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How to cite:

Turner, Claire; Knobloch, Henri; Richards, John; Richards, Peter; Mottram, Toby T. F.; Marlin, David and Chambers, Mark A. (2012). Development of a device for sampling cattle breath. *Biosystems Engineering*, 112(2), pp. 75–81.

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Version: Accepted Manuscript

Link(s) to article on publisher's website:

<http://dx.doi.org/doi:10.1016/j.biosystemseng.2012.03.001>

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## Development of a device for sampling cattle breath

Claire Turner<sup>1\*</sup>, Henri Knobloch<sup>1</sup>, John Richards<sup>2</sup>, Peter Richards<sup>2</sup>, Toby Mottram<sup>3</sup>,  
David Marlin<sup>4</sup> and Mark Chambers<sup>5</sup>

<sup>1</sup>Cranfield Health, Cranfield University, MK43 0AL, UK; <sup>2</sup>Solutions for Research, Wrest Park, Silsoe, MK45 4HP; <sup>3</sup>eCow Ltd, Innovation Centre, University of Exeter, EX4 4RN; <sup>4</sup>David Marlin Consulting Ltd, PO Box 187, Newmarket, CB8 1BA; <sup>5</sup>Veterinary Laboratories Agency, Woodham Lane, New Haw, Addlestone, Surrey, KT15 3NB.

\*Present address & author for correspondence: Department of Life, Health and Chemical Sciences, The Open University, Walton Hall, Milton Keynes, MK7 6AA UK, e-mail: c.turner@open.ac.uk.

**Keywords:** breath sampling, non-invasive disease diagnosis, VOC monitoring, cattle monitoring

### Abstract

Diagnostic tests for some conditions affecting cattle, such as tuberculosis, are often expensive and of long duration, requiring diagnostic tests involving more than one visit by a qualified vet. An alternative rapid and non-invasive diagnostic test would be desirable. One possibility is the use of breath testing, which has been shown to have diagnostic potential in humans. The development of a device for taking a representative breath sample from a bovine animal is described. Six devices using different configurations were assessed over three separate testing days for their ability to take a representative breath sample which does not cause undue stress to the animal and which is easy for an operator to use. The main factors affecting the sample integrity was dead space, however temperature played a role. The best samples causing the lowest stress to animals were taken using a nostril sampler. The nostril samplers were then used to take breath samples from cattle with and without tuberculosis which were then analysed using selected ion flow tube mass spectrometry and gas chromatography-mass spectrometry to demonstrate proof-of-principle.

### 1. Introduction

Breath analysis in its most basic form has been around for a long time. It has been known for centuries that the odour present on the breath of humans may indicate the presence of disease and early physicians used this in their diagnoses. For instance, it has long been known that the sweet smell of rotting apples on breath indicates diabetes; it is now known that such an odour is as a result of ketones in the breath. Tuberculosis (TB) sufferers were also found to have nasty smelling sputum and the odours became more intense after the sputum was pyrolysed (Pease, 1940). This knowledge has led to attempts to use odour, or the volatile organic compound (VOC) signature for the diagnosis of tuberculosis. Fend et al. (2005) analysed the headspace of cattle and badger sera using electronic nose and found discrimination between sera derived from tuberculous and non-tuberculous animals using principal components analysis (PCA). Philips et al. (2007) found markers for TB in human breath, as did Syhre et al. (2009) and Syhre and Chambers (2008), indicating that it is likely that various pulmonary or systemic VOC markers are present that can then be transported around in blood and pass across the alveolar membranes prior to being exhaled. Breath analysis is now becoming accepted as having diagnostic potential for a number of conditions (Amann and Smith, 2005). Taking human breath samples is a desirable sampling medium because it is non-invasive, can be rapid and there is usually little patient reluctance. But how can breath samples be taken from cattle and how can they be made

representative and reproducible with little distress for the animal whilst being safe for the person taking the samples?

Attempts have been made to take breath samples from cattle in order to monitor specific conditions. Elliott-Martin et al. (1997) developed a nasal breath sampling device for use with dairy cattle, which supplied breath for analysis by gas sensors, gas chromatography-mass spectrometry (GC-MS) and Fourier transform infra red (FTIR). This breath sampler was later used to detect hyperketonaemia in dairy cows using GC-MS (Mottram et al. 1999) and then subsequently, using electronic nose technology (Gardner et al., 1999). Methane production has also been monitored in ruminants by sampling breath from near the nose and mouth of animals and then analysed by GC-FID (Lassey et al., 1997). However, it can be difficult to distinguish volatiles derived from the lungs from those of the rumen headspace, coming from eructation.

Because of the difficulties in sampling whole breath, attempts have been made to sample and analyse exhaled breath condensate (EBC) from cattle. Reinhold et al. (2006) evaluated factors affecting EBC in cattle. Many of the compounds present in EBC are non-volatile, however may provide important information on the health of an individual (Kharitonov and Barnes, 2002). Equipment required to sample EBC is different from whole breath and requires a cooling system to condense the material.

The aim of this study was to design and test a sampler capable of taking representative and reproducible breath samples from cattle safely and with minimal stress to the animal. The sampler was then tested on animals infected with *Mycobacterium bovis* to take breath samples to seek potential breath biomarkers for *M. bovis*.

## **2. Materials and methods**

### *2.1 Design of breath samplers*

The cow breath samplers tested fall into two categories: firstly a large breath sampler designed to go over the whole nose of the animal (covering both nostrils) and secondly, a nostril sampler to take breath from a single nostril. Two basic whole nose samplers and four nostril samplers were tested.

The whole nose samplers are shown in figure 1. The samplers are moulded from clear food grade polyethylene fitted with a silicone rubber seal which may be cut to size for each animal to enable a comfortable seal to be made around the animal's mouth and nostrils. Both samplers have the same length and width but a different height; the larger a height of approximately 12 cm and the smaller a height of approximately 6 cm. Both have on the underside two simple one way valves inside plastic tubes, one enabling the animal to inhale and the other for exhalation into a breath sampling bag which may be attached to the outside of the valve. The key difference between these two devices is the dead volume: the large sampler has a dead volume of twice that of the smaller one but is easier to fit over an animal's snout.

The nostril samplers were designed to fit over a single nostril of an animal. They consisted of an autoclavable glass reinforced polypropylene tube with a simple one way valve for the animal to exhale through. The tube is fitted with soft malleable silicone rubber seals (to seal around the nostril) identical to those used in the whole nose samplers. Figure 2 shows all nostril samplers. Four nostril samplers were constructed with slightly different dimensions as follows: "Long-narrow", with diameter 3cm and length 8cm; "Long-thick", 4.5 cm diameter, 8.5cm long; "Short-narrow" 3.5 cm diameter, 4cm long; and "Short-thick", 4.5 cm diameter and 4cm long.

## 2.2 Sampling protocol for sampler testing

Samplers were developed by assessing a cow's tolerance of having them applied. Cattle were sampled on two separate occasions to assess ease of use and to decide which sampler gave the most representative samples. Breath samples were taken into 135mm diameter Nalophan bags (made of polyethyleneterephthalate) (Kalle, UK) and analysed immediately and then after several hours to assess sample stability. Taking a sample from the full nose samplers involved holding the sampler over the animal's nose until the breath bag was filled. The nostril sampler involved holding the nostril cup over one nostril and covering the other nostril with the free hand, being aware of when the cow needed to inhale so that the sampler could be removed from the nostril. Figure 3a shows the nostril sampler being used on a haltered and compliant animal. Animals unused to being handled should be (and were in other experiments) sampled while in a crush, but the principle of holding the sampler gently over the nostril is the same.

The first sampling occasion involved 5 sampler setups: The full snout sampler as shown in figure 1a; the full snout sampler pre-warmed to 50°C prior to sampling; the full snout sampler pre-flushed with breath before the sample was taken; the smaller dead volume full snout sampler and finally a basic nostril sampler consisting of a one way valve connected to a sampling bag. Five replicate samples were taken for each of the sampler setups described above; three were used for CO<sub>2</sub> analysis and two were used for selected ion flow tube mass spectrometry (SIFT-MS) analysis. CO<sub>2</sub> analysis was undertaken to act as a surrogate for sampling efficiency – if samples contain levels of CO<sub>2</sub> consistent with breath, then this implies that the samples are representative; if values are low then the breath sample is not representative. The second sampling occasion involved use of the 4 nostril samplers as described above and six replicate samples were taken with each device. On this occasion, the sampling was undertaken at the same location as the analytical equipment, hence 0 hour and 6 hour sampling points were available.

## 2.3 Sampling protocol for sampling cattle experimentally infected with *M. bovis*

Breath samples were taken from a cohort of ten animals housed in ACDP Containment Level 3 facilities at times before and after infection with *M. bovis* into Nalophan bags via the long-thick nostril sampler. The sample bags were placed inside a sealed box and transported to the laboratory. They were heated to 37°C inside a specially constructed incubator placed inside a Biosafety cabinet to ensure a consistent temperature and generate volatiles. The bags were then directly connected to conditioned thermal desorption tubes (TD tubes) via a brass Swagelok fitting. Tubes had been previously conditioned before use by purging with helium carrier gas for 2 min at room temperature followed by 1 hour at 320 °C and then sealed with locking caps until required for use. 500ml of sample was drawn across the tube using a constant-flow pump (Flec, Markes, UK). The TD tubes used were of standard stainless-steel, containing dual packing comprising 50% Tenax TA and 50% Carbotrap (Markes International Limited, Llantrisant, UK). A filter was placed between the tube and pump (so that the air was pumped from the bag, through the TD tube, then filter and then pump) to prevent contamination of the pump (figure 3b). After exactly 5 mins (corresponding to 500 ml) of flow, the pump was switched off, the TD tube removed, and brass caps fitted immediately to either end to seal the tube, which was then incubated at 80°C for 1 hour to kill any *M. bovis* that may be present. The treated tubes were then sent to the analytical laboratory for analysis by TD-gas chromatography-mass spectrometry (see below).

## 2.4 Cattle used

In this study, cattle from two different sources were used. In sampler testing, healthy Holstein dairy cows were used as they were easily handled. They tolerated the use of the samplers very well and only required haltering during sampler use. Further breath samples were obtained from cows experimentally infected with *M. bovis* as part of on-going studies at AHVLA. Samples of breath were taken from each animal prior to experimental infection with *M. bovis*, and then at two, three, and five weeks post-infection using the approach described above. These animals were chosen as they were unused to being handled and thus representative of most animals that may need to be monitored. They were put into a cattle crush to enable breath samples to be taken safely.

## 2.5 Analysis

Volatile compounds in breath samples were analysed using SIFT-MS for identification and quantification of the components of bovine breath. CO<sub>2</sub> was analysed using a Servomex CO<sub>2</sub> analyser. Gas chromatography – mass spectrometry with thermal desorption (ATD-GC-MS) was used to sample and subsequently analyse the composition of cattle infected with *M. bovis*; SIFT-MS was not used at this point due to the difficulties in sterilising breath samples for SIFT-MS to avoid contaminating the equipment with the pathogenic *M. bovis*.

### SIFT-MS

SIFT-MS was used to assess the efficacy of the samplers as it provides rapid, quantitative analysis of many compounds present in breath. Details of how it works are described in detail elsewhere (Smith and Spanel, 2005) so will not be given here. Analysis of cattle breath samples was carried out by attaching the Nalophan bag containing the breath sample to the capillary inlet of the SIFT-MS. After a few seconds, the absolute, on-line real-time analyses of selected components of breath were obtained down to the parts-per-billion by volume (ppbv) level (ppbv describes gas concentration on a volume-per-volume ratio) and the concentrations of volatile compounds calculated using the kinetics database in the SIFT-MS on-board computer. The sensitivity limit is approximately 5 ppbv for most compounds, and the mass to charge ratio (m/z) upper limit in this study is 160.

### Thermal desorption-gas chromatography-mass spectrometry (ATD-GC-MS)

Volatiles captured on TD tubes were analysed using an AutoSystem XL gas chromatograph equipped with an ATD 400 thermal desorption system and TurboMass mass spectrometer (Perkin Elmer, Wellesley, MA). CP grade helium (BOC gases, Guildford, UK) was used as the carrier gas throughout, after passing through a combined trap for the removal of hydrocarbons, oxygen and water vapour. Cartridges were desorbed by purging for 2 min at ambient temperature then for 5 min at 300 °C. Volatiles purged from the cartridge were captured on a cold trap which was initially maintained at 30 °C. Once desorption of the cartridge was complete, the trap was heated to 320 °C using the fastest available heating rate and maintained at that temperature for 5 minutes whilst the effluent was transferred to the gas chromatograph via a heated (180 °C) transfer line coupled directly to the chromatographic column.

A Zebron ZB624 chromatographic column was used (Phenomenex, Torrance, CA). This is a wall-coated open tubular column (dimensions 30m × 0.4mm × 0.25mm ID), the liquid phase comprising a 0.25 µm layer of 6% cyanopropylphenyl and 94% methylpolysiloxane. The gas chromatograph oven was maintained at 50 °C for 4 min following injection and was then raised at

10 °C.min<sup>-1</sup> to 220 °C for 9 min. Separated products were transferred by heated line to the mass spectrometer and ionised by electron bombardment. The spectrometer was set to carry out a full scan from mass/charge ratios (m/z) 33 to 350 using a scan time of 0.3s with a 0.1s scan delay. The resulting mass spectra were combined to form a total ion chromatogram (TIC) by the GCMS integral software (TuboMass ver 4.1). Analysis of individual compounds was undertaken using AMDIS software and the NIST mass spectral library.

### 3. Results and discussion

#### 3.1 Assessment of sampler efficacy and sample storage

A summary of the results from the analysis of the breath from a single animal on two separate occasions using SIFT-MS and the CO<sub>2</sub> analyser is given in table 1. SIFT-MS results for water, methanol, acetone and ammonia are given as they are felt to be most representative of the other compounds present and are readily measurable, being abundant in breath. Results given are the mean of a minimum of two samples; if the samples gave results different from each other by more than 20%, then a further replicate was taken. The samples were kept in the bags and were re-analysed six hours later. This was to test the affect of storage time on the stability and retention of the breath VOCs in the bag, as it is not always possible to analyse samples immediately. By knowing the effect of storage time on the results, it was possible to ensure that the correct decision could still be taken about the efficacy of each sampler.

Table 1. Breath analysis values averaged from two (H<sub>2</sub>O and VOCs using SIFT-MS) or three (CO<sub>2</sub>, using a Servomex CO<sub>2</sub> analyser) samples taken from the whole snout and nostril samplers on two separate occasions. Numbers 1 to 5 were tested on the first sampling occasion and 6 to 9 on the second.

Sampler description	CO <sub>2</sub> (%)		H <sub>2</sub> O (%)		Methanol ppbv		Acetone ppbv		Ammonia ppbv	
	0h	6h	0h	6h	0h	6h	0h	6h	0h	6h
1.Full snout sampler	0.57	0.47	*	2.52	*	338	*	284	*	137
2.Full snout sampler pre-heated	0.6	0.57	*	2.73	*	307	*	229	*	176
3.Full snout sampler with pre-flush of breath	1.23	1.17	*	2.83	*	277	*	330	*	172
4.Small sized snout sampler	0.93	0.87	*	2.75	*	291	*	315	*	117
5.Simple nostril sampler	2.13	2.00	*	3.32	*	286	*	407	*	175
6.Long narrow nostril sampler	2.75	2.7	4.06	1.5	200	242	2808	2297	90	134
7.Long thick nostril sampler	2.85	2.7	3.3	1.52	212	268	2216	2073	95	159
8.Short narrow nostril sampler	2.45	2.15	3.66	1.65	216	296	2625	2343	94	163
9.Short thick nostril sampler	3.05	2.55	3.19	1.62	277	363	1961	1935	92	185

\*Only analysed after 6 hours due to location of instrument

The results of CO<sub>2</sub> analysis show that none of the whole snout samples taken during the first sampling day are in the range expected of exhaled breath (3-5%); results from the second sampling day demonstrated that all the nostril samplers had significantly better performance. The

sampler giving the highest mean CO<sub>2</sub> value was the short thick nostril sampler, although there was not much difference between all the nostril samplers. The results indicate that there was some loss of CO<sub>2</sub> (an average of about 8%) after 6 hours storage in the bag. The ambient CO<sub>2</sub> level was normal. A human volunteer filled a clean evacuated sampling bag with his own breath, which was subsequently measured using the CO<sub>2</sub> monitor. The concentration of CO<sub>2</sub> was as expected for exhaled breath demonstrating the CO<sub>2</sub> monitoring set up was fit for purpose.

Results shown in table 1 demonstrate that the simple nostril breath sampler from the first sampling occasion (5.) gave the highest concentrations of acetone but not necessarily of the other VOCs, some of which had condensed onto the large surface area of the whole snout samplers along with exhaled water vapour visible on the surface. Thus the nostril samplers give the most representative samples. It can also be seen from the second sampling occasion using the nostril samplers that that methanol and ammonia are increased in concentration after six hours. Results from analyses of other VOCs (data not shown) show the same trend. The exceptions to this are water, which is reduced from a mean value of 3.43% to a mean value of 1.58% and acetone, which decreases slightly. This is a counterintuitive result. We speculate that this arises in the following way: water is lost from the bags through pores as the Nalophan is slightly porous, and this rate of loss is increased at higher temperatures. Some of the VOCs remaining in the bag do not stick to the bag surface after water loss and hence increase in concentration. Previous unpublished studies have shown that acetone is stable in sample bags over longer periods and this is supported by these data, where little loss of acetone is apparent.

The most stable compounds from breath are acetone and water. Ammonia is more difficult to store and analyse as it tends to stick to surfaces and the six hour delay between sampling and analysis renders this a less reliable marker. Ammonia also is present in the environment ubiquitously and levels detected here are not much different from background levels. Methanol and ethanol may be present in rumen gases and presumably eructation may affect levels present in breath, although no obvious eructation was observed during collection of the samples. For this reason, the most reliable markers of sampling efficiency are acetone and water. Acetone in human breath has a mean value of approximately 480 ppb (Turner et al, 2006). No data have been found on the mean level expected in the breath of healthy cattle, although it is known that ketotic cows may have high levels of acetone in milk (de Roos et al., 2007). The animal sampled on the first sampling occasion (bullock) had a low level of acetone, however, the animal sampled on the second sampling occasion was a lactating dairy cow, and had a much higher level of breath acetone as supported by the literature (Mottram et al., 1999). In conjunction with this, the lactating cow also had concentration of 2-butanone of 400 parts-per-billion, whereas the 2-butanone could not be detected in the breath of the bullock (i.e. below 10 ppb). Breath samples from neither animal showed any evidence of rumen contents headspace, with low concentrations of the volatile fatty acids: acetic, propanoic and butanoic acids. Very little dimethyl sulphide or dimethyl disulphide were detected in either animal in contrast to that reported by Elliott-Martin (1997), who noted high levels (80-100ppm) of dimethyl sulphide on the breath of dairy cattle.

Water concentration in the breath of healthy humans at 37°C is about 6%, which implies that the breath is fully saturated with water (6% is the amount of water in air at 37°C if fully saturated). Bovines have greater “dead volume” in their breath and as a result it is possible that their breath is less humid than human breath. However, only the nostril samplers gave water concentrations higher than 3%, supporting the CO<sub>2</sub> data showing the nostril sampler to give more representative breath samples. It should be noted that after 6 hours of sample storage, the water concentration in the bags on the second sampling occasion (using the nostril samplers) was much lower than on the first sampling occasion. This is probably due to the storage temperature of the bags, which was lower on the first occasion as bags were stored outside during November. On the second

occasion they were stored during the summer (May). Hence the increased ambient temperature appeared to increase water loss from the bags.

### 3.2 *Sampling the breath of tuberculosis-infected cattle*

TD-GC-MS analysis identified more than 100 compounds present in the breath of each animal at different times before and after infection with *M. bovis*. In this experiment, the most abundant compounds in breath detected using TD-GC-MS were acetone, dimethyl sulphide and 2-butanone. However, our studies have indicated the presence of different concentrations of compounds than found in other studies, for example Mottram et al. (1999). This indicates that there is a large variation of the composition of cattle breath depending upon the type of animal, its gender, diet etc. For this reason, any study involving the use of breath sampling to diagnose disease should ensure that the breath profile of healthy animals of that type and kept under those conditions is well characterised in a statistically significant number of animals before sampling diseased animals to identify biomarkers.

## 4. Conclusions

In taking breath from cattle, the outcome of the testing of several types of sampler indicates that the most representative breath samples are obtained from using a simple device which takes breath from a single nostril. The person taking the sample needs to monitor the breathing of the animal to ensure the sampler is gently moved away from the animal during inhalation. This proved simple, and the animals were far happier having this applied than the whole snout samplers. Apart from being less well tolerated, the considerable dead space of the whole snout samplers rendered the samples obtained in this way much less representative.

Results of the repeated analysis of samples showed a loss of water vapour after 6 hours, however many of the other VOCs did not change substantially. Although it is obviously desirable to analyse samples as soon as possible, these results indicate that a six hour delay is probably acceptable.

The nostril samplers were then used to take samples of breath from cattle experimentally infected with TB for analysis by TD-GC-MS. Successful sampling and analysis demonstrated the potential of this technique for the analysis of breath from animals. It is hoped that this may one day be used to develop diagnostic tests for infectious diseases. This may, in future, offer the possibility of a non-invasive method for diagnosing infectious disease with minimal risk to the operator.

## Acknowledgements

This work was funded by the Department for Environment, Food and Rural Affairs (Defra) as part of project SE3221. Thanks are due to Martin Vordermeier and Derek Clifford for assistance with providing and sampling the cattle.

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Figure 1. Photograph of whole nose samplers. 1a: Full size sampler; 1b: reduced headspace sampler

Figure 2. Photograph of nostril samplers. Photograph of nostril samplers. Top left of the picture is sampler 1 (long narrow), then going clockwise is 2 (long thick), 4 (short thick) and 3 (short narrow).

Figure 3. a) Use of nostril sampler in taking a breath sample from a lactating cow; b) Photograph demonstrating how breath samples are pumped across TD tubes prior to analysis by GC-MS. The sample bags containing breath are kept within a specially adapted incubator, with the adapter protruding. The adapter is attached to a thermal desorption tube via a pump and filter. The pump is enclosed in a bag; the filter and bag are to protect the equipment and operators from potential TB release.



1a



1b



Figure 2.



Figure 3a



Figure 3b