bases to allow air to freely enter the chambers. The polycarbonate sheeting was sealed to the frame using standard draft excluder tape and silicone sealant. Chambers were designed to be airtight, to prevent air entering or leaving the chamber in places other than an air inlet at the bottom of the chamber doors and the outlet pipe in the roof. The chambers were sited inside a sheep shed and were constructed directly on the shed's concrete floor, and the floor inside each chamber was covered with removable rubber matting for animal comfort and easy cleaning. The outlet of the chamber was in the roof, towards the back wall of the chamber, to draw air through the whole volume of the chamber.

A fan in the outflow pipe drew air through the chamber and the speed of the fan can be altered to control airflow through the chamber using a fan speed controller (ME1.1, Fläkt Woods UK Ltd, Colchester, UK). This ensures that airflow through chambers is relatively constant and that animals have a sufficient supply of fresh air. Airflow must be also controlled in order to ensure that it methane concentrations remain within the detection limits of the gas analyser used. For the studies carried out for this thesis, the concentration of methane in the air analysed had to be between 0.5 ppm and 50 ppm, which were the minimum and maximum limits of analysis.

The chamber method assumes that majority of expired gases were drawn out of the chamber via the outflow pipe. Some expired air may be lost through the inlet and around the seals of the doors, which are not completely airtight, but this was not evaluated. Instead, each chamber was calibrated to assess methane recovery. Sample collection tubes, which sampled air from each chamber's outflow pipe, were connected to an 8-port single channel gas analyser (MGA-3000 multi-gas analyser, ADC Gas Analysis Ltd, Hoddeston, UK), which was set to measure methane concentration in rotation from each of the four chambers and two ambient air sampling pipes. Sampled air was drawn through a home-made in-line desiccator comprising self-indicating silica gel (SiO_2) desiccant in a small screw-top plastic bottle. The silica gel extracted water from the sampled air sample to prevent interference in the gas analyser. In-line dust filters were also fitted to the sampling pipes to prevent dust contamination of the analyser. The gas analyser was set to record the methane concentration from each chamber or ambient inlet after three minutes dwell time; this allowed adjustment of the gas analyser between samples to ensure that the previous sample taken into the analyser did not have an effect on the next measurement.

3.1.3. Airflow and environmental measurements

In order to calculate the daily methane output from animals, the rate of airflow through the chambers is required. This was measured using mini vane anemometers (MiniAir 6, Schiltknecht Messtechnik AG, Gossau, Switzerland) inserted into each of the outflow pipes, connected to a 4-channel MSR145 mini data logger (MSR Electronics GmbH, Seuzach, Switzerland). Airflow measurements for each chamber were collected every 30 seconds throughout the time sheep spent in the chambers. The airflow through chambers changed slightly throughout the day, making it necessary to take these regular measurements in order to calculate an average airflow. Noticeably, the airflow changed when lights were turned on and off in the building because this affected the power supply to the chamber fans. The datalogger also recorded ambient air temperature and pressure, which are required to convert methane concentrations in ppm to volume, should an actual (rather than standardised) volume be needed. Periodically throughout experiments, approximately once per week, the data logger was connected, via a USB cable, to a computer. The airflow and environmental data were then downloaded onto the computer.

3.1.4. Chamber measurement calibration factor

Although it is assumed that all gases released from animals in the methane chambers is extracted via the outlet pipe, it is possible that some are lost via either the chamber inlet or around the doors of the chamber. Also, a major component of the calculation of methane emissions from the chamber is the airflow measurement in the outflow pipe, and it is well known that laminar flow of gases in a pipe means that airflow at the centre of that pipe is faster than airflow close to the pipe walls. To improve the accuracy of the methane measurements, it was necessary to quantify these potential errors and correct for them. As part of Defra-funded project AC0115, one of the Defra Greenhouse Gas Platform projects, representatives from the National Physical Laboratory (NPL) visited IBERS in December 2011 in order to calibrate the chambers, i.e. check measured values against a known amount of methane released into the chamber, and create a correction factor to minimise any differences. To do this, a device that accurately released methane at a known rate of 2.19 L/hour, was used. The device was placed inside methane chambers, as a sheep would be, and set to release methane at a constant concentration and rate, which was similar to that which would be expected from a sheep. The volume of methane released into the chamber was calculated and compared with the volume of methane measured by the chamber equipment. As expected, there were small differences in methane measurement compared to methane release; the apparent chamber capture efficiency was 0.928 (±0.115) (NPL, 2013). The NPL were able to provide a calibration factor of for each of the four methane chambers, which could be applied to the daily methane calculations to increase the accuracy of methane measurements by the chambers. The calibration factors for each chamber are shown in Table 1.

Table 1: Chamber calibration factors (NPL, 2013)

Chamber	Calibration factor	Factor uncertainty
1	1.199	±0.082
2	1.158	±0.029
3	1.124	±0.074
4	1.110	±0.152

3.1.5. Chamber measurements

Since there were four calibrated methane chambers available, sheep were placed in chambers in groups of four in each experiment. Animals were kept in the chambers for a three day period. During this time, they were released from chambers twice daily to allow the chambers to be cleaned and fresh feed and water to be placed in chambers. Chamber events, detailing exact times at which doors were opened and animals left and entered chambers, were recorded. This was

important as any methane concentration data recorded when animals were not present in chambers or when chamber doors were open was later discarded; it would not be a true representation of methane emissions from the animals. Dates and times that sheep entered and left chambers were also recorded. Methane concentration data was checked and saved twice daily to minimise any data loss due to potential problems with the gas analyser or the computer that was recording measurements.

The gas analyser was set up to take samples from the sample outlet tubes of each chamber in sequence, along with two ambient samples. When a new batch of animals were put in the chambers, the methane concentrations were carefully monitored at the start and the fan speeds were adjusted if necessary to ensure that the concentrations were in the analyser's measurement range. Once three days' worth of methane concentration data was recorded for each set of four sheep, the animals were released from the chambers, which were then thoroughly cleaned before the next group entered them.

3.1.6. Calculation of daily methane output

Daily methane output (g/d) was calculated using the methane concentrations measured by the gas analyser and measurements of the flow of air leaving the chambers. This was done by firstly converting the airflow (m^3/s) into flow (L/d). This was multiplied by a 'true methane concentration' (ppm), which is a mean of the chamber methane concentration measurements minus the ambient methane concentration measurements. Flow (L/d) was multiplied by true methane concentration (ppm) and the product was divided by one million (because concentration is measured in ppm) to give daily methane output (L). All methane data were calculated to standard temperature and pressure of 0°C and 101.325 kPa regardless of the actual temperatures and pressures measured during the experiments, and methane emissions were reported in grams. The molar volume (22.414 L/mol) was calculated by multiplying Avogadro's universal gas constant (8.314462) by the standard temperature and dividing the product by the standard atmospheric pressure. Daily methane output (g) was then calculated by dividing daily methane output (L) by molar mass (L/mol) and multiplying the product by the molar mass of methane (16.04246). The daily methane output was then divided by the calibration factor for the relevant chamber in order to correct for methane losses from chambers.

3.1.7. Chamber experiments completed

Small scale experiment

An initial small scale experiment was conducted using four Cheviot wethers fed on an *ad libitum* diet of grass silage. Animals were adapted to the diet for a two week period in a group pen. They were then placed in individual pens for three days before entering methane chambers for a period of five days. Animal weights were recorded upon entering and leaving chambers. Feed was offered twice daily and water was constantly available.

Large scale experiment

Over the course of 4 large scale chamber experiments, 32 sheep of 4 different breeds (Welsh mountain, Scottish blackface, Welsh mule and Texel) were used. Many of these animals were used throughout the experiments, or in more than one of the experiments, although there were some changes. However, in each of the experiments, the number of sheep of each breed was eight. Animals were weighed upon entering and leaving the methane chambers.

Chamber experiments were carried out as part of the Defra AC0115 project and IBERS technicians were responsible for collecting data from these experiments. I assisted with the data collection and used the data for my own subsequent comparisons.

3.2. The Laser Methane Detector

3.2.1. LMD principle

The LMD used throughout experiments was the SA3CO6A LMD from Tokyo Gas Engineering Co. Ltd. (supplied by Crowcon Detection Instruments Ltd., Abingdon, UK). This relies on infrared spectroscopy, using a semiconductor laser to measure the concentration of methane between the LMD and the source of methane. The laser beam is transmitted towards the methane source and a fraction of the diffusely reflected beam from the target point is measured by the LMD. The measurement obtained is the methane column density (ppm-m), which is the methane concentration (ppm) multiplied by the thickness of the column (m). The LMD measurements should not be affected by gases other than methane (Tokyo Gas Engineering Co. Ltd., 2006).

3.2.2. Using the LMD

The LMD was used by simply pointing it at the methane source, pulling the trigger. The trigger could be held in place with an additional button next to it. To stop measurements the trigger was released. The LMD was set to take one measurement per second for all experiments. The LMD shown in Plate 1. The display screen, also shown in Plate 1, shows the real-time column density (ppm-m) of methane between the LMD and the point source of methane. As the data is displayed on the screen, it is saved on the machine and can later be transferred to a computer via an SD card.

Plate 1: The Laser Methane detector (LMD) used for measuring real-time methane concentrations at a distance.

3.2.3. Units of measurement

The LMD measured methane column density in units of parts per million-metres (ppm-m). This means that concentration (ppm) was measured assuming that the distance between the LMD and source of methane was one metre. Therefore, if the distance between the LMD and source of methane was either more or less than one metre, the data needed to be corrected for the distance. This was done by multiplying the LMD measurement by the actual distance (m); for example, if the LMD was 0.85 metres away from the source, the data was multiplied by 0.85. In most of the experiments using the LMD, measurements were taken from approximately one metre away from the methane source to avoid the need to correct the data.

3.2.4. Battery life

Two Ni-MH rechargeable batteries (4.8V, 2700m Ah) were supplied with the LMD. Each battery life was approximately 1.5 hours and recharging each battery took approximately 3 hours. Protocols for individual experiments were therefore adapted to allow for the battery life and recharging time. If the LMD was left to run for the duration of the battery life, it automatically saved any data taken before it turned off, preventing any data losses.

3.2.5. Correcting for background methane

The LMD has a setting to offset background methane concentration, which involves briefly pointing the LMD away from methane sources whilst pressing the offset button. However, this was tried and, due to the large number of animals in the vicinity and the presence of a muck heap near the experimental facilities, it was decided to correct data after measurements were taken rather than offsetting the LMD prior to measurements. This was done by subtracting the minimum LMD measurement from all measurements within each measurement period as this was assumed to be the background methane concentration.

3.2.6. Calculation of daily methane emissions

As the output of the LMD was methane concentration, assuming that the measurement distance was one metre and no adjustment for this was required, it was necessary to calculate daily methane emissions (g) from individual sheep. The calculations developed were based on those used to calculate daily methane emissions from the methane chamber results.

Airflow equivalent

The value used as an equivalent to airflow (used in methane chamber calculations) was respiratory rate, which is calculated by multiplying tidal volume (L) with breaths per minute. Tidal volumes for sheep were estimated based on body weight (kg): it was assumed that tidal volume (L) amounted to 12ml per kilogram of body weight, as average tidal volumes range from 10 to 15ml/kg of body weight (Kohn *et al.*, 1997). Breathing rate was assumed to be constant, using 20 breaths per minute as a normal breathing rate for sheep (University of Adelaide, 2009); this was scaled up to

breaths per day (28800). Respiratory rate (L/d) was calculated by multiplying tidal volume (L) by breaths per day.

Integration

The integration, or area under the curve, of the LMD measurement was calculated in order to represent both the heights and lengths of peaks. Integration was calculated using the following formula in Microsoft excel, assuming that the time (seconds) was in column A, the methane concentration (already corrected for background methane) was in column B, and the length of the measurement period was ten minutes (600 seconds):

Integration = SUMPRODUCT(A3:A601-A2:A600, (B3:B601+B2:B600)/2)

The integration values for all measurement periods for a particular sheep for one day were added up and, assuming that the total measurement time was thirty minutes per day for each sheep, this value was multiplied by 48 calculate a daily value. The square root of the 'daily integration' was then taken as the integration value is an area.

Calculation of daily methane (L)

Daily methane (L) was then calculated by multiplying the respiratory rate (L/d) with the square root of the daily integration and dividing the product by one million, as the units of methane concentration are parts per million (ppm).

Calculation of daily methane (g)

The molar volume of an ideal gas at standard temperature and pressure (273.15K and 100kPa respectively) is 22.4L/mol. Ambient temperature and pressure were not measured during LMD measurements, and as for the calculation of methane emissions using the chambers, this value was used, along with the molar mass of methane (16.04246 g/mol), to calculate daily methane emissions. This was achieved by dividing daily methane (L/d) by molar volume and multiplying the product by the molar mass of methane. This gave the daily methane emissions (g/d) from each animal.

3.2.7. Experiments completed

Small scale experiment

An initial small scale experiment was completed using 4 Cheviot wethers, as described in Section 3.1.7. LMD measurements were taken three times per day per sheep, for periods of ten minutes over the course of three days, while animals were in individual pens. The first measurement period was in the morning (8:30am-9:30am), forty minutes after feeding. The second was in the early afternoon (12:30pm-13:30pm), and the third was later in the afternoon (16:00pm-17:00pm), forty minutes after the afternoon feed. The measurements were taken from approximately one metre away from the animal, pointing the LMD directly at the nostrils of animals. Animals were free to move within individual pens and the operator of the LMD moved around to keep the LMD pointing

at the nostrils and to maintain the same distance between the LMD and the sheep as much as possible.

Large scale experiments

Two larger scale experiments were also conducted using 32 sheep of 4 different breeds as described in Section 3.1.7. LMD data were collected in conjunction with chamber experiments in which sheep were fed grass nuts and *Molinia caerulea* (Section 3.1.7).

3.3. *In vitro* gas production

3.3.1. Overview of the *in vitro* gas production method

The *in vitro* gas production technique used in the experiment was a semi-automated method based on the method outlined by Theodorou *et al.* (1994) and Davies *et al.* (2000). This is a relatively quick and simple way of analysing samples in a simulation of rumen fermentation by incubating them with rumen fluid and measuring the amount of gas (and in particular methane) produced. Serum bottles containing samples and a digestion medium were prepared the day before inoculation with rumen fluid to allow the digestion medium to be reduced by the reducing agent and to allow the bottles to be heated to 39^0C, to simulate the temperature of the rumen. The France *et al.* (1993) model was then fitted to the data, providing gas production curves and allowing comparison between plants and feeds based on the model parameters.

The preparation of gas production bottles was completed over two days before inoculation with rumen fluid. This allowed time to incubate the samples, which was necessary in order to allow for deoxygenation of the digestion medium and for increasing the temperature of the solutions to 39°C. It was important that this reduction reaction occurred as the microbes contained in rumen fluid require warm (39°C) anaerobic conditions.

3.3.2. Samples used

Samples of feeds offered to sheep were collected during four large scale methane chamber experiments (Section 3.1.7), including perennial ryegrass, permanent pasture (containing a variety of grass species [e.g. perennial and Italian ryegrass, cocksfoot, and timothy], clovers, and some weeds), grass nuts, and *Molinia caerulea*. Perennial ryegrass, permanent pasture, and *Molinia caerulea* were offered using zero grazing. Plants were cut using a Haldrup Harvester in IBERS fields (perennial ryegrass and permanent pasture) or at Pwllpeiran, an upland site (*Molinia caerulea*). Plants were harvested daily or as required and stored in a large walk-in refrigerator next to the sheep shed. Grass nuts were also kept in this refrigerator during the grass nuts experiment.

These experiments each used 32 sheep of four different breeds (Welsh mountain, Scottish blackface, Welsh mule, and Texel). The majority of the sheep remained the same throughout the four experiments, though there were some changes. During the experiments, sheep were fed either perennial ryegrass, permanent pasture, grass nuts, or *Molinia caerulea*. Four sheep entered the chambers for three days at a time. There were, therefore, eight runs of sheep being put through chambers. Samples of feed offered were taken on a daily basis and bulked for each three day period so that there was a feed sample per run of sheep entering the chambers. This meant that there was a total of 32 samples, eight samples of each of the feeds offered, and methane emission data from the sheep that consumed these feeds. These samples were used along with a standard silage sample and blanks.

3.3.3. Day 1

Solutions

The following solutions were made up using distilled water boiled in a microwave oven to remove oxygen:

- Buffer solution (g/L distilled boiled water): 4g ammonium hydrogen carbonate (NH_4HCO_3), 35g sodium hydrogen carbonate (Na_2HCO_3).
- Macromineral solution (g/L distilled boiled water): 9.45g di-sodium hydrogen orthophosphate ($Na_2HPO_4.12H_2O$), 6.2g anhydrous potassium di-hydrogen orthophosphate (KH_2PO_4), 0.6g magnesium sulphate 7-hydrate ($MgSO_4.7H_2O$).
- Micromineral solution (g/100ml distilled boiled water): 13.2g calcium chloride 2-hydrate ($CaCl_2.2H_2O$), 10.0g manganese chloride 4-hydrate ($MnCl_2.4H_2O$), 1.0g cobalt chloride 6-hydrate ($CoCl_2.6H_2O$), 8.0g ferric chloride 6-hydrate ($FeCl_3.6H_2O$).
- Resazurin solution (g/100ml distilled boiled water): 0.1g resazurin (redox indicator).
- Reducing agent (g/100ml distilled boiled water): 0.625g cysteine HCl, 4ml 1M sodium hydroxide (NaOH).

Stock solutions of micromineral and resazurin solutions were made up and could kept refrigerated as very little was required for each gas production experiment.

Sample preparation

Samples to be used were freeze dried and ground to pass through a 1mm sieve. Approximately 1g of each sample was added to 160ml serum bottles and the weight added was recorded. Samples were analysed in triplicate so three serum bottles were used for each sample. Three bottles were left empty to act as blanks, containing no substrate. In all experiments, a standard sample of dried and ground grass silage was used to allow comparison between experiments.

Digestion medium preparation

The digestion medium was prepared by mixing the prepared solutions with boiled, distilled water in the following proportions:

- 1000ml boiled distilled water.
- 0.2ml micromineral solution.
- 400ml buffer solution.
- 400ml macromineral solution.
- 2ml resazurin solution.

The volume of medium required for each gas production experiment was mixed and then CO_2 was passed through it for 1.5-2 hours to remove oxygen.

Preparation of serum bottles

An automatic dispenser was used to add 85ml of digestion medium to each serum bottle, while gassing the bottle with CO_2. A 4ml volume of reducing agent was added to each bottle immediately after the digestion medium and bungs were put in bottles as quickly as possible to reduce the amount of oxygen entering. Bottles were then sealed with aluminium seals and placed in an incubator set at 4^0C. The incubator temperature was set to automatically change to 39^0C early on day 2, a few hours before inoculation with rumen fluid. Bottles were ready for inoculation when the pink/purple colour of the redox indicator had faded, indicating that the solution was reduced.

3.3.4. Day 2

Collection of rumen fluid

An empty Dewar flask (2L volume) was taken to Trawscoed farm, approximately 20 minutes drive from IBERS, Gogerddan. The flask was filled with hot water to pre-warm it. The rumen contents were removed from three ruminally fistulated cows, which were being fed on grazed grass and grass silage. Rumen contents were squeezed through a wire sieve to separate the large solid feed particles from the rumen fluid. Rumen fluid from all animals was mixed together. The Dewar flask was emptied of water before being immediately filled with rumen fluid. The flask of rumen fluid was then transported back to IBERS, Gogerddan as quickly as possible to avoid cooling and placed in an incubator set at 39^0C until needed.

Inoculation of serum bottles with rumen fluid

In a fume cupboard, rumen fluid was strained through a double layer of muslin into a beaker, whilst being gassed with CO_2. Fluid was mixed using a magnetic stirrer in the beaker of strained rumen fluid. A 10ml syringe fitted with a hypodermic needle was used inject 10ml of the filtered rumen fluid through the bungs into each serum bottle. A hypodermic needle was then used to equalise the pressure, by releasing any gases, in the bottles before replacing them in the incubator at 39^0C.

3.3.5. Data collection

The gas analyser (ADC 5000 series, ADC Gas Analysis Ltd., Hoddesdon, UK) used to determine proportions of carbon dioxide and methane in samples was first calibrated with standard gases, one containing 80% carbon dioxide and the other containing 80% methane, the remainder being nitrogen in both cases. The gas production bottles were removed from the incubator and placed in a water bath heated to 39^0C. A three way valve was used to connect a detachable pressure transducer and LED digital readout voltmeter (Bailey & Mackey Ltd., Birmingham, UK), encased in plastic (length 200mm, depth 145mm, height 75mm; R.S. Components, Northampton, UK; constructed at IBERS Gogerddan), to a 60ml syringe and a hypodermic needle (23 gauge x 3.8cm). The needle was inserted into one bottle at a time through the bung. The syringe plunger was held in place to prevent the pressurised gas leaving the bottle while a pressure reading was taken (this was displayed by the voltmeter connected to the pressure transducer). The syringe

plunger was then released and pulled out until the pressure reading reached zero, i.e. atmospheric pressure. The volume of gas removed from each bottle was recorded. The needle was withdrawn from the bottle and the gas in the syringe was injected into the gas analyser. The gas analyser provided values for percentages of carbon dioxide and methane in each sample, which were recorded. A minimum of approximately 15ml of gas was required to produce an accurate reading from the gas analyser. If there was little gas produced by samples (less than 15ml), the gas from the three triplicate samples was pooled to inject into the gas analyser. The samples were then replaced in the incubator. Measurements were taken over a period of about 120 hours. The first measurements were taken after about 3 hours after inoculation with rumen fluid. Measurements were then taken every 3-4 hours until fermentation began to slow down and the rate of gas production decreased. The length of time between measurements was gradually increased, eventually to about 12 hours between measurements. When the volumes of gas produced by a sample became too small to analyse, the experiment was stopped and samples were refrigerated at 4^0C. Times at which measurements were taken were recorded.

3.3.6. Vacuum filtration of gas production products

Crucibles for vacuum filtration were placed in a dryer for fifteen minutes to ensure that they were completely dry. They were then weighed and labelled with sample names. A rubber bung, with a hole large enough to accommodate a crucible through it, was placed in the top of a side-arm conical flask and a crucible was placed in the top of the bung. The side-arm of the flask was then connected to a water pump, creating a vacuum. A sample was then poured into the crucible. The liquid fraction of the sample was sucked into the conical flask and the solid part remained in the crucible. Once most of the liquid was removed, the crucibles were placed in a freezer. Once frozen, the samples in crucibles were freeze-dried to remove any remaining moisture. The crucibles were then weighed again to determine the weight of samples left in them. This allowed an apparent DM digestibility to be calculated; apparent digestibility calculations include the possible presence of particles and bacteria from the rumen inoculum in the filtered samples, which may have had a slight impact on the weight of filtered samples, as opposed to true digestibility, which would not include contributions to DM from sources other than the feed itself, but true digestibility could not be calculated easily by this method.

3.3.7. Calculation of cumulative methane production

Carbon dioxide and methane volumes were calculated from the total gas volumes and percentages of each gas. Cumulative total gas, carbon dioxide and methane production values were calculated for each sample. These values were multiplied by the apparent DM digestibility values to give cumulative total gas, carbon dioxide and methane values per gram of apparently digested DM.

3.3.8. Data analysis

GenStat 16th edition (2013) was used to fit the France *et al.* (1993) model ($y = A - BQ^t Z^{\sqrt{t}}$, where $Q = e^{-b}$, $Z = e^{-c}$, and $B = e^{bT+c\sqrt{T}}$) to the data. In this model, y represents cumulative methane production (ml), t is the incubation time (h), A is the asymptote for the methane pool size (ml), T is the lag-time before the rate of methane production increases, and b (h^{-1}) and c ($h^{-0.5}$) are rate constants. Estimates of the model parameters A, Q, T and Z were determined by fitting the the France *et al.* (1993) model to the cumulative methane production data. These estimated parameters were then used to determine fractional rates of degradation (Equation 1), total methane production in the system (asymptote), and cumulative methane production at any time point within the 120 hour measurement period (Equation 2).

Equation 1:
Fractional rate of degradation (h^{-1}) = $b + c/(2\sqrt{t})$

Equation 2:
Cumulative methane production *at t* hours (ml) = Constant($1-Q^t$)

GenStat 16th edition (2013) was used to perform repeated measures analysis of variance to compare samples in terms of total methane production potential in the system (ml/g apparently digested DM), cumulative methane production at 16, 24, 36 and 48 hours, and fractional rate of degradation. Functional bisector regression was performed using GenStat to correlate total methane production potential, cumulative methane production at 16, 24, 36 and 48 hours and fractional rate of degradation with analytical chemistry results, such as feed neutral detergent fibre (NDF) and water soluble carbohydrate (WSC) concentration values.

3.4. Fourier-transform infrared (FTIR) spectroscopy

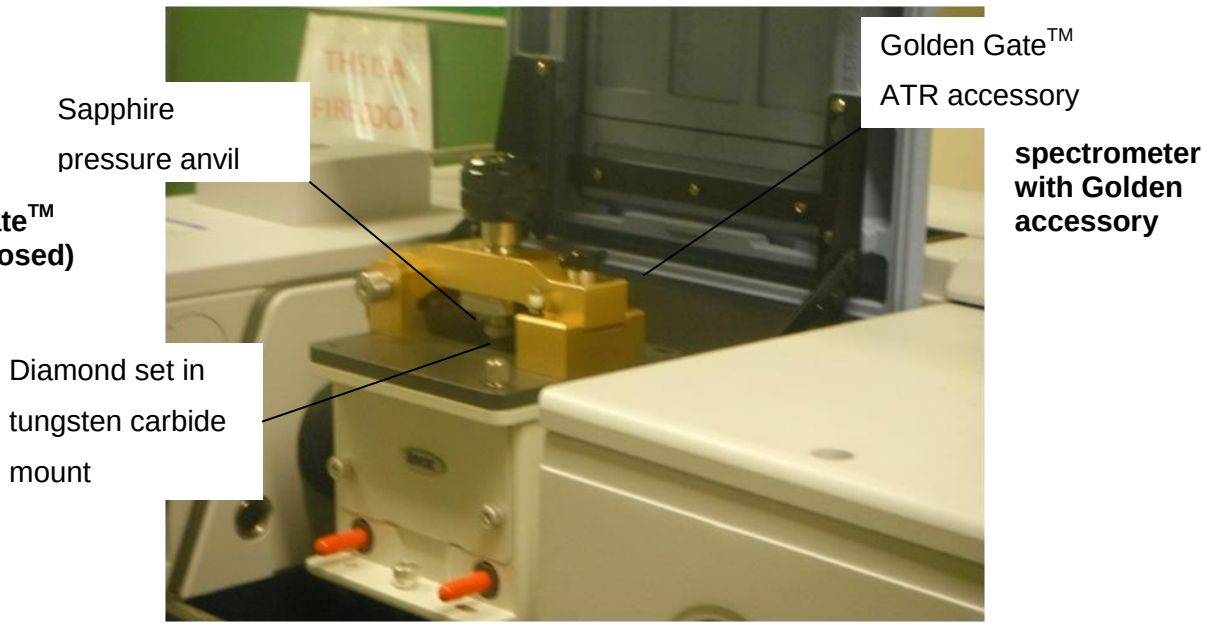
3.4.1. Samples used

The samples used in the experiment were the samples used in *in vitro* gas production experiments (Section 3.3.2), which were those fed to animals during methane chamber experiments (Section 3.1.7). These consisted of eight samples each of grass nuts, perennial ryegrass, permanent pasture and *Molinia caerulea*, and one sample of silage. Average daily methane emissions from sheep from which the feed samples were taken were calculated and used as the observed values for the PLS regression of the FTIR data on methane chamber values.

3.4.2. FTIR spectrometer

The FTIR spectrometer used in the experiment was the Equinox 55 from Bruker UK Ltd., Coventry, UK fitted with a Golden Gate™ (Specac Ltd., Slough, UK) attenuated total reflectance (ATR) accessory (shown in Plate 2). This accessory uses a trapezoid crystal of type IIIa diamond. The sample, which was dried and ground, was pressed against the surface of the diamond using the inert sapphire pressure anvil. The IR beam was passed through the diamond using mirrors and hits the diamond at a very shallow angle. The IR beam was reflected within the diamond, which formed a wave that extended into the sample on the sample-covered surface of the diamond, before passing out of the other side to the detector. The spectrometer operates in a wavenumber range of 4000-600cm^{-1}. The software used to collect the FTIR measurements was Opus (Bruker UK Ltd., Coventry, UK).

Plate 2: FTIR spectrometer with Golden Gate™ accessory (closed)



3.4.3. Measurement method

The Golden Gate™ ATR accessory was fitted into the FTIR spectrometer. This signal was checked using the 'Validate' menu, and the screws controlling the height of the accessory plate were adjusted to achieve a maximum amplitude (around 2000) and the peak position was saved. Bellows were then fitted either side of the accessory, between the accessory and the potassium bromide beamsplitters. The file pathway for saving the data collected was entered into the 'Advanced' section of the 'Advanced Measurements' option from the 'Measure' menu.

A background measurement was taken by leaving the accessory open as shown in Plate 3. The 'Measure/ Advanced Measurements/ Basic' menu was used, and the 'Background Single Channel' option was selected. A background measurement was taken for every new sample.

The samples used were freeze dried and finely ground to pass through a 1mm sieve. Using a spatula, a small amount of sample, enough to cover the exposed surface of the diamond, was placed on the plate (see Plate 3). The accessory was then closed and the sapphire anvil was screwed down to crush the sample against the surface of the diamond. The 'Measure/ Advanced Measurements/ Advanced' menu was used to input a file name consisting of the sample number. The 'Measure/ Advanced Measurements/ Basic' was then used, and the 'Sample Single Channel ' option was selected. This produced a spectrum for the sample (Figure 2) and saved the data to the file pathway previously selected. The anvil was then unscrewed and the accessory opened. The sample was removed from the plate using a vacuum cleaner and replaced with another sample of the same plant/feed material, which was measured in the same way. For every few samples analysed, acetone was wiped over the plate to remove any sample remnants. All samples were analysed in triplicate.

Plate 3: Golden Gate™ accessory whilst open, with a sample on the plate.

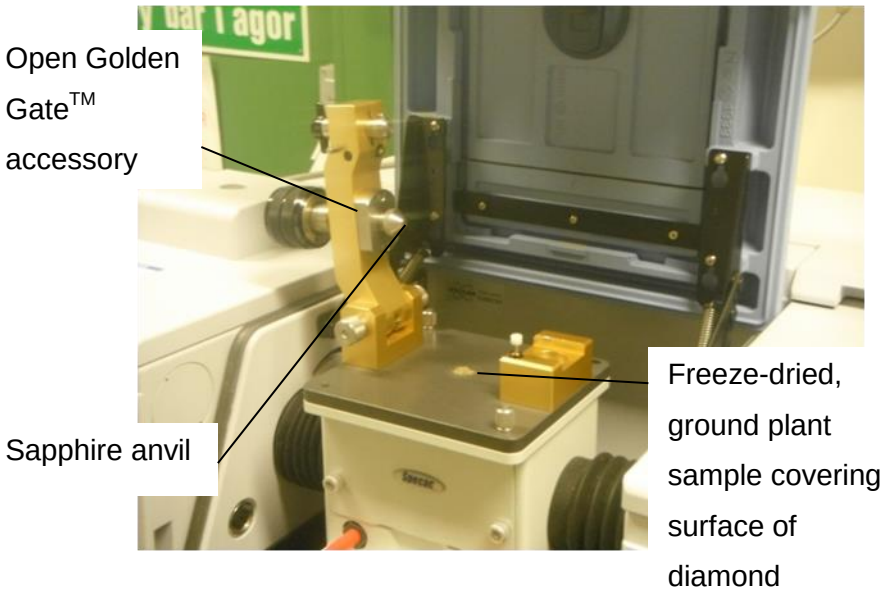
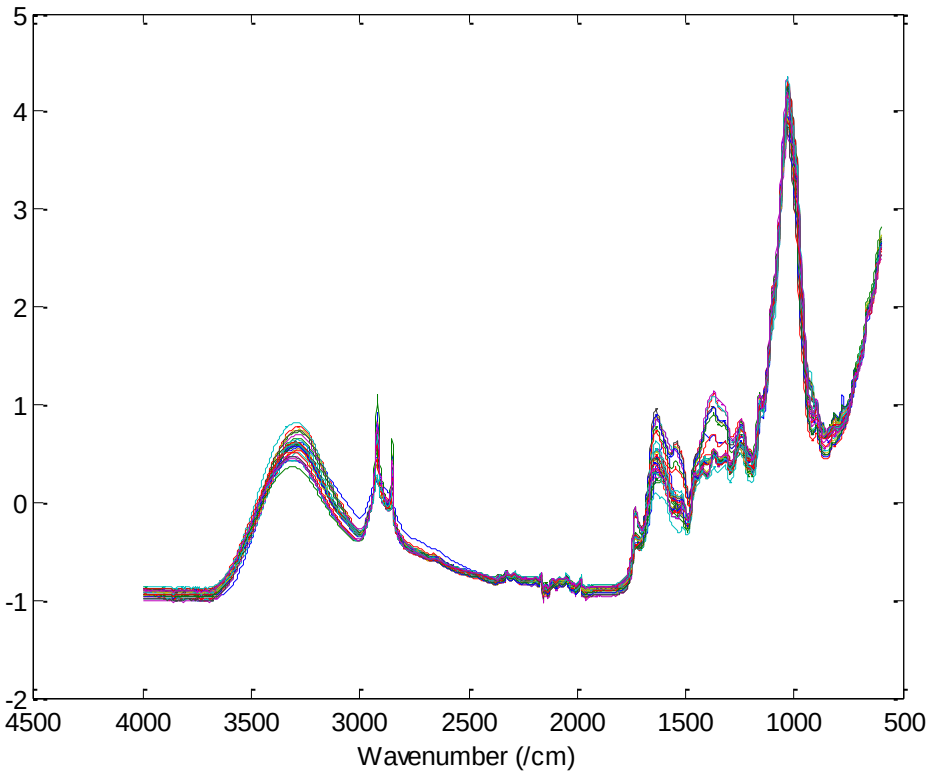


Figure 2: Example FTIR spectra from various plant samples. These data have been normalised to a mean absorbance of 0 and a standard deviation of 1 to account for differences in sample thickness.



3.4.4. Data conversion

In order to analyse the data, it was necessary to convert the data to xy data. This was done by creating a new folder entitled 'xy data'. This was done using the 'Macro' menu in the Opus software.

3.4.5. Data analysis

Initially, principal components analysis (PCA) and partial least squares (PLS) regression was performed in GenStat 16th edition. However, GenStat was not particularly intuitive for these analyses and, to ensure that results were accurate, I received help from my supervisor (Dr. Jon Moorby) to conduct PCA, PLS regression and cross validation using Matlab (R2013a, MathWorks Inc., Cambridge, UK).

An initial visual check of the data was carried out to ensure that all spectra appeared normal. Any spectra that appeared to be odd were discarded. Mean spectra were calculated for each sample from the original data collected in triplicate; any spectra that appeared not to be normal were excluded from the mean values calculated. Absorbance spectra from individual samples varied in amplitude because of difference in the thickness of the samples analysed. To remove this

component of variation from the dataset, all spectra were mean-centre normalised using the MAPSTD function of Matlab, which normalises data to a mean of zero and a standard deviation of 1. This mean-centred FTIR data was then used for subsequent analyses.

Principal components analysis

A large number of variables were identified using FTIR spectroscopy of feed samples. Principal components analysis was conducted in Matlab using the PCA function: this technique was used to simplify the data by replacing groups of variables with single variables (principal components). Each principal component identified was a linear combination of the original variables. The principal components with combined variances were equal to at least 80% of the total variance of the original data set were plotted. By examining the plotted data, it was possible to determine whether the FTIR data could be used to distinguish between the different feeds.

Partial least squares regression

Partial least squares regression was also conducted in Matlab in order to determine whether the FTIR dataset from feeds offered to sheep could be used to predict methane output by sheep as measured in methane chambers. The PLS technique combines the principles of multiple linear regression and principal component analysis, using correlated predictor variables (in this case from the FTIR dataset) to construct new predictor variables or "components", which are linear combinations of the original predictor variables. The components are then constructed using combinations of predictors that have a large covariance with the response values (in this case the daily methane emissions measured from sheep in methane chambers), leading to a model with reliable predictive power. The PLSREGRESS function of Matlab was used to perform PLS regression with the same number of components as predictors and to plot the percentage of variance explained in the response data (chamber daily methane emissions) as a function of the number of components. It was then necessary to determine the number of components that should be used in the PLS model: using all components would result in an over-fitted model that would not fit well to an independent data set. Mean-squared errors (MSEs) for predictors and response were calculated using the PLS procedure, which includes an optional parameter for cross-validation type and the number of Monte Carlo repetitions. The method used for the cross validation of model created using the FTIR data was *k*-fold cross validation (10-fold cross validation was used in this case), which involves partitioning the original data set into equal *k* sized subsamples. All but one of these subsamples are used to train the model, with the remaining subsample being used to validate the model. All of the subsamples are used in both the training and the validation; each is used once as the validation data. This method was therefore appropriate for the data as the data set was relatively small. Mean square errors of response and prediction were plotted in order to determine the number of PLS components that should be used in the model.

3.5. Summary of experiments completed

The methane chamber data used in this project was largely taken from experiments undertaken as part of the Defra AC0115 project. During these experiments, LMD measurements were taken from sheep over 3 days while sheep were kept in individual pens prior to entering methane chambers. In addition, samples of feed offered to each sheep were taken during methane chamber experiments, to be used for *in vitro* gas production and FTIR analysis.

3.5.1. LMD experiments

Following initial trials to determine the optimum methods for obtaining LMD measurements, a small scale LMD experiment was conducted using four Cheviot wethers. Measurements were taken from these animals over a 3 day period, 3 times per day for 10 minute measurement periods. Methane chamber measurements were then taken from the same 4 sheep for a further 3 days to provide a comparison for the calculated daily methane emissions from the LMD measurements taken. Sheep were offered grass silage during this experiment and feed samples were later for further analyses.

Two large scale experiments were then conducted using the same methods as the small scale experiment. Thirty-two barren ewes of four different breeds (Welsh Mountain, Scottish Blackface, Welsh Mule and Texel) were used in both experiments. In the first of these experiments animals were offered grass nuts and 10 minute LMD measurements were taken three times per day over three days. In the second experiment, animals were offered *Molinia caerulea* and 5 minute LMD measurements were taken six times per day over two days. The change in measurement times was due to the results of the previous experiment, which showed that measurements from the same sheep varied significantly depending on the time of day that they were taken but not depending on the day that they were taken.

Estimates of daily methane emissions calculated using LMD measurements were compared with daily methane emissions calculated using methane chamber measurements using functional bisector regression in GenStat 16th edition (2013).

3.5.2. *In vitro* gas production experiments

To validate the use of *in vitro* gas production as a proxy indicator for methane output by sheep, samples of feeds were taken during all methane chamber/LMD experiments described in Section 3.5.1 and in during two additional methane chamber experiments using 32 sheep of four different breeds fed on *Festuca spp.* or permanent pasture. As DM intake was measured during methane chamber experiments, it was possible to estimate methane output by each sheep using DM intakes and methane produced per gram of DM digested obtained from the *in vitro* analysis. These estimates were then compared with estimates of methane output obtained using methane chamber measurements using functional bisector regression conducted in GenStat 16th edition (2013).

3.5.3. FTIR experiments

Feed samples collected during the methane chamber/LMD experiments described in Sections 3.5.1 and 3.5.2 were used for FTIR experiments, in order to determine whether the FTIR spectra of feeds could provide a means of predicting the methane emissions of sheep fed on particular feeds. Firstly, principal components analysis (PCA) was conducted in MatLab to determine whether feed samples could be distinguished by their FTIR spectra. Partial least squares (PLS) regression was then conducted in MatLab to determine the extent to which methane emissions from sheep given each feed could be predicted using FTIR spectra of feeds offered.

4. Results

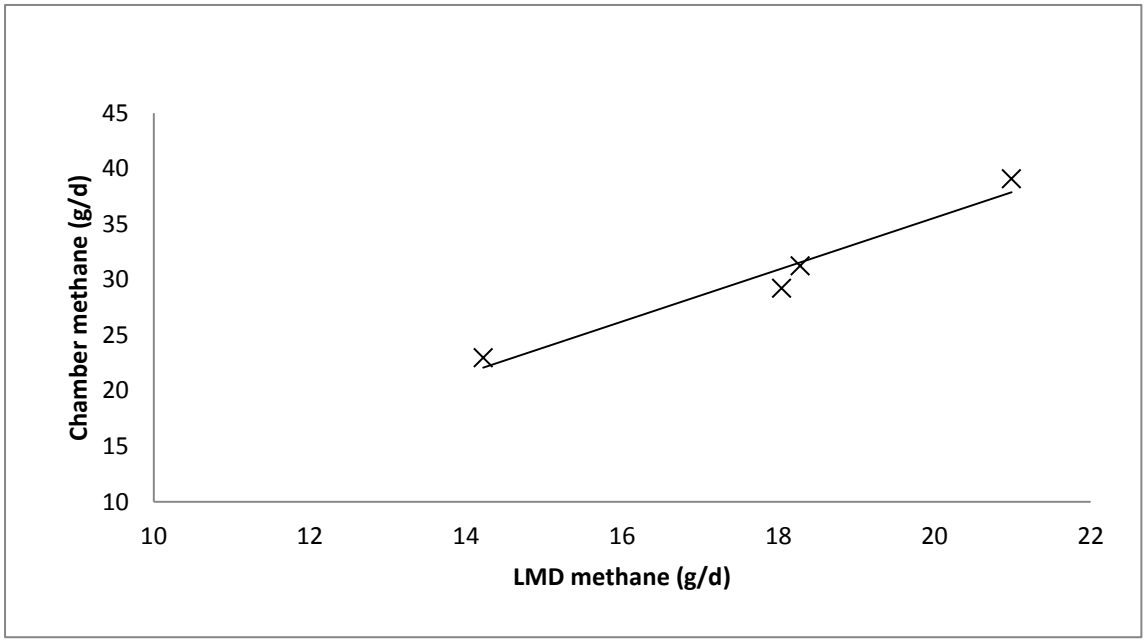
4.1. LMD experiments

4.1.1. Small scale experiment

Comparison between daily methane emissions obtained using LMD vs methane chamber measurements

The LMD values appear to underestimate methane emissions as measured in methane chambers, though there is a significant correlation (R=0.98; P<0.05) between the LMD and chamber measurements, as shown in Figure 3. Therefore, the LMD measurements accurately predicted the ranks of animals in terms of methane emissions according to chamber measurements, though they underestimated the actual values. On average, the factor required to scale the LMD daily methane measurements to the chamber daily methane measurements was approximately 1.7, ranging from 1.62 to 1.86.

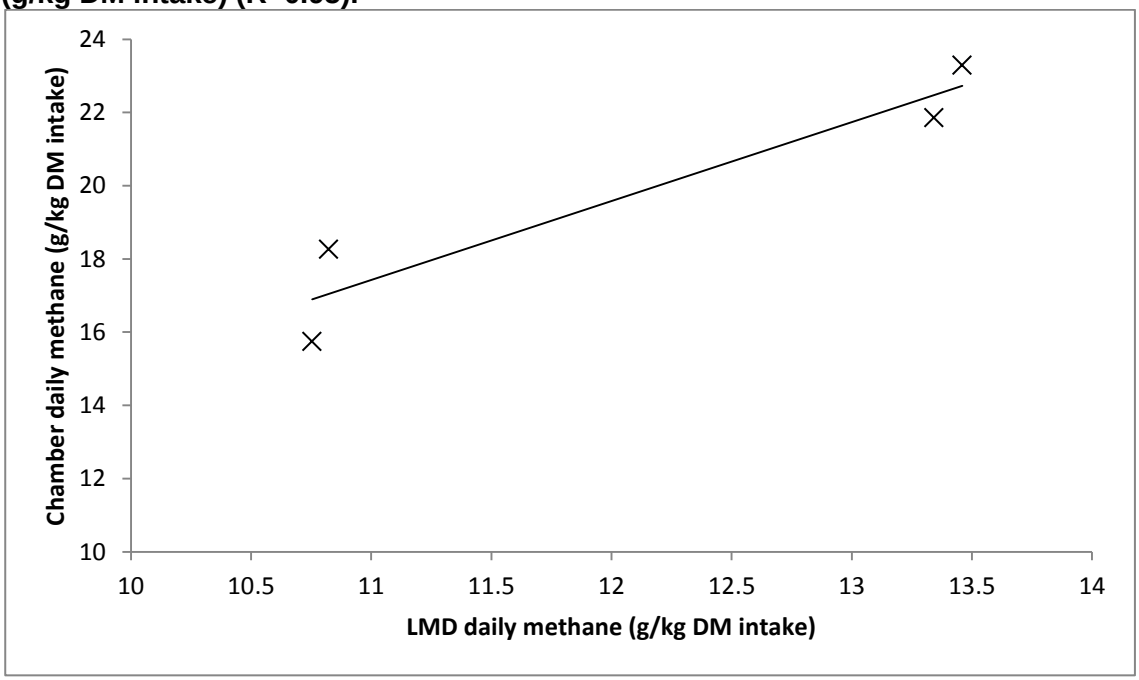
Figure 3: Simple linear regression between chamber and LMD daily methane emissions (R=0.98).



Methane emissions per gram DM intake

Once again, the LMD appears to underestimate methane emissions in comparison to the methane chambers. Though the ranking of the animals in terms of their methane emissions per gram of DM intake are the same using both the chamber and the LMD measurements, in this case, the correlation between the LMD and chamber is not significant (P>0.05). There is, however, a definite trend towards a positive correlation (R=0.95), as P=0.051. The small sample size may be partly responsible for the lack of significance in this data. Figure 4 shows the correlation between daily methane emissions per gram of DM intake from the two types of measurement.

Figure 4: Simple linear regression between chamber and LMD daily methane emissions (g/kg DM intake) (R=0.95).



4.1.2. Large scale experiment with sheep offered grass nuts

Calculated daily methane emissions and DM intakes

Table 2 shows the calculated methane emissions for each sheep using the LMD and chamber measurements, along with respective DM intakes and daily methane emissions per gram of DM intake for each of the two measurement methods. Chamber daily methane emissions have been previously calculated and reported as part of Defra AC0115 project.

Table 2: Methane emissions and yields by breed as measured using LMD and Chamber data

Breed	LMD daily methane emissions (g)	LMD DM intake (kg/d)	Chamber daily methane emissions (g)	Chamber DM intake (kg/d)	LMD methane yield (g/kg DM intake)	Chamber methane yield(g/kg DM intake)
WM	9.63	0.57	12.63	0.57	17.03	22.07
SB	12.51	0.73	18.50	0.73	17.18	25.34
M	15.45	0.84	17.30	0.82	18.29	21.08
T	16.61	0.87	19.84	0.85	19.02	23.35

Abbreviations: DM, dry matter; LMD, LaserMethane Detector; M, Mule; SB, Scottish Blackface; T, Texel; WM, Welsh Mountain.

Functional relationship using the bisector method

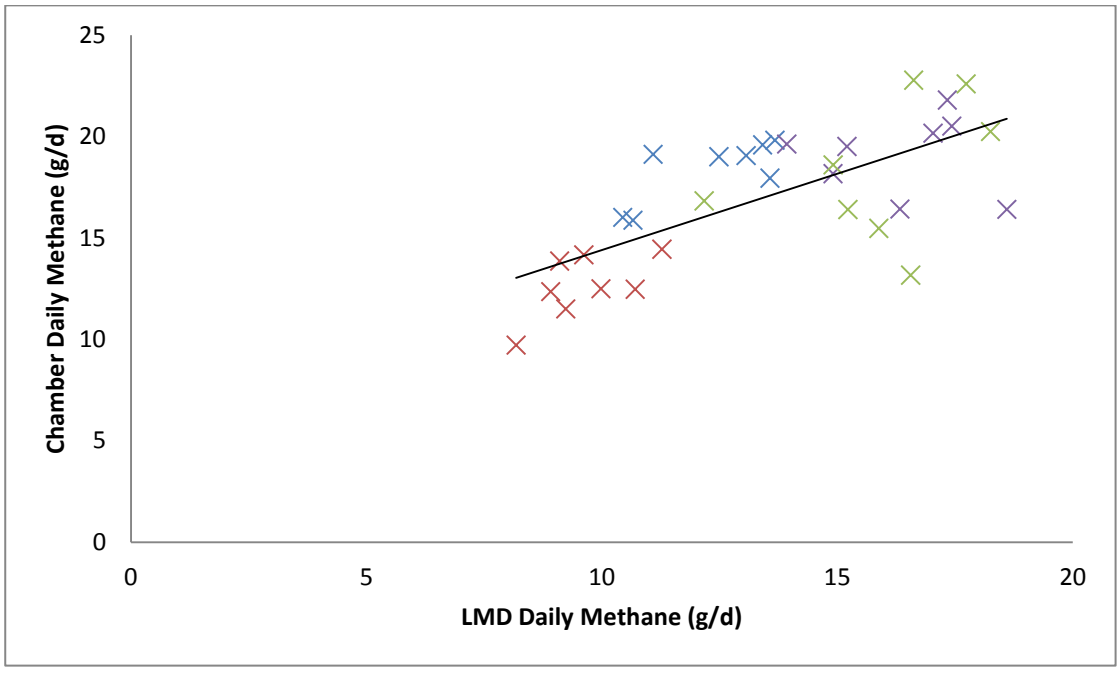
The parameters for all functional relationships are shown in Table 3. Figure 5 shows a significant positive correlation (R=0.70, P<0.001) between daily methane emissions estimated using the LMD and measured in methane chambers. The LMD data, therefore, successfully predicted daily methane emissions measured by methane chambers. There was no significant relationship between the methane emissions per gram of DM intake as measured using either the LMD or chamber measurements; the correlation was close to zero (R=0.04, P>0.05). However, there was

a significant positive relationship (R=0.94, P<0.001) between the DM intake and the daily methane emissions estimated using the LMD. Similarly, there was a significant positive correlation (R=0.76, P<0.01) between DM intake and daily methane emissions measured in methane chambers. The relationships between daily methane emissions from the sheep estimated by LMD and methane chambers and the DM intakes are shown in Figure 6 and Figure 7, respectively.

Table 3: Relationship parameters

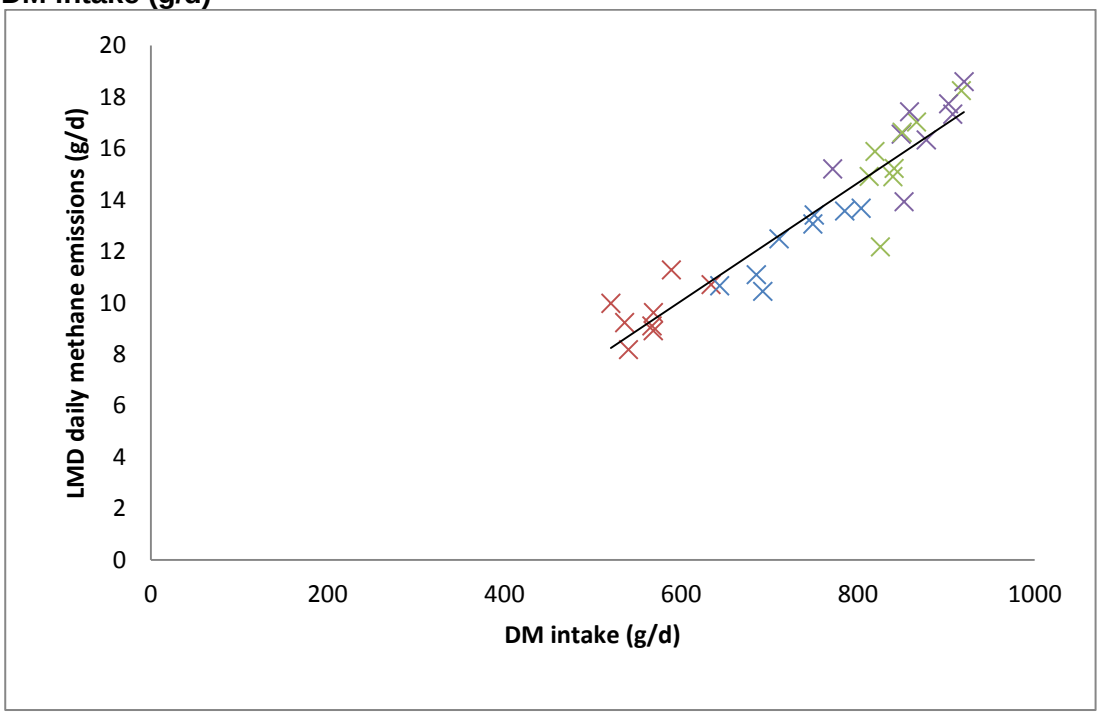
Relationship	Constant	s.e.	Slope	s.e.	Lower	s.e.	Upper	s.e.
LMD methane vs chamber methane	2.50	1.085	1.075	0.0882	0.070	0.8513	4.89	1.23
LMD methane/g DM intake vs chamber methane/g DM intake	0.0042	0.02120	1.051	1.1864	-0.002	-1.594	0.052	1.396
LMD methane vs DM intake	-4.81	1.258	0.024	0.0015	-7.40	0.0218	-2.78	0.0274
Chamber methane vs DM intake	-5.24	2.022	0.030	0.0026	-10.20	0.0261	-2.33	0.0361

Figure 5: Functional bisector relationship between daily methane emissions (g/d) calculated from LMD measurements and methane chamber data.



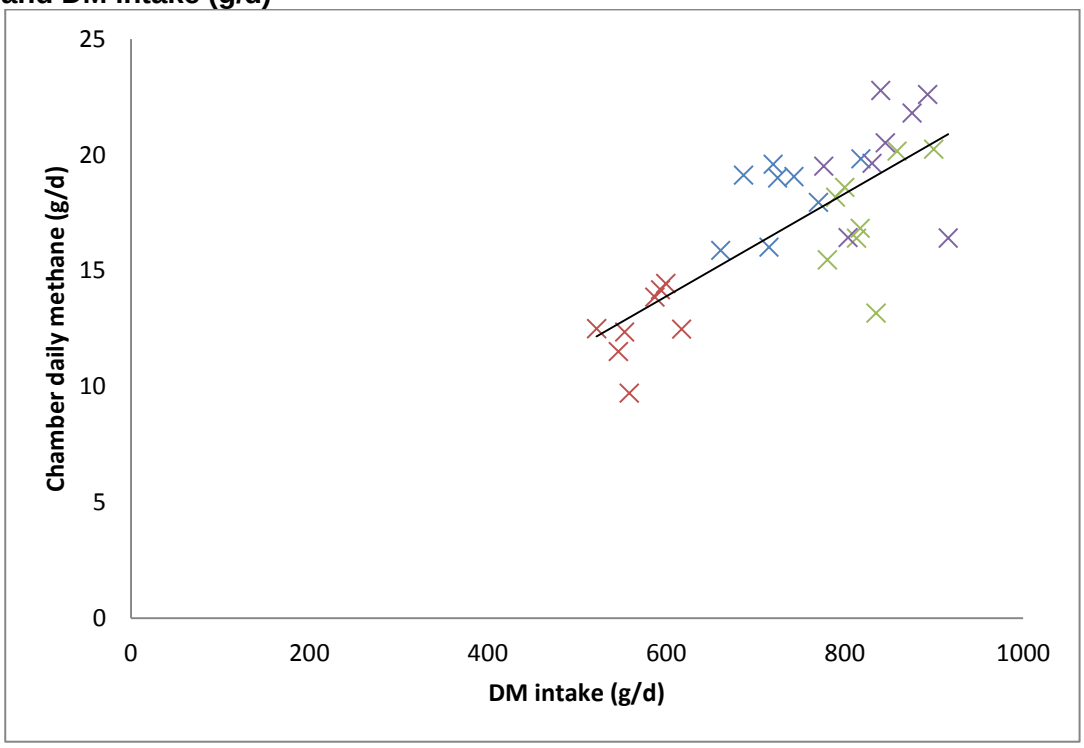
Red=Welsh Mountain; Blue=Scottish Blackface; Green=Welsh Mule; Purple=Texel.

Figure 6: Functional bisector relationship between LMD daily methane emissions (g/d) and DM intake (g/d)



Red=Welsh Mountain; Blue=Scottish Blackface; Green=Welsh Mule; Purple=Texel.

Figure 7: Functional bisector relationship between chamber daily methane emissions (g/d) and DM intake (g/d)



Red=Welsh Mountain; Blue=Scottish Blackface; Green=Welsh Mule; Purple=Texel.

Repeated measures analysis of variance (ANOVA)

Repeated measures ANOVA, using a treatment structure of Sheep with Breed as blocks, showed significant differences (P<0.001) between mean methane concentrations measured at different times of day (Table 4). The least significant difference (L.S.D.) at the one percent level was 1.245. When breed was used as the treatment structure, with data at different times as repeated measures, breed did not significantly affects differences between LMD measurements at different times of day (Table 5). Repeated measures ANOVA also showed that there were no significant differences (P>0.05) between mean methane concentrations, as measured using the LMD, in the first and second halves of the ten minute measurement periods, or between the overall daily methane concentrations for each sheep, shown in Table 6 and Table 7 respectively.

Table 4: Mean LMD methane concentrations at different times of day (repeated measures ANOVA)

Measurement period	08:30am-09:30am	11:30am-12:30pm	15:30pm-16:30pm	SED	P
Mean methane concentration (ppm-m)	12.64[a]	9.38[b]	8.51[b]	0.456	<0.001

Abbreviations: ppm-m, parts per million-metres; SED, standard error of the difference.
Values in the same row with different superscripts differed significantly (P<0.01).

Table 5: Mean LMD methane concentrations at different times of day, using a treatment structure of Breed (repeated measures ANOVA)

Time/Breed	WM	SB	M	T	SED	P
08:30-09:30	13.24	11.71	12.87	12.73	1.107	0.872
11:30-12:30	9.49	8.52	9.40	10.09		
15:30-16:30	9.38	8.06	7.96	8.62		

Abbreviations: ppm-m, parts per million-metres; SED, standard error of the difference.

Table 6: Mean LMD methane concentrations on different days (repeated measures ANOVA)

Measurement period	Day 1	Day 2	Day 3	SED	P
Mean methane concentration (ppm-m)	9.72	10.31	10.49	0.543	0.341

Abbreviations: ppm-m, parts per million-metres; SED, standard error of the difference.

Table 7: Mean LMD methane concentrations in first and second halves of the measurement period (repeated measures ANOVA)

Measurement period	0-5 minutes	5-10 minutes	SED	P
Mean methane concentration (ppm-m)	13.00	12.29	0.453	0.129

Abbreviations: ppm-m, parts per million-metres; SED, standard error of the difference.

4.1.3. Large scale experiment with sheep offered *Molinia caerulea*

Calculated daily methane emissions and DM intake

Table 8 shows the mean calculated daily methane emissions for each sheep breed from both the LMD and chamber measurements, the DM intakes during the respective measurement periods for the LMD and chambers, and the calculated methane emissions per gram of DM intake. As in the previous experiments, the LMD tended to underestimate methane emissions when compared to the methane chamber results. The chamber data have been previously reported as part of the Defra AC0115 project.

Table 8: Methane emissions and yields by breed as measured using LMD and Chamber data

Breed	LMD daily methane (g)	LMD DM intake (kg)	Chamber daily methane (kg)	Chamber DM intake (kg)	LMD methane yield (g/kg DM intake)	Chamber methane yield (g/kg DM intake)
WM	8.34	0.70	10.69	0.64	12.38	17.53
SB	11.51	0.94	14.29	0.82	12.62	18.86
M	15.01	0.96	14.04	0.92	16.69	15.96
T	15.74	1.01	18.87	1.05	16.15	20.03

Abbreviations: DM, dry matter; LMD, Laser Methane Detector; M, Mule; SB, Scottish Blackface; T, Texel; WM, Welsh Mountain.

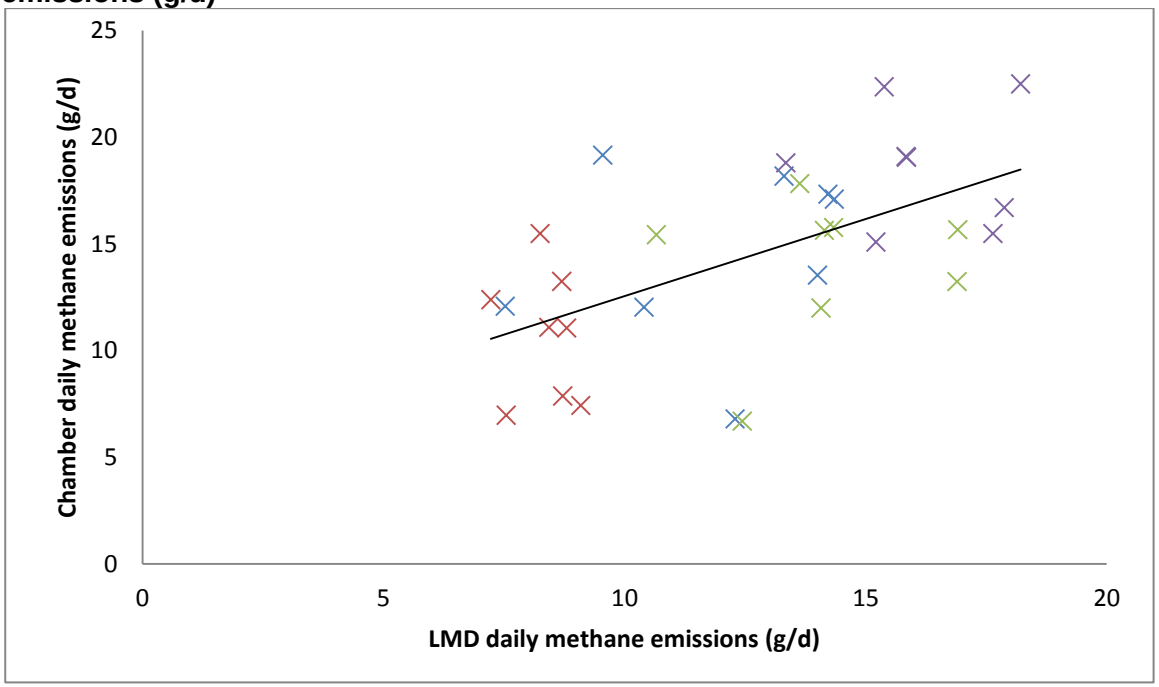
Functional relationship using the bisector method

The parameters of all functional relationships are presented in Table 9. Figure 8 shows a significant positive relationship (R=0.57, P<0.001) between the calculated LMD and chamber daily methane emissions. Figure 9 shows a significant positive relationship (P<0.01) between the daily LMD and chamber methane emissions per gram of DM intake. This relationship was not, however, particularly strong (R=0.48). As in the previous experiment there were significant relationships between DM intake and daily methane emissions as calculated using LMD and chamber data (R=0.59, P<0.001 and R=0.40, P<0.05, respectively). These relationships are shown in Figure 10 and Figure 11.

Table 9: Relationship parameters

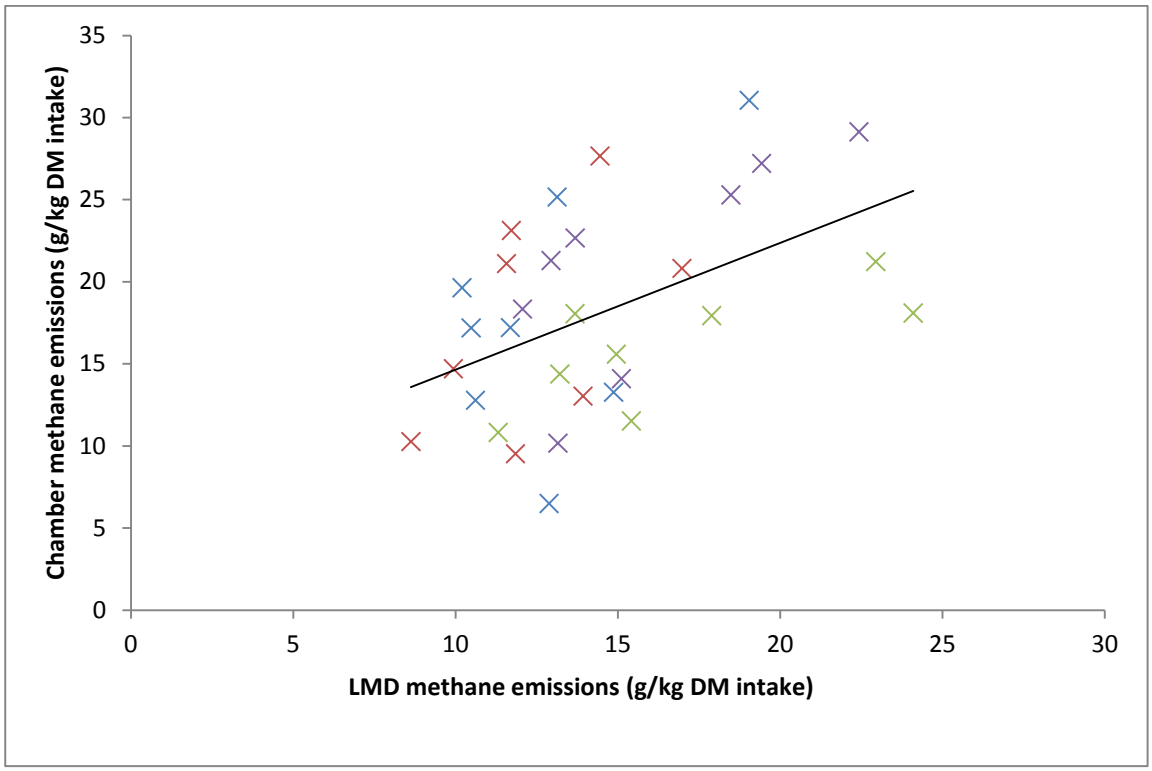
Relationship	Constant	s.e.	Slope	s.e.	Lower	s.e.	Upper	s.e.
LMD methane vs chamber methane	-0.972	2.027	1.22	0.1371	-4.85	0.9644	2.40	1.483
LMD methane/g DM intake vs chamber methane/g DM intake	-0.003	0.0031	1.45	0.2258	-0.001	1.0701	0.002	1.929
LMD methane vs DM intake	-1.76	3.204	0.016	0.0038	-10.16	0.0126	1.18	0.0265
Chamber methane vs DM intake	-3.46	17.119	0.0209	0.0194	-31.44	0.0137	2.51	0.0555

Figure 8: Functional bisector relationship between LMD and chamber daily methane emissions (g/d)



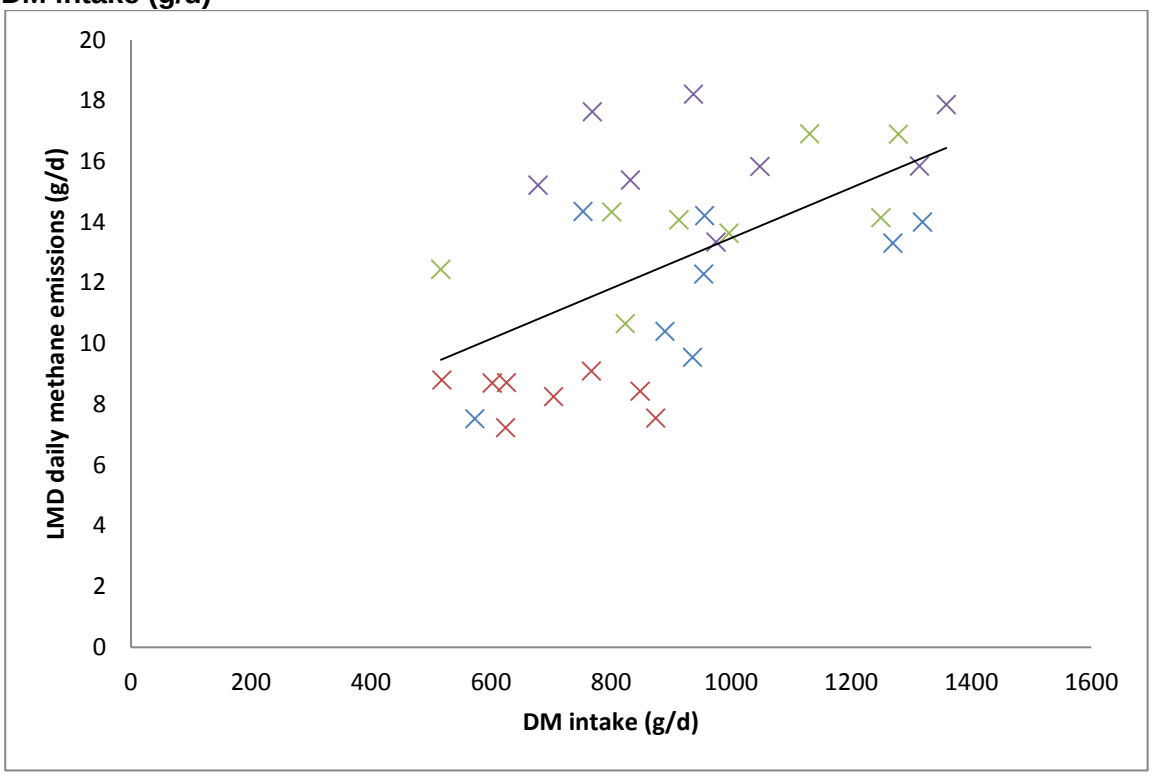
Red=Welsh Mountain; Blue=Scottish Blackface; Green=Welsh Mule; Purple=Texel.

Figure 9: Functional bisector relationship between LMD and chamber methane per kg DM intake



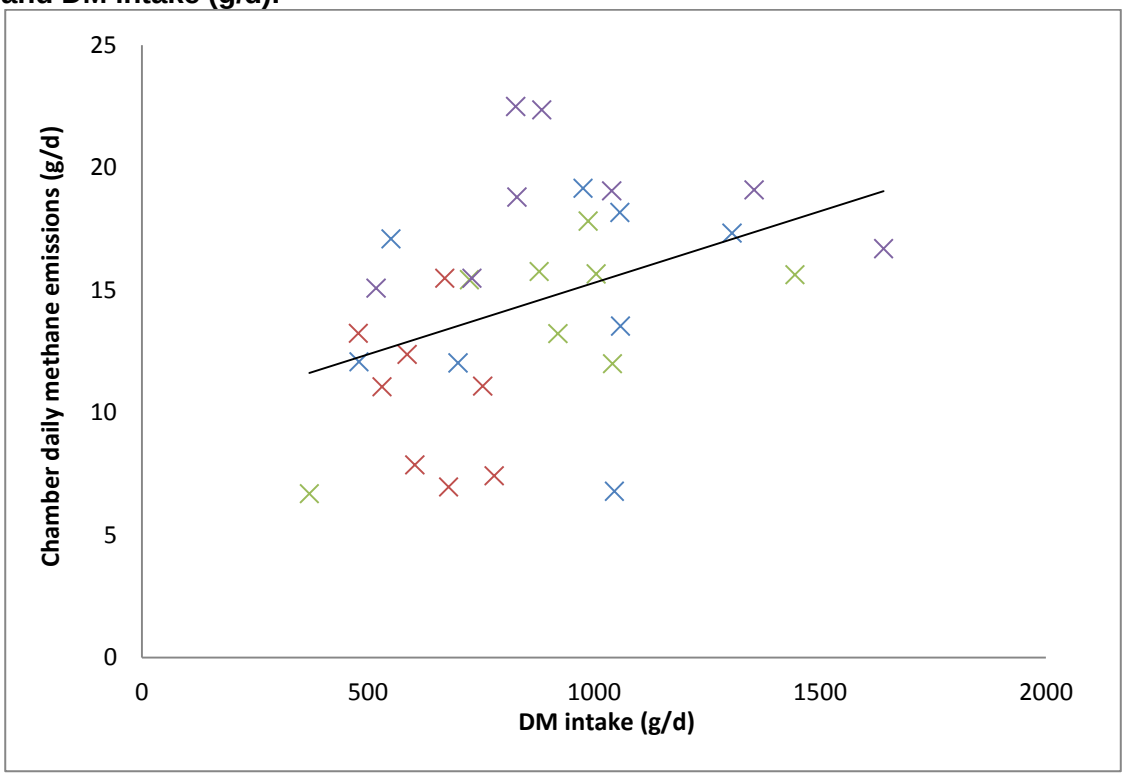
Red=Welsh Mountain; Blue=Scottish Blackface; Green=Welsh Mule; Purple=Texel.

Figure 10: Functional bisector relationship between LMD daily methane emissions (g/d) and DM intake (g/d)



Red=Welsh Mountain; Blue=Scottish Blackface; Green=Welsh Mule; Purple=Texel.

Figure 11: Functional bisector relationship between chamber daily methane emissions (g/d) and DM intake (g/d).



Red=Welsh Mountain; Blue=Scottish Blackface; Green=Welsh Mule; Purple=Texel.

Repeated measures ANOVA

There were no significant differences (P>0.05) between mean methane concentration measurements taken using the LMD on different days (Table 12). There were, however, significant differences between mean methane concentrations measured at different times of day (P<0.001, Table 10). When sheep breed was used as the treatment structure for the repeated measures ANOVA, breed was shown to have no significant effect on differences between LMD measurements taken at different times of day (Table 11). Table 10 shows the mean methane concentrations, taken over the two day measurement period, from different times of day. The least significant difference (L.S.D.) between mean methane concentrations at a one percent level was 1.645 ppm-m. The highest methane concentrations were recorded in the second and sixth measurement periods, which were the two measurements taken directly after feeding.

Table 10: Repeated measures ANOVA (different times of day)

Measurement period	08:30-09:00am	10:30-11:00am	12:00-12:30pm	13:30-14:00pm	15:00-15:30pm	16:30-17:00pm	SED	P
Mean methane concentration (ppm-m)	9.54^{a}	11.68^{b}	10.13^{ab}	9.13^{a}	9.48^{a}	11.31^{b}	0.597	<0.001

Abbreviations: ppm-m, parts per million-metres; SED, standard error of the difference.
Values in the same row with different superscripts differed significantly (P<0.01).

Table 11: Repeated measures ANOVA using breed as treatment structure (different times of day)

Time/Breed	WM	SB	M	T	SED	P
08:30-09:00am	9.22	8.79	8.76	11.39	1.349	0.140
10:30-11:00am	10.20	11.59	11.60	13.35		
12:00-12:30pm	9.22	10.02	10.94	10.34		
13:30-14:00pm	8.17	9.50	9.63	9.23		
15:00-15:30pm	10.70	9.11	9.18	8.95		
16:30-17:00pm	9.29	11.33	11.08	13.54		

Abbreviations: ppm-m, parts per million-metres; SED, standard error of the difference.

Table 12: Repeated measures ANOVA (different days)

Measurement period	Day 1	Day 2	SED	P
Mean methane concentration (ppm-m)	10.21	10.21	0.002	0.983

Abbreviations: ppm-m, parts per million-metres; SED, standard error of the difference.

4.1.4. Group LMD measurements

The calculated daily methane emissions for each group of sheep and the mean daily methane emissions per sheep in each group are shown in Table 13. The figures obtained compare well with typical daily methane emissions calculated using both individual LMD measurements and methane chamber data, though the results are not directly comparable. The results are also consistent for each group of sheep; this would be expected as the total body weights of sheep were similar for each group.

Table 13: Calculated daily methane emissions (g/day per sheep) as measured using the LMD data obtained from groups of sheep

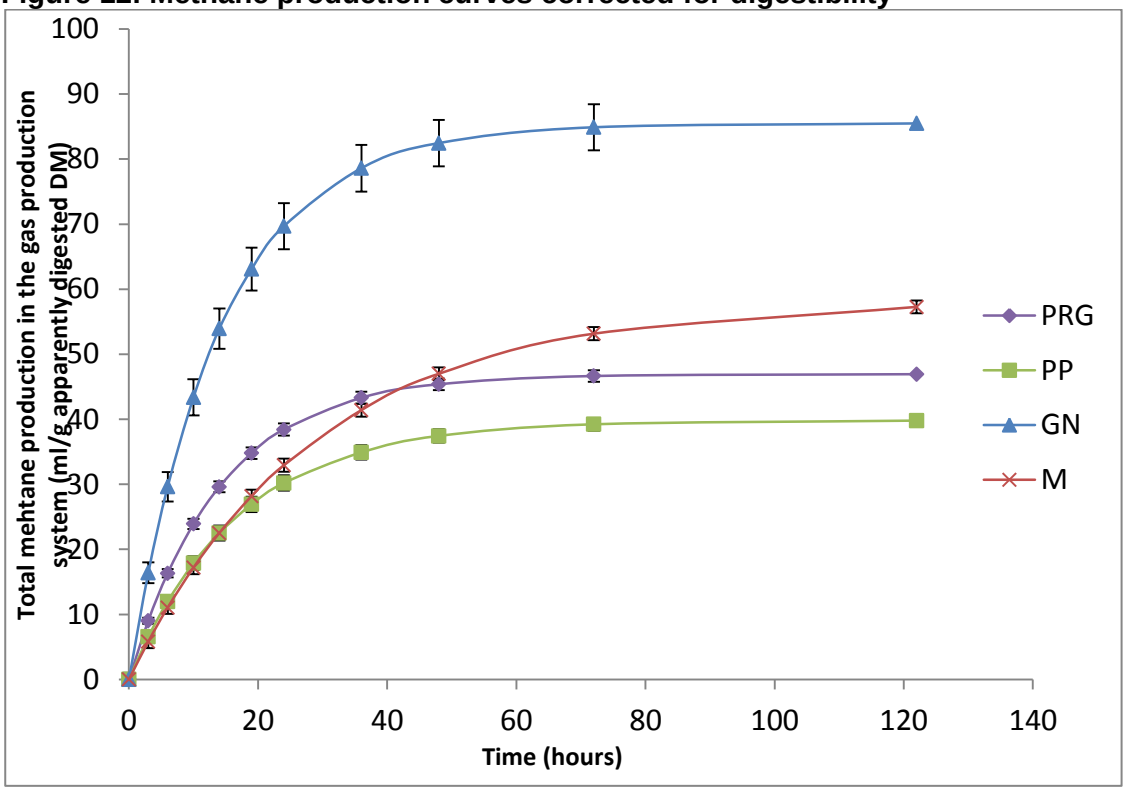
Sheep identity numbers in groups	Combined sheep weight (kg)	Daily methane emissions from group (g)	Mean daily methane emissions per sheep (g/d per sheep)
5,12,17,30	239	93	23
3,13,19,25	232	97	24
4,9,18,31	237	96	24
6,15,24,28	249	92	23
2,20,65,69	200	104	26
7,23,32,66	247	78	19
1,16,21,68	223	100	25
8,10,22,67	226	84	21

4.2. *In vitro* gas production

4.2.1. Methane production curves of four different feeds

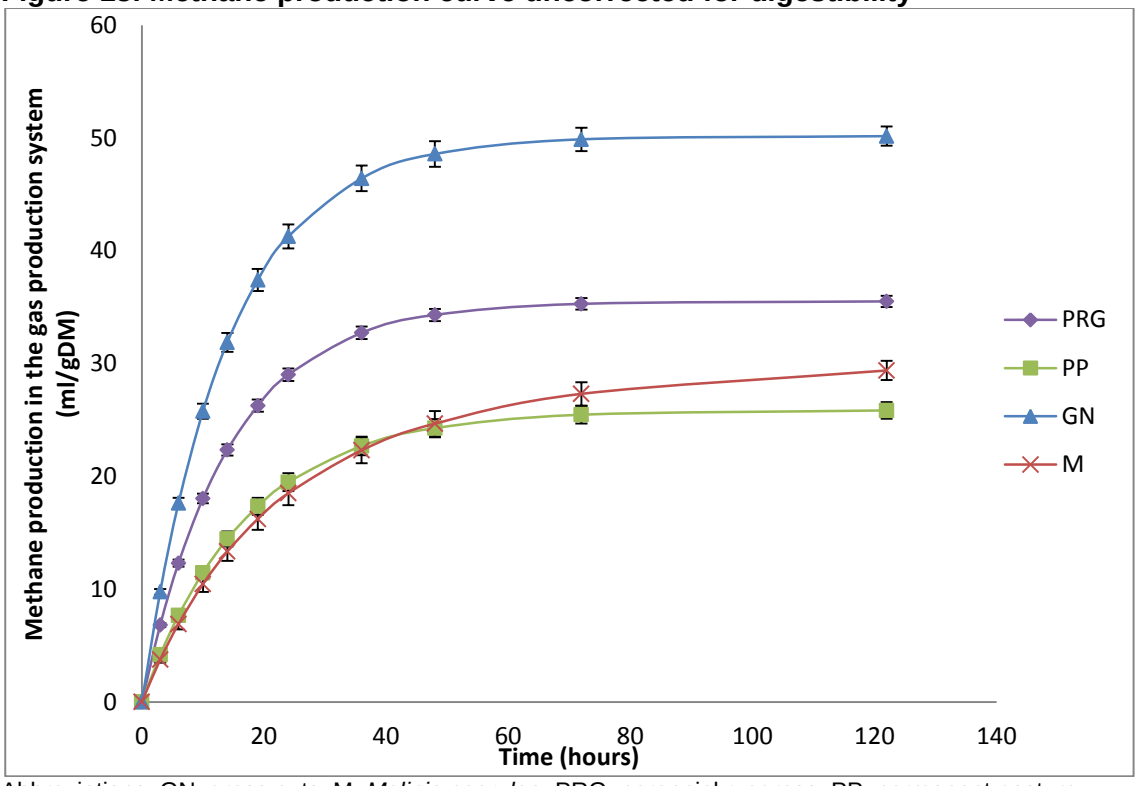
Average methane production curves, corrected for digestibility, for each of the different types of feed are presented in Figure 12. Grass nuts and *Molinia caerulea* results were scaled up to those of the perennial ryegrass and permanent pasture based on correction factors obtained using standard silage samples. Figure 13 shows average methane production curves for the different feeds, uncorrected for digestibility, but corrected according to differences between standard silage samples.

Figure 12: Methane production curves corrected for digestibility



Abbreviations: GN, grass nuts; M, *Molinia caerulea*; PRG, perennial ryegrass; PP, permanent pasture.

Figure 13: Methane production curve uncorrected for digestibility



Abbreviations: GN, grass nuts; M, *Molinia caerulea*; PRG, perennial ryegrass; PP, permanent pasture.

4.2.2. Repeated measures analysis of variance for methane production parameters

There were significant (P<0.01) differences between different feed materials in terms of total methane production in the system, fractional rate of degradation, and cumulative methane production at 16, 24, 36 and 48 hours (Table 14). Grass nuts and perennial ryegrass samples degraded at a significantly faster rate (P<0.01) than permanent pasture, which degraded at a significantly faster rate (P<0.01) than *Molinia caerulea*. Grass nuts samples produced significantly more methane (P<0.01) than all of the other feeds at 16, 24, 36 and 48 hours and in terms of total methane production in the system. Similar results were observed using data not corrected for digestibility (Table 15) although perennial ryegrass samples produced significantly more methane than *Molinia caerulea* in this case (P<0.01).

Table 14: Means total methane (ml/g apparently digested DM), fractional rate of degradation (g/h), and methane produced at 16, 24, 36 and 48 hours (ml/g apparently digested DM) for the feeds tested using the gas production technique using data corrected for digestibility

Methane production parameter	Feed means				S.E.D	P
	Perennial ryegrass	Permanent pasture	Grass nuts	Molinia		
Total methane (ml/g apparently digested DM)	47.0[a]	39.8[a]	85.5[c]	58.4[b]	2.78	<0.001
Fractional rate of degradation (g/h)	0.066[c]	0.054[b]	0.068[c]	0.034[a]	0.0025	<0.001
Methane at 16 hours (ml/g apparently digested DM)	32.0[b]	24.4[a]	57.9[c]	24.9[a]	2.45	<0.001
Methane at 24 hours (ml/g apparently digested DM)	38.5[b]	30.2[a]	69.7[c]	32.9[ab]	2.68	<0.001
Methane at 36 hours (ml/g apparently digested DM)	43.4[b]	35.1[a]	78.6[c]	41.4[ab]	2.83	<0.001
Methane at 48 hours (ml/g apparently digested DM)	45.4[b]	37.4[a]	82.5[c]	47.0[b]	2.84	<0.001

Superscript letters denote significant differences (P<0.01) between feed samples.

Table 15: Means total methane (ml/g DM), fractional rate of degradation (g/h), and methane produced at 16, 24, 36 and 48 hours (ml/g DM) for the feeds tested using the gas production technique using data uncorrected for digestibility

Methane production parameter	Feed means				S.E.D	P
	Perennial ryegrass	Permanent pasture	Grass nuts	Molinia		
Total methane (ml/g DM)	35.5[c]	25.8[a]	50.2[d]	29.4[b]	1.08	<0.001
Fractional rate of degradation (g/h)	0.066[c]	0.053[b]	0.068[c]	0.041[a]	0.002	<0.001
Methane at 16 hours (ml/g DM)	24.1[b]	15.7[a]	34.3[c]	14.6[a]	1.06	<0.001
Methane at 24 hours (ml/g DM)	29.0[b]	19.5[a]	41.3[c]	18.5[a]	1.21	<0.001
Methane at 36 hours (ml/g DM)	32.7[b]	22.7[a]	46.4[c]	22.3[a]	1.28	<0.001
Methane at 48 hours (ml/g DM)	34.3[b]	24.3[a]	48.6[c]	24.7[a]	1.26	<0.001

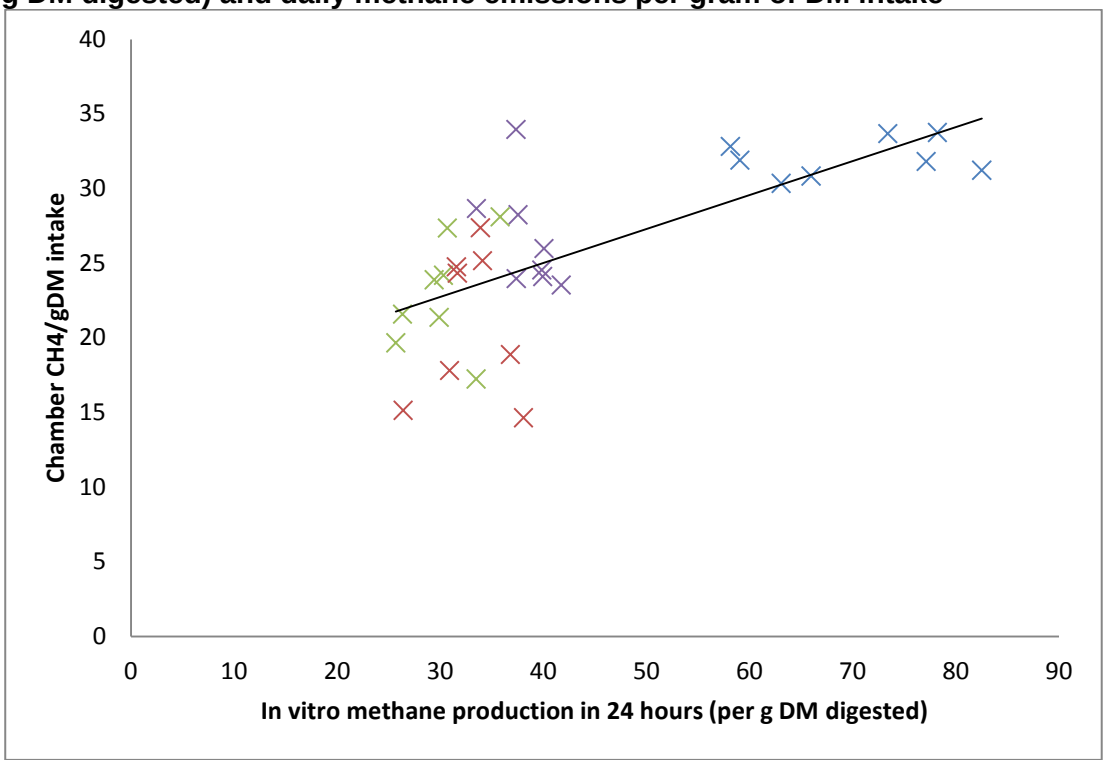
Superscript letters denote significant differences (P<0.01) between feed samples.

4.2.3. Regression between *in vitro* methane production and daily methane emissions measured using methane chambers

There was a significant positive correlation (R=0.68; P<0.001) between *in vitro* methane production per gram of DM digested in 24 hours and daily chamber methane emissions by sheep per gram of DM intake (Figure 14), suggesting that *in vitro* gas production data can be used to predict methane emissions provided that the DM intake, or an estimate of the DM intake is known. There was also a

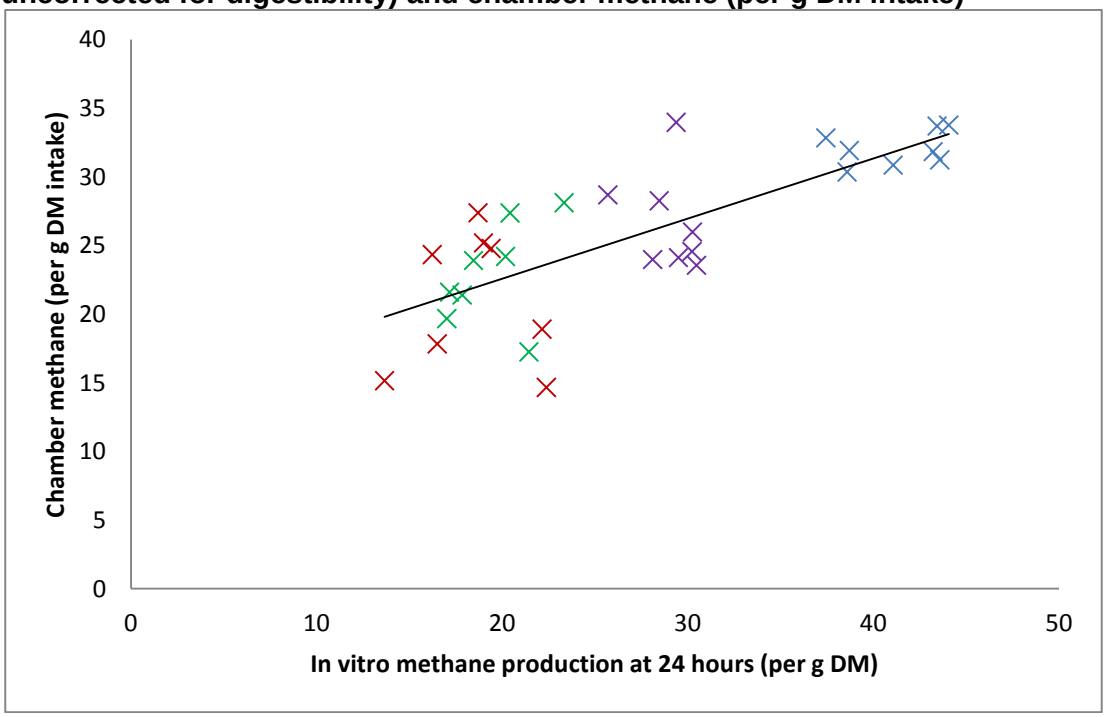
significant positive correlation (R=0.77; P<0.001) between *in vitro* methane production per gram of DM in 24 hours (uncorrected for digestibility) and daily chamber methane emissions by sheep per gram of DM intake (Figure 15).

Figure 14: Functional bisector relationship between *in vitro* gas production at 24 hours (per g DM digested) and daily methane emissions per gram of DM intake



Red=*Molinia caerulea*; Green=permanent pasture; Purple=perennial ryegrass; Blue=grass nuts.

Figure 15: Functional bisector relationship between *in vitro* methane production (per g DM, uncorrected for digestibility) and chamber methane (per g DM intake)



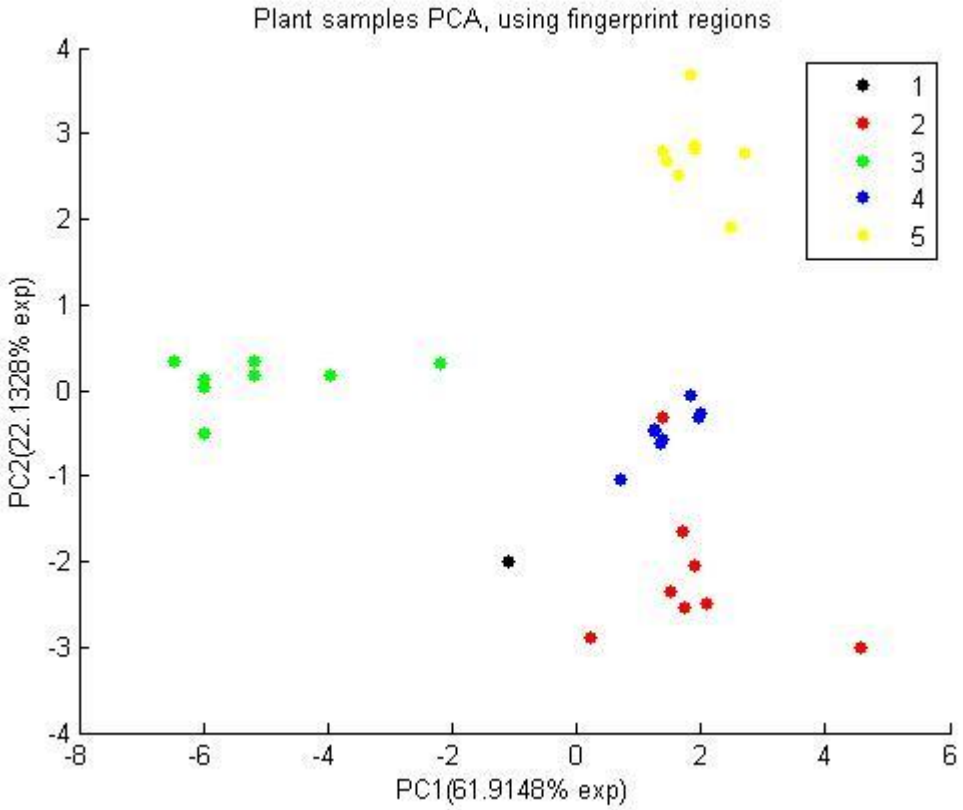
Red=*Molinia caerulea*; Green=permanent pasture; Purple=perennial ryegrass; Blue=grass nuts.

4.3. Fourier-transform infrared spectroscopy

4.3.1. Principal components analysis

The first two principal components accounted for 84% of the total variation within the data set (PC1=61.9%; PC2=22.1%). There was generally good grouping of feeds based on these two principal components, though there was a slight overlap between one of the perennial ryegrass samples and the grass nuts samples; these sets of samples grouped relatively closely together. It was, therefore, possible to distinguish between different feeds based on the FTIR spectra. The grass silage sample also grouped more closely to the perennial ryegrass samples than to other feeds (Figure 16).

Figure 16: Principal components analysis of plant samples based on FTIR analysis

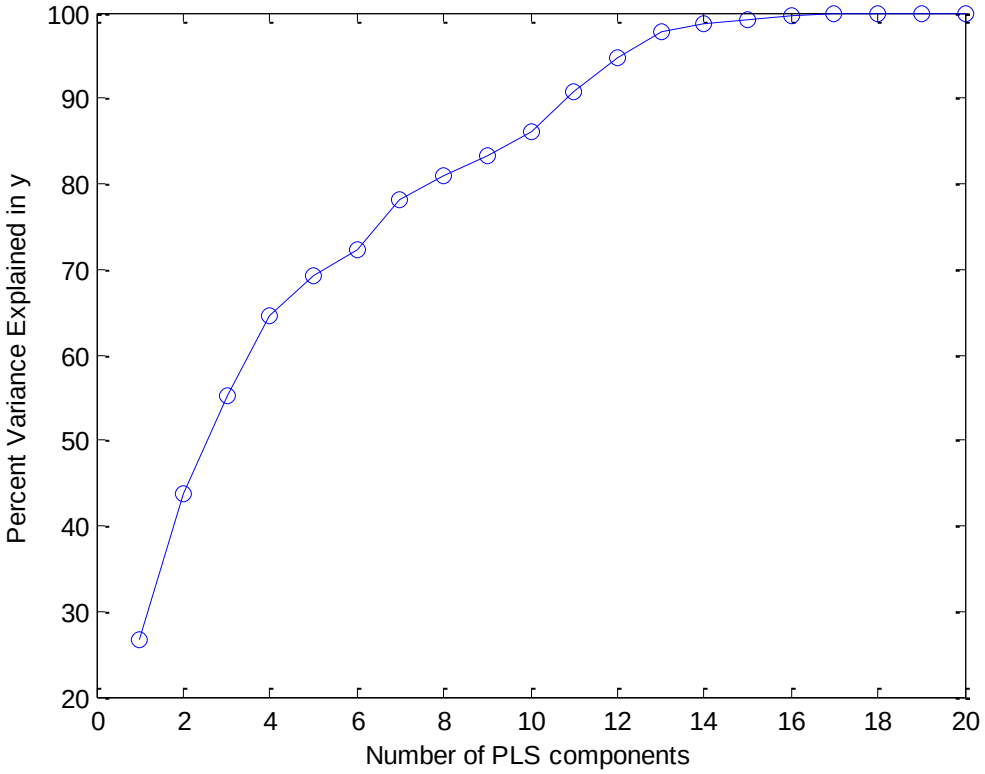


1=standard silage sample, 2=perennial ryegrass, 3=permanent pasture, 4=grass nuts, 5=*Molinia caerulea*.

4.3.2. Partial least squares regression

The results of the PLS regression showed that 100% of the variation in daily methane emissions measured in methane chambers was explained by 17 components produced using the FTIR data set (Figure 17). However, using all 17 components in a model would have meant that it would likely have been over-fitted and unlikely to work well with an independent data set.

Figure 17: Percentage of variation in daily methane emissions (measured in methane chambers) explained using PLS components of the FTIR data.



The PLS regression using 10-fold cross validation generated MSEs of response, which show the goodness of fit of the model to the test data, and the MSEs of predictors, which show the goodness of fit of the model to the cross validation data. The MSEs of response and predictors are plotted in Figure 18. The MSE of response is significantly reduced by 3–4 components, beyond which little improvement is evident. The MSE of prediction increases considerably after the first 3–4 components, showing divergence from the prediction model. The optimal model would therefore use approximately 4 components. Using a PLS model with 4 components, the relationship between observed values (daily methane emissions from methane chamber data) and predicted values (using FTIR data) is shown in Figure 19.

Figure 18: Effect of increasing the number of PLS components included in the model on the estimated MSEs of predictor and response

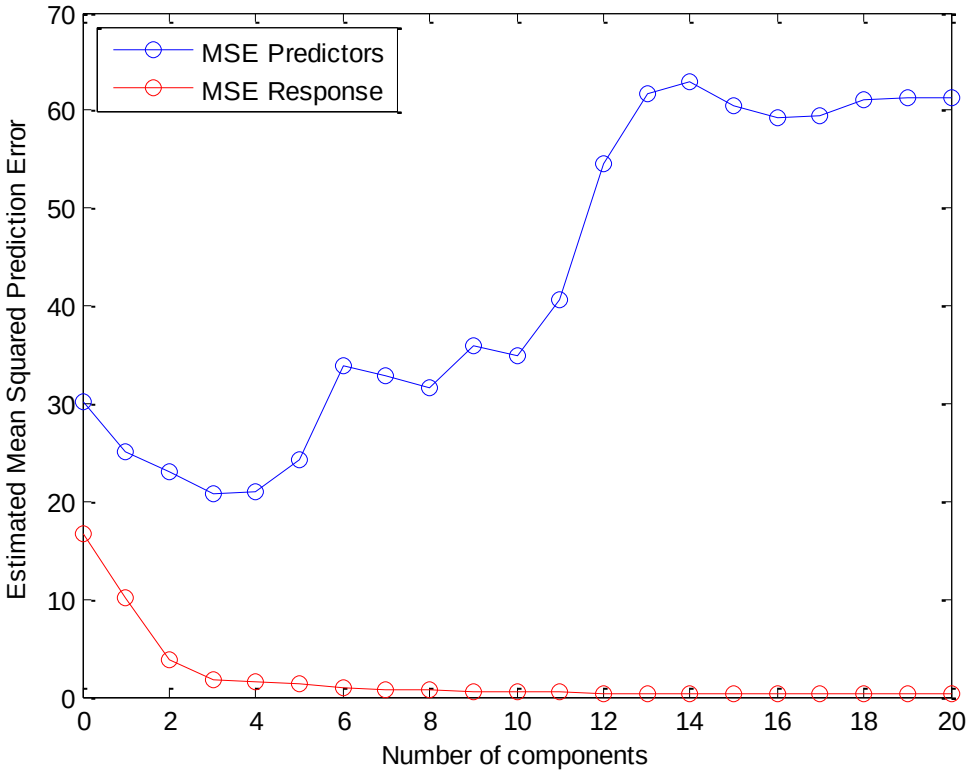
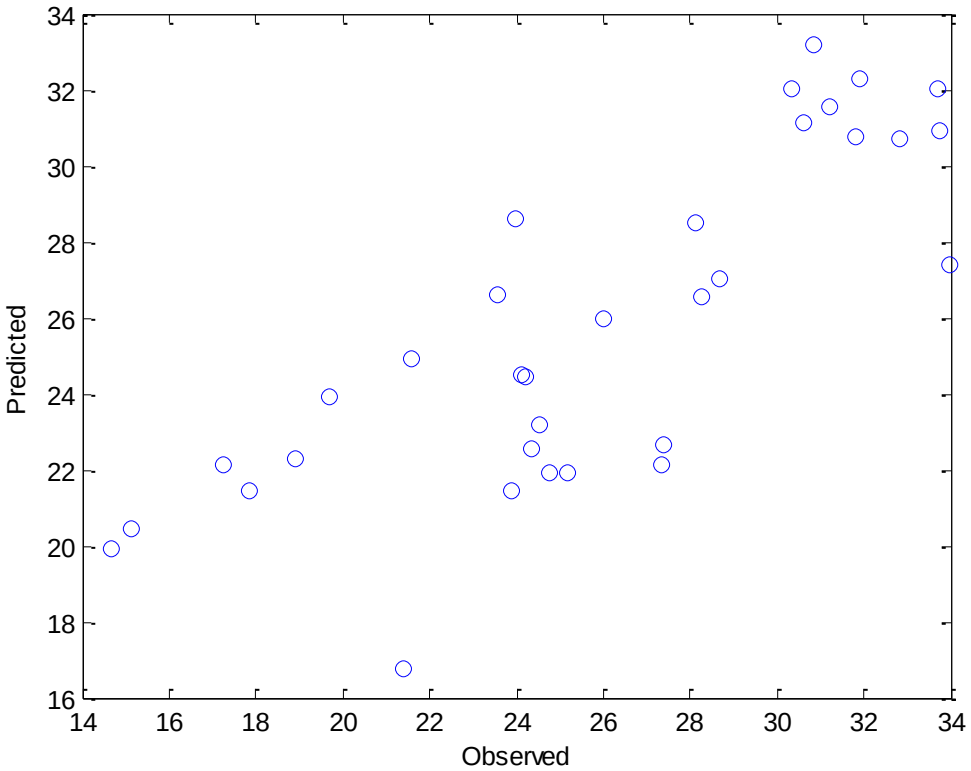


Figure 19: Relationship between observed and predicted values using a PLS model with 4 components



5. Discussion

5.1. Laser Methane detector

The use of the LMD as a potential means of estimating methane output by ruminant livestock is a relatively new concept (Chagunda *et al.*, 2009). The focus of the experiments carried out in this project, and of other experiments (Chagunda *et al.* 2011; 2013), has been to determine the potential of the LMD to accurately estimate methane output by animals and to begin the development of a method to use the LMD in the simplest and most time-effective way possible to achieve the desired results. The results presented in Section 4.1 suggest that the LMD could potentially be used to estimate daily methane output (g) by sheep.

In this project, a novel approach was taken to calculating daily methane emissions from animals that have undergone short periods of measurement using the LMD. This approach was used to successfully estimate daily methane emissions from animals, which were not only realistic in terms of magnitude, but also representative of data obtained from the same animals using "gold standard" methane chamber measurements. Using this calculation method, significant positive correlations were achieved between daily methane outputs obtained using LMD and chamber data. The correlations achieved were considerably stronger than the correlation between LMD and chamber data from sheep published by Chagunda *et al.* (2013).

Due to the simplicity of the LMD method and assumptions necessary to calculate daily methane emissions using LMD measurements, there is potential for inaccuracy in the estimation of daily methane output using LMD measurements. However, the LMD consistently provided a means of calculating daily methane output by sheep that not only fell within the range that would be expected but that also significantly correlated with daily methane emissions as measured using methane chambers. Given the simple method of measurement, this is an impressive feat that would justify further exploration of the technique. In addition, the LMD demonstrated potential as a tool for ranking of sheep in terms of methane production. This could potentially allow for the selection of sheep for breeding based on their rank as high or low methane producing animals (Hegarty *et al.*, 2007).

In addition to the individual methane output measurements obtained using the LMD, an initial experiment to determine the potential of the LMD to estimate methane output from groups of sheep was conducted. There is a lack of published data regarding the use of the LMD the estimation of methane output from groups of sheep. However, the use of open-path lasers for this purpose has been investigated with some success, although the method used overestimated methane emissions as compared to those measured in calorimetric chambers (Tomkins et al., 2011). The preliminary results of group measurements used to provide estimates of methane outputs by individual sheep within the group (g/d) (presented in Section 4.1) are in the magnitude that would

be expected from individual sheep. Further work would be required to develop and validate methods for taking LMD measurements and calculating methane output from groups of sheep.

5.2. *In vitro* gas production

Previous studies have used the *in vitro* gas production technique to evaluate feed characteristics, such as digestibility (Blümmel and Ørskov, 1993; Brown *et al.*, 2002). However, there is a lack of published data regarding the use of the technique specifically used to predict *in vivo* methane output by sheep.

The significant relationship (Section 4.2) between *in vitro* methane production (both ml/g apparently digested DM and ml/g DM) at 24 hours and mean methane produced by sheep fed on the same samples, as measured using methane chambers, suggests that the technique could potentially be used to predict methane output by sheep, provided that DM intake is known, or can be reasonably estimated. The correlation coefficient was not particularly high when *in vitro* methane production per gram of apparently digested DM was used, and slightly higher when *in vitro* methane production per gram of DM was used. It may be possible to improve the fit to the correlation by altering the time taken to be the rumen retention time for different feeds.

It is likely that the feeds analysed using the gas production technique all have different average rumen retention times. If the retention times were known for each feed, it would be possible to take methane production at different times, depending on the feed offered, as estimates of methane production per gram of DM intake. Rumen retention times depend on a variety of factors, including feeding level, particle size and dietary composition (Sriskandarajah *et al.*, 1981). Feeding level is something that is easily measured in intensive systems, although it is more complicated for grazing animals, as is dietary composition. The feeds were all ground to the same particle size in the gas production experiments. Measuring rumen retention times is possible using dietary markers; however, this is too complicated to do at a large on-farm scale. It may be possible to introduce correction factors for rumen retention times, based on NDF content of feed and feeding level.

5.3. FTIR spectroscopy

The results of the PCA show that the feed samples analysed were clearly discernible based on their FTIR spectra. This suggests that FTIR spectroscopy can be used to distinguish between plant samples, which may have implications for the potential use of FTIR spectra to predict methane potentials of ruminant feeds. The results are in accordance with those of other studies, which have demonstrated that it is possible to distinguish between faecal samples of sheep fed on different diets (Moorby *et al.*, 2010; Parveen *et al.*, 2008). The perennial ryegrass and grass nuts samples, and the standard silage sample, grouped closely together. The grass nuts may have been made from a perennial ryegrass sward and the silage was made from an improved perennial ryegrass. This may explain why these samples grouped together, suggesting that the variation present in the

samples was inherent to the grass species, given that the silage sample still grouped with the perennial ryegrass samples, despite having been ensiled.

The results of the PLS regression and 10-fold cross validation demonstrate that is was possible to predict daily methane emissions as measured from sheep in methane chambers using the FTIR spectra of feeds offered to these sheep. As FTIR and NIR spectroscopy are similar techniques, this is in accordance with the results presented in the Moss and Givens (2000) publication, which suggested that NIR spectroscopy was a good predictor of ruminant methane emissions. In this experiment, as in the Moss and Givens (2000) publication, the dataset was relatively small, and larger datasets, with a wider variety of feed samples, may provide a more comprehensive idea of the factors of FTIR or NIR spectra that allow for the prediction of daily methane emissions. However, even with a relatively small dataset, the results presented in this chapter demonstrate that FTIR is reasonably good predictor of methane emissions by measured *in vivo* using methane chambers. Mid-infrared spectroscopy and PLS regression has also been used to successfully predict methane emissions from dairy cows using milk samples (Dehareng *et al.*, 2012), providing further evidence of the potential of spectroscopy techniques as proxy indicators for ruminant methane output.

While this experiment demonstrated that FTIR can be used to predict methane emissions from sheep based on their diets, the elements of the FTIR spectra that differentiated between feeds and made this prediction possible are not clear. It is likely that a combination of factors contributed to the ability of the PLS components that were predictive of methane emissions. These could include aspects of the compositional properties of the feed samples, which are likely to affect digestibility, and have been shown to be well-predicted by NIR spectroscopy (Landau *et al.*, 2006) and, to some extent, by FTIR spectroscopy (Belanche *et al.*, 2013; Belanche *et al.*, 2014). Another factor that may have contributed to the ability of the PLS components, produced using the FTIR dataset, to predict methane emissions by sheep was the identification of properties of the feed sample that may influence feed intake by sheep. Silage intake by cattle can be successfully predicted using NIR spectroscopy (Steen *et al.*, 1998), suggesting that a factor or combination of compositional factors of the silage, which is detected by NIR spectroscopy, affects animal intake. Feed intake is known to be predictive of methane emissions (Lassey *et al.*, 1997; Molano and Clark, 2008). Therefore, if spectroscopy techniques can be used to predict feed intake, this may partially explain how spectroscopy techniques are able to predict *in vivo* methane emissions.

Previous studies have found that FTIR spectra of faecal samples from sheep can be used to differentiate between sheep based on their diets (Moorby *et al.*, 2010; Parveen *et al.*, 2008), and that FTIR of feed samples can provide information about the chemical composition of feeds (Belanche *et al.*, 2013) and some information about feed digestibilities (Belanche *et al.*, 2014). However, to my knowledge, there are no published data to date, which demonstrate the

relationship between FTIR spectroscopy of feed samples and *in vivo* methane output by sheep offered these feeds.

6. Industry messages from this research

One of the challenges faced in the reduction of methane emissions by ruminants is the successful implementation of any practices that are shown to be beneficial. Hegarty *et al.* (2010) argue that there is a lack of policy to motivate farmers to reduce their emissions, with emphasis being entirely on productivity and profit. Without a means of accurately estimating methane output by sheep, or the effects of any measures taken to reduce methane emissions by sheep at a large on-farm scale, it is difficult to introduce incentives for farmers to take measures to reduce methane emissions. Pinares-Patiño *et al.* (2013) also highlighted the need for shorter and alternative methane emissions measurements in order to facilitate the establishment of selection lines of low-methane-producing animals, as methane output has been shown to be a heritable trait.

The aim of this project was to develop and validate proxies that could potentially be used to obtain accurate estimates of methane output by sheep. Proxy indicators for methane output by sheep could provide a useful tool for accurately estimating methane output at an on-farm level, as well as measuring the impact of introducing different management systems or diets on methane emissions. A brief discussion of the implications for industry of the results for each potential proxy investigated during this project is provided in the following sections.

6.1. Laser Methane detector

The results obtained during this project suggest the LMD has potential to be used as a means to estimate methane output by sheep. Although the methods used to collect LMD data were simple, they were relatively time consuming, required close contact with animals on a daily basis, and required expensive equipment (i.e. the LMD). However, the work presented in this thesis demonstrates that LMD measurements can be used to estimate daily methane output by sheep that is in the expected magnitude and correlates with daily methane output as measured in methane chambers. Furthermore, the LMD could potentially be used to rank animals in terms of their methane production, thereby facilitating the introduction of breeding programmes for animals that are low methane producers (Hegarty *et al.*, 2007; Pinares-Patiño *et al.*, 2013).

Further work would be required in order to establish methods for taking LMD measurements that could be used at a large on-farm scale. The LMD can be used at a range of up to 150m, which could allow for collection of LMD measurements from grazing animals, without causing any disruption to animals. However, there is a paucity of published data regarding LMD measurements taken from ruminants at long distances. Similarly, there is a lack of published data regarding the use of the LMD the estimation of methane output from groups of sheep. However, the use of open-path lasers for this purpose has been investigated with some success (Tomkins *et al.*, 2011). The results of this initial experimentation presented in 4.1.4 suggest that further work regarding group

LMD measurements should be pursued as a means of quickly and simply estimating methane output from animals, making the LMD potentially useable at a large on-farm scale.

6.2. *In vitro* gas production

The *in vitro* gas production technique could provide a quick and simple means of estimating methane emissions by sheep based on the methane production potential of the feeds offered and intake measurements or estimates of feed intake. The technique requires small amounts of feed material, which is freeze-dried, allowing analyses to be conducted several weeks or months after collection. Multiple samples can be analysed at the same time, making the technique practical for use on a reasonably large scale.

The results presented in Section 4.2 demonstrate that there were significant positive correlations between methane production per gram of apparently digested DM and per gram of DM using the *in vitro* method and those obtained using the *in vivo* methane chamber experiments. The gas production technique, therefore, has the potential to be used as a proxy to predict methane emissions from different animals given different diets, provided a reasonable estimate of the DM intake of animals can be made. The challenge posed by measuring DM intake may limit the usefulness of the in vitro gas production technique, particularly as DM intake does not correlate particularly well with body weight (Lassey et al., 1997). However, in cases where animal intake is already monitored, taking samples to be used for in vitro gas production analysis would be easily manageable. The technique requires collection of rumen fluid, which may present a problem as it requires either fistulated animals or stomach tubing, both of which are subject to Home Office regulation and require careful management. However, if the technique were to be used as a means of predicting methane potentials of diets from farms, the samples would be collected on-farm and sent to a research facility for analysis; this would also reduce any variation in results due to laboratory conditions and staff performing analyses.

6.3. FTIR spectroscopy

Fourier-transform infrared (FTIR) spectroscopy is a rapid-throughput laboratory technique that requires a very small amount of plant or feed material (Allison *et al.*, 2009), making it ideal as a proxy indicator for methane output by sheep provided that it can be used to successfully predict methane emissions using feed samples or faecal matter. The data presented in Section 4.3 demonstrate that it is possible to distinguish between different feed samples on the basis of their FTIR spectra. Furthermore, there is potential for FTIR spectra of feed samples to successfully predict daily methane emissions from sheep as measured in methane chambers.

The main limitation of the data presented in this thesis is that the data set is relatively small compared with data sets presented in the literature: for example, Belanche *et al.* (2013) used 663 samples, representing 80 feed types. Conclusions are therefore limited from this small data set regarding the aspects of the FTIR spectra that enable the prediction of methane emissions. Further

work should, therefore, involve using larger data sets, with contrasting feed samples, to perform a similar analysis. This may provide a more comprehensive idea of the properties of feeds that cause animals to produce more or less methane, whether this is related to feed composition, or perhaps whether certain properties of feeds affect feed intake by animals.

6.4. Conclusion

While further work is required to optimise the methods used to estimate or predict methane output by sheep, the data collected during this project provide evidence for the potential of three proxy indicators for this purpose.

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