# Analytical approaches for detection of breath VOC biomarkers of cattle diseases -A review

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**Keywords:** volatile organic compounds, animal agriculture, bovine health, sampling and sample preparation, non-invasive analysis, precision agriculture.

## Abstract

Diagnosis of diseases in cattle at early stages is of significance both economically and clinically. Non-invasive diagnostic samples such as breath are preferred since they cause minimum inconvenience or pain to the animals. In this review, different sampling devices, sample preparation techniques, instrumentation, and statistical analysis approaches that have been designed and tested are described and compared in terms of their applicability in the diagnosis of common cattle diseases. The sample preparation techniques used include solid-phase microextraction (SPME), sorbent extraction, and needle trap device (NTD). The collected volatile organic compounds (VOCs) are determined using gas chromatography-mass spectrometry (GC-MS) and the electronic nose (e-nose) technology. The majority of studies are focused on the diagnosis of ketosis and

bovine respiratory disease (BRD). The common diseases and potential biomarkers are summarized and discussed. Due to the differences in the number of subjects and the type of animals used in different studies, the results are not consistent. Acetone, although detected in almost all studies and subjects, has elevated concentrations in cattle suffering from ketosis. The results of currently available studies were not indicative of specific biomarkers for BRD, and further investigation is required. The current studies have shortcomings in regards to defining useful VOC profiles, the impact on animal welfare, and the practical application at the producer level. While the presented approaches are promising, more controlled, standardized clinical studies need to be conducted before breath analysis can be routinely performed on cattle.

**Introduction**

A disease is defined as a disorder or incorrect function of an organ, structure, or system of an animal's body, which can be caused by nutrient deficiencies, pathogens, and genetics [1]. Cattle have been raised by farmers for millennia as livestock for meat, milk, and as draft animals [2], and are economically significant to many local, regional, and national communities around the world. Cattle health is significant both from the economic point of view and the threat to human health by zoonotic diseases, i.e., diseases that can be transmitted from vertebrate animals to humans [3]. The World Organization for Animal Health (OIE) has listed the following as diseases reportable for cattle in 2020: bovine anaplasmosis, bovine babesiosis, bovine genital campylobacteriosis, bovine spongiform encephalopathy, bovine viral diarrhea, enzootic bovine leukosis, hemorrhagic septicemia, infectious bovine rhinotracheitis/infectious pustular vulvovaginitis, infection with lumpy skin disease virus, infection with *Mycoplasma mycoides* subsp. *mycoides* SC (contagious bovine pleuropneumonia), theileriosis, trichomonosis, trypanosomosis (tsetse-transmitted) [4].

Bovine respiratory disease (BRD) is the costliest disease problem in the cattle feeding industry. This complex is the most commonly diagnosed disease in feedlot cattle, affecting approximately 16.2% of all cattle on feed in the United States [5]. This is true for other major cattle-producing countries as well [6, 7]. Losses have been estimated at just under $1 billion in the United States alone [8]. Reduced profitability is due to treatment, labor, reduced feeding performance, reduced carcass value, and increased death loss. Severe lung lesions due to BRD found at slaughter are associated with decreases in average daily gain, hot carcass weight, less internal fat, and lower marbling scores [7, 9]. Either untimely treatment of or undiagnosed BRD can lead to these lesions and economic losses associated with them.

The segmented structure and management practices of the cattle industry in the United States contribute to BRD development at the feedlot level. There are over 800,000 cow/calf operations in the US where calves are initially born and raised until they are weaned and marketed. The vast majority of these operations have less than 50 cows and serve only as a source of supplemental income for the cow/calf producer [10]. Since this large number of independent producers are located in all regions of the country, there is tremendous variation in the genetic makeup, health management, feeding programs, and cattle selection at the cow/calf level. In addition, most feedlots are located in the upper Midwest and Plains states in the US, and this may require cattle to be trucked long distances to reach their final destination. After leaving the cow/calf operation, most calves are marketed through a central public sale facility ("sale barn"), sorted into more consistent groups, and transported to their next destination. It is not unusual for this process to take 2-5 days from when calves leave the farm of origin until they reach the feedlot. This combination of comingling of calves from multiple sources, potential inclement weather, long transit times, and incomplete health management often results in high levels of BRD once these

calves reach the feedlot. The feedlots facilitate the final stage of cattle production. Cattle are put on a specialized intensive diet, confined to pens to gain weight before slaughter.

Finding cattle affected with BRD is a challenge in all feedlots. Traditionally, feedlots have relied on a two-step approach for diagnosing BRD. The first step is identifying sick animals in their home pens. Clinical evaluation or pen riding/walking is the most common means of identifying sick animals in their home pens. Clinical signs such as depression (D), decreased appetite (A), abnormal respiration (R), nasal and or ocular discharge, and weight loss are commonly used to evaluate animal health. The second step is confirming that those identified animals are truly sick and establishing a cause for their illness. Traditionally, rectal temperature (T) has been the most common means of confirming illness. This combination of clinical signs and temperature is referred to as 'DART'.

Various diagnostic tests have been developed and investigated to improve the accuracy of diagnosing BRD in feeder cattle. All diagnostic tests used in a feedlot setting can be categorized as either detection tests (used to identify sick cattle in their home pen) or confirmatory tests (used to determine if BRD is the cause of their symptoms). A lack of an antemortem 'gold standard' for BRD diagnosis has made establishing the sensitivity and specificity of the diagnostic tests difficult. Diagnostic test analyses are often difficult to perform and lend themselves to bias. With a lack of a gold standard, classification bias is a major issue. Classification bias occurs when the reference test is not 100% accurate. This results in the disease state not always being correctly identified [11]. When diagnostic test accuracy varies based on the severity of the disease, spectrum bias becomes an issue [11]. Comparability of studies to each other becomes limited due to the number and variation of the diagnostic tests that are being used as gold standards in each study.

Clinical evaluation is not a perfect test but is the standard by which other diagnostic tests are measured due to its wide application in commercial feeding operations. Most of the symptoms noted during evaluation are nonspecific physiologic reactions to any infection and subsequent fever. Symptoms such as depression, lethargy, rough hair coat, anorexia, and dehydration are often noted [12]. However, these symptoms are not specific to BRD and can arise with a fever of any origin [12]. The respiratory rate has also been described as a symptom noted during clinical evaluation. However, one study was not able to demonstrate a significant change in respiratory rate for calves subjected to a known infectious challenge [13].

Bayesian models have compared clinical evaluation of the live animal at the feedlot with the presence of lung lesions at slaughter [14, 15]. White et al. (2009) [14] calculated the sensitivity and specificity of clinical evaluation to be 61.8% and 62.8%, respectively. Timsit et al. (2016) [15] calculated the sensitivity and specificity of clinical evaluation to be 27% and 92%, respectively. They also noted that their calculations had wide credible intervals and found significant heterogeneity among those studies that were evaluated in their meta-analysis. This indicates that caretakers' live animal evaluation for BRD in the feedlot is a relatively poor way to identify animals with BRD. This leads to the improper application of treatment protocols and over usage of antibiotics. The relatively low level of sensitivity and specificity achieved by using DART as a diagnostic tool has dramatically increased the interest in more accurate ancillary diagnostic tests from cattle producers and veterinarians.

Laboratory diagnosis of veterinary infectious diseases has been traditionally performed by detecting the pathogens by culture or antibodies using various techniques such as serum neutralization, enzyme-linked immunosorbent assay (ELISA), agar gel immunodiffusion, and complement fixation [16]. For instance, the diagnosis of bovine tuberculosis (bTB) relies on a

combination of screening and confirmatory tests, based on tuberculin skin testing, and interferon-gamma assay, an enzyme-linked immunosorbent assay, respectively [17, 18]. However, these methods are often time-consuming and expensive, require multiple animal handling, are labor-intensive and need to be conducted by certified veterinarians, and in some cases lack the required accuracy and reliability [17, 19-21]. An alternative *in vitro* assays includes serologic assays, lymphocyte proliferation assay, and polymerase chain reaction (PCR), which have limitations in terms of cost, sensitivity, accuracy, and being invasive [20, 22]. Clinical symptoms of some infectious diseases do not appear until two years or more after the infection has started, and the available techniques are not sensitive enough to diagnose these diseases at early stages [21]. On the other hand, due to lack of accurate and rapid diagnostic practices to distinguish between infectious diseases caused by bacterial infection from those caused by viruses and from non-infectious diseases, unnecessary antibacterial therapy is initiated upon observation of clinical symptoms and temperature (DART), which are often not specific to bacterial diseases [23, 24]. Therefore, the development of rapid on-field tests, which are sensitive, specific, and non-invasive and can identify the animals at the early stages of the disease, are desirable and beneficial to the livestock industry [17, 18, 25, 26].

It has been known for many years that certain diseases could cause a change in body odor or in the profile of volatile organic compounds (VOCs) emitted from the body and body excretions (e.g., breath, feces, urine, sweat, milk, and blood) indicating that there are relationships between the diseases and the volatile chemicals liberated into the air, making body odor a diagnostic tool for diseases and infections [27, 28]. The significant change in the VOCs composition in the mammalian body is known to be caused by structural changes. One example includes peroxidation of the cell membrane, which may occur both in the host and the invading cell, resulting in the emission of

certain VOC patterns unique to each disease or infection [18, 27]. It should be noted that besides diseases, the VOCs found in biological samples are affected by other variables, including age, breed, gender, reproductive status, genetics, and environmental factors, such as diet, climate, and husbandry [21]. The volatile gases present in the environment can be inhaled or absorbed through the skin and appear in exhaled breath [29]. Understanding the different factors that can influence the VOC profiles is significant in designing the experiments and interpreting the obtained data.

Considering the evidence of the influence of diseases and infections on VOC contents of biological samples, a new area of clinical biochemistry is focused on the diagnosis of infectious diseases based on variations in the volatile organic compound (VOC) profiles, with the purpose of early detection of diseases in veterinary medicine. The main goals of the available studies were:

1. To design, build and test non-invasive sampling devices for breath analysis [19, 20, 30-32].
2. To identify the VOCs in the headspace of bacteria cultures [21, 26, 33].
3. To investigate the possibility of using tested sampling devices to study the VOCs in samples of breath for the diagnosis of infectious diseases by either identifying unique biomarkers of respiratory diseases or by studying the variations in VOC profiles in:
    a. breath [18 - 20, 22, 34, 35, 36, 37]
    b. the headspace of feces [22, 35, 38],
    c. serum [17, 27, 39, 40], and
    d. nasal secretions [38].
4. To find correspondence between the VOCs identified in breath and headspace of feces and investigate if the previously identified VOCs in the headspace of cultures can be identified in the *in vivo* samples [22].

5. To study the effect of growth and metabolism on the variations of VOC compositions in the *in vivo* samples, to be considered before defining biomarkers for diagnosis of diseases [41].
6. To present strategies for data analysis to help identify the disease biomarkers and VOCs indicative of diseases [42].

Besides biological samples, VOCs have also been monitored in real-time in barn air by Gierschner et al. (2019) using proton transfer reaction time of flight mass spectrometry (PTR-MS) for different groups of dairy cows prior to milking [43]. It should be noted that besides VOCs, nonmetal oxides, i. e. nitric oxide and carbon dioxide, have also been measured in bovine exhaled breath for health screening purposes of cattle, using tunable diode laser absorption spectroscopy [44, 45], which is not the focus of the current review.

The main purpose of the current review is to demonstrate the development of analytical approaches investigated for sampling and determination of VOCs in cattle breath and to discuss the feasibility of these approaches in the diagnosis of cattle diseases at an early stage.

**Exhaled breath analysis**

Although exhaled breath analysis has been studied for a long time and has been successful in diagnosing several diseases in human studies [46], it is still at an early stage in animal studies. It is known that the VOCs that compose the exhaled breath have both endogenous and exogenous sources [18]. The endogenic VOCs are formed through metabolism in the cells and are released into the blood, circulated in the body, and excreted through breath and body fluids. Therefore, any changes that occur in biochemical reactions in cells, including the changes caused by diseases, change the blood chemistry and are reflected in the breath through the exchange of VOCs in the lungs [18]. Breath analysis is preferred over the analysis of other biological samples such as blood, urine, and feces since breath is a non-invasive sample, easier to obtain, and has the potential to

provide real-time monitoring [18]. Breath is also a less complex matrix compared to blood, urine, and feces, which makes the sample preparation and/or data analysis less complicated. As a result, most volatilomic analyses in cattle have been focused on breath analysis and specially dedicated to the diagnosis of ketosis [30, 32, 34], BRD [19, 31], or other bacterial infections [18, 20, 22].

Exhaled breath analysis in cattle is more challenging compared to human breath analysis. Humans can be trained to cooperate with sample collection. Unlike humans, for which the breath samples are collected under clinically clean conditions such as hospitals, cattle breath is collected in the field and can be contaminated by the VOCs and particles present in the environment. Unlike humans, cattle eructate, and the "burp", which consists of the VOCs generated through rumination, is mixed with the exhaled breath making the sample not representative of only VOCs in blood but also enzymatic activities in the rumen. The VOC composition of ruminal gas and the effect of eructation on VOC composition in breath has been investigated and needs to be considered in cattle breath analysis [47]. Moreover, while the deep exhaled breath is representative of blood VOC concentrations, animals cannot be instructed to blow their breath deeply into the sampling device. Therefore, the first steps that were taken in exhaled breath analysis in cattle were towards designing sampling devices that would address these challenges. Similar to human exhaled breath analysis, breath sampling in animals is either followed by gas chromatography-mass spectrometry, which provides separation, identification, and quantitation of VOCs, as well as VOC profiles or, is combined with e-Nose technology [33], which produces a profile of selected classes of VOCs present in the breath samples. Considering the number of VOCs present in biological samples, the data interpretation is also another challenging step in all volatilomic studies, which are addressed by applying statistical approaches, e.g., principal component analysis (PCA). In this section, we first

discuss the evolution of the sampling devices designed and tested for breath analysis in cattle, followed by a discussion of the analyses and interpretation of the obtained results.

**Breath Sampling Devices and Designs**

To our knowledge, the first sampling device for breath analysis in cattle was designed and patented by Mottram (1992) [48] and was later applied to bovine breath analysis by Dobbelaar et al. (1996) [32]. Martin et al. (1997) designed a hand-held device version of the device for sampling breath directly from the nostrils, with the purpose of eliminating cross-contamination from odors emitted from the mouth [30].

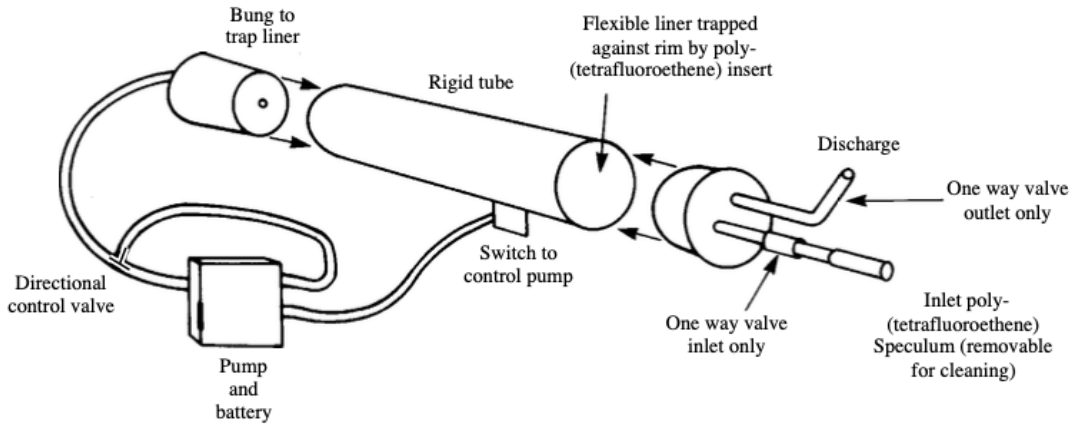


Fig. 1. Exploded diagram of the breath sampling device. (Elliott-Martin et al. 1997; [30]; published with publishers permission 5067230115864).

Fig. 1. illustrates the schematics of the device, which consists of a flexible, disposable gas sampling bag made from polyethylene terephthalate film (PET, also known as Nalophan), with a capacity of 0.55 to 0.60 L, placed in a rigid tube. The sampling bag is passed through the rigid tubing and is sheathed at one end around the lip of a sampling head, trim fitted to the rigid tubing. The other end of the sampling bag is enfolded around the rear end of the rigid tubing, which is then

sealed by a bung. A duct, housed in the bung, connects to a pump, the annular volume between the sampling bag and the rigid tubing, to maintain a partial vacuum or positive pressure during the breath sampling. Before each sampling, a clean inlet speculum is mounted on the sampling head, and the sampling head is fitted to the rigid tubing containing a new sampling bag. The inlet is then placed in the animal's nostril; once the animal starts exhaling, which is decided by the operator by observing the breath mist or whisker movements, the operator manually activates the pump. Once the pump is turned on, a partial vacuum is generated in the space between the sampling bag and the rigid tubing, causing the bag to fill and expand while the animal is exhaling. Once the animal starts inhaling, the pump is turned off. The sampling bag is discharged through the outlet by pumping air into the space between the sampling bag and the rigid tubing and creating positive pressure on the bag. The sampling time was generally achieved within 30 s, and the gas discharge was accomplished within 10 to 30 s [30].

The main advantage of the described nasal sampling device is portability. As illustrated in Fig. 1, the pump is operated by a battery, and the switch is conveniently placed near the sampling head so that the operator can easily move the whole device and turn the pump off and on even when the animal is moving, causing less discomfort to the animal. The disadvantage of the device is that sampling when the animal is exhaling or inhaling is decided by the operator, who can get distracted or make mistakes during the operation. Moreover, in this study, it was assumed that Nalophan (PET) sampling bags are impermeable to VOCs and water vapor, and are neutral in odor and do not contain a plasticizer, and therefore do not produce background interferences. Research published later on, on the inertness and permeability of Nalophan (PET) sampling bags partially supports these assumptions [49]. Koziel et al. (2005) [49] tested Nalophan (PET) sampling bags, among other commercially available air sampling bags, for recovery and background interferences, using

standard gas generators, pure air, and solid-phase microextraction (SPME), and found that there were very low background interferences in Nalophan bags. However, the average sample recovery, although the best among other sampling bags, was 71.7% and 47.2% for 0.5- and 24-h sample storage time, respectively [49]. In general, air sampling bags have been shown to have low recoveries and are likely not suitable for collecting/storing breath samples for determination of low concentrations of potential biomarkers present in complex breath matrices. While generally not recommended, some sampling bags could be reused under proper cleaning and QC/QA protocols that address or account for background impurities and carry over [49]. The safest approach to minimize carryover is to use new, properly conditioned bags for each sampling.

The described device was later improved and applied to determine the feasibility of detecting hyperketonaemia in dairy cows in another study [34]. Background samples were taken from a 1-m distance in front of the cow at the cow's head level. The repeatability of sampling was tested by taking multiple breaths and background samples every 2 min from a control and a treatment cow [34]. The VOCs in the collected samples were concentrated on silica and carbon-based adsorbents and were subsequently introduced into the GC-MS system with a thermal desorption unit [34]. The acetone concentration was monitored in breath samples of treatment cows before and after reducing feed and was compared with those in breath samples from controlled cows and background samples.

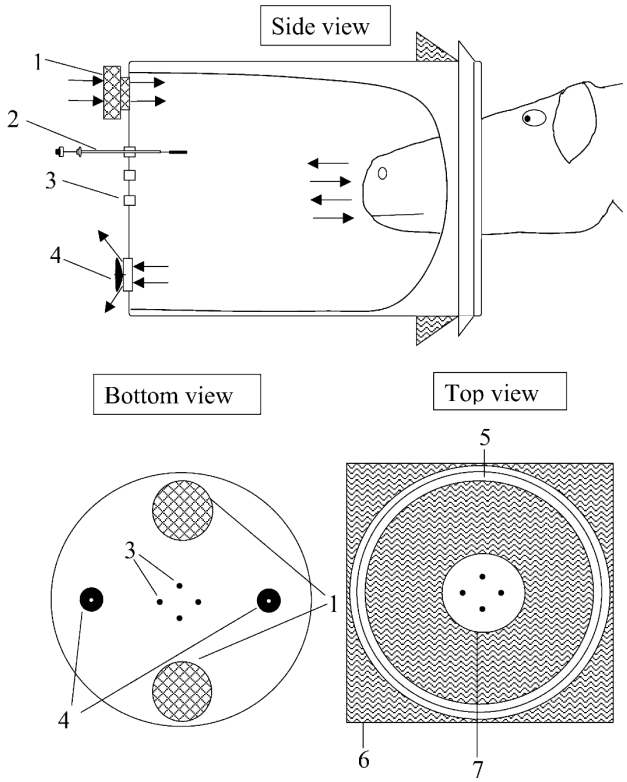


Fig. 2. Schematic of fast on-site breath measurements of VOCs from bovine using solid-phase microextraction (SPME) fibers and sampling chamber (18.9 l HDPE bucket); ambient air enters the system through (1) filter cartridges, is sampled with (2) SPME fiber through (3) septa, and exits the system through (4) one-way valves; the (5) sealing ring of the bucket holds the (6) silicone sheet in place; the bottom of the bucket can be seen from the (7) hole in the silicone sheet that provides access for animal's nose and mouth [31]. (Spinhirnie et al., 2003; [31]; Published with publisher's permission 5123270740887).

The next on-site, non-invasive animal breath sampling device was designed, built, and tested by Spinhirne et al. (2003), illustrated in Fig. 2 [31]. In this sampling system, real-time breath sampling was achieved with SPME for the first time in cattle. Previous studies with standard gas sampling had demonstrated that SPME fibers had excellent recoveries of VOCs, i.e., 106% (±20.2%) for 0.5-h storage time and 98% (±18.6%) for 24-h storage time [48]. Moreover, SPME combines sampling and pre-concentration and facilitates sample introduction to a GC. The sampling system was made of an 18.9 L high-density polyethylene (HDPE) bucket. The bucket lid was replaced with a 1.5 cm thick silicone sheet with a 15 cm hole in the center to allow the nose

and mouth of large animals to be placed inside the bucket while separating the breath from the ambient air. The air entering the bucket was filtered by installing one-way valves and filters at the base of the face mask to remove background gases. Therefore, the animal inhaled the air which was passed through the filter cassettes (adsorbing the VOCs in ambient air) before entering the device. On the other hand, as the animal exhaled, the air from inside the device exited through the two one-way valves, allowing the animals to breathe normally [31]. During the sampling, SPME fibers were exposed to the air inside the sampling device through half-hole LB-1 septa, placed in the center of the bottom of the bucket. The sampling system was reusable and was cleaned and heated before use to minimize the background interferences from the materials used in the system and carry-over between sampling events. Blank samples were taken using SPME for 15 min to determine the possible residual compounds released from the HDPE bucket and other plastic parts after heating [31]. The device was used to sample exhaled breath from two heifers on three different days from 5 to 15 min, using Divinylbenzene/Carboxen/polydimethylsiloxane (DVB/Carboxen/PDMS) 50/30 μm and PDMS 100 μm SPME fibers. The samples collected on SPME fibers were preserved using tightly fitting polytetrafluoroethylene (PTFE) plugs and placed in a cooler of ice immediately after sampling and were analyzed using GC-MS within several hours after collection. To eliminate the background interferences, ambient air samples were also taken simultaneously as the breath samples [31].

This device was further improved in another study by the same group [19]. The main modification was replacing the HDPE bucket with a stainless-steel bucket to reduce the background interferences. The leak around the sealing membrane caused by the animal head movement due to hyperventilation was decreased by increasing the number of filters and outlet valves to assure that the animal received an adequate breathing air supply. The sampling devices were cleaned, and the

background was checked in the laboratory using SPME. Trip blanks and ambient air samples were also collected to investigate possible interferences originating from the process and the ambient air [19]. The collected samples were refrigerated and analyzed after 8 h using GC-MS [19].

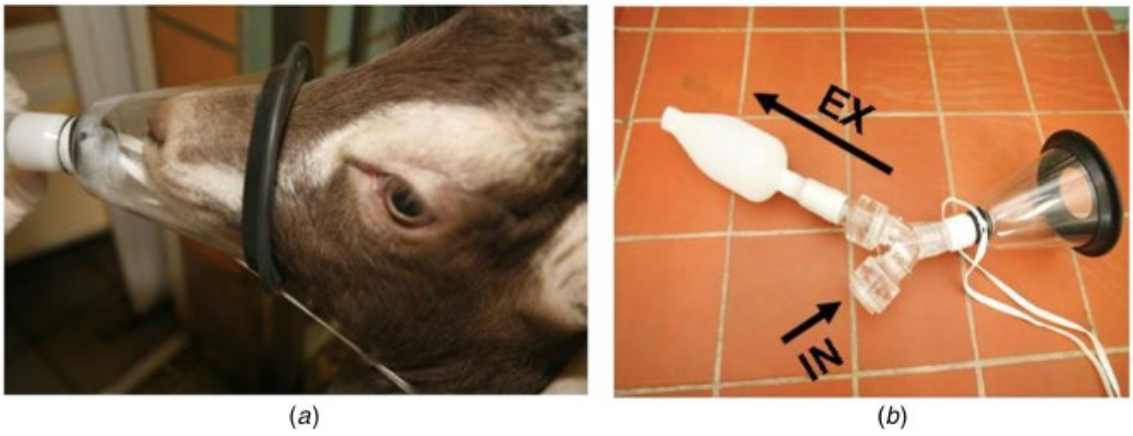


Fig 3. Methodology to analyze exhaled breath from spontaneously breathing goats using a tightly fitting face mask (a) and a spacer adapted to the expiratory valve (b). Photos: Wolfram Maginot (FLI, Jena, Germany). (Purkhart et al. 2011; [35]; Published with publishers permission 1138955-1).

Purkhart et al. (2011) [35] performed breath sampling using a tightly fitting mask (Fig. 3a) and a spacer (Fig. 3b), and the expired breath was collected and suctioned into a differential ion mobility spectrometer (DMS) for analysis [35]. The rationale for breath (and headspace feces) chemical analyses with DMS was the characteristic smell ('smellprint') of certain animal diseases [35].

Peled et al. (2012) [18] studied the VOC patterns in breath samples collected from cattle infected with *Mycobacterium bovis* (*M. bovis*) and a control group. Breath sampling was performed using the device illustrated in Fig. 4.

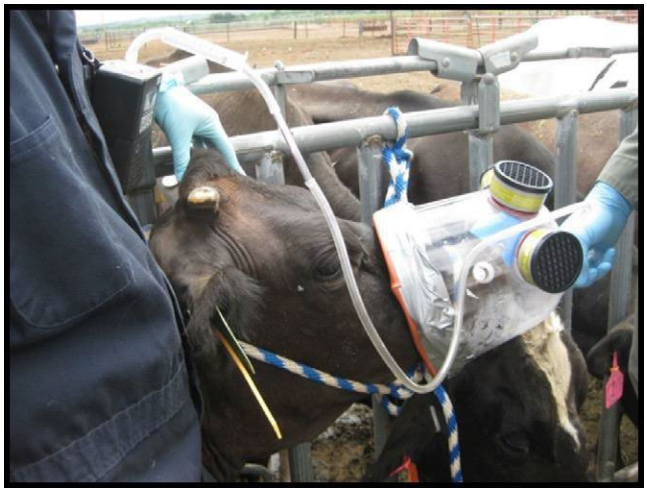

Fig. 4. Photo illustrating the system employed for breath sample collection in the cattle. Inspired air first passes into the mask through three charcoal filters and one-way valves to remove environmental VOCs. Expired air passes out of the mask through two one-way valves and through the tubing inserted into a hole in the front of the mask. Air in the tubing passes through a glass cartridge containing sorbent material (TenaxTM) and is exhausted through the hand-held suction pump. (Peled et al. 2012; [18]; Published with publishers permission 5123281456631).

A mask designed for delivering nebulized medication to horses was modified, so that inspired air was passed through charcoal filter cartridges to remove the confounders and contaminants from the sample. The exhaled breath was passed through an inert sorbent material (Tenax) using a hand-held pump. The sorbent material, which contained VOCs extracted from 2 L of exhaled breath, was then sealed and stored at -70 °C, before analysis by GC-MS and the specially designed NA-NOSE. The compounds were thermally desorbed from Tenax before analysis with GC-MS and NA-Nose [18].

Turner et al. (2012) [20] designed two sets of breath samplers, one large sampler to cover the whole nose and mouth (for taking samples from both nostrils) (Fig. 5), and a single-nostril sampler (Fig. 6). Two whole-breath samplers were made with different headspace depths. Therefore, different dead volumes, from a transparent food-grade polyethylene fitted with a silicone

rubber seal, were cut to size for each animal for the comfortable seal around the animal's mouth and nostrils. The two valves on the sampler allowed the animal to breathe, and the exhaled breath was collected into a Nalophan sampling bag [20].

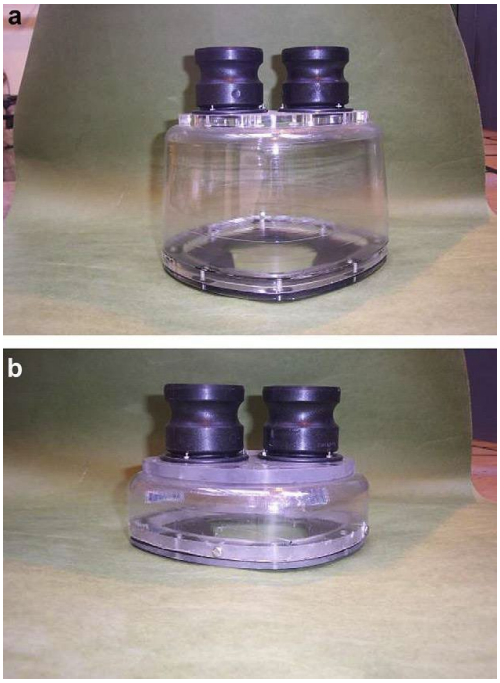


Fig. 5. Photograph of whole nose samplers. a: Full-size sampler; b: reduced headspace sampler. (Turner et al. 2012; [20]; Published with publishers permission 5123290725432).

Fig. 6. Photograph of nostril samplers. The top left of the picture is sampler 1 (long narrow), then going clockwise is 2 (long thick), 4 (short thick), and 3 (short narrow). (Turner et al. 2012; [20]; Published with publishers permission 5123290725432).

Four nostril samplers of different sizes were made of autoclavable glass-reinforced polypropylene tubes with a one-way valve to collect exhaled breath as the sampler was sealed around the nostril with a soft, malleable silicone rubber seal, identical to those used in the whole nose samplers (Fig. 6) [20]. The full nose samplers were held over the animal nose until the sampling bag was filled. Taking a sample with the nostril sampler was done by holding the sampler cup over one nostril while covering the other nostril with the second hand (illustrated in Fig. 7). Sampling was made possible by carefully observing when the animal would inhale to remove the sampler and only collect the exhaled breath. The collected samples were incubated at 37 ºC before connecting them to conditioned stainless-steel thermal desorption tubes (TD) containing 50% Tenax TA and 50% Carbotrap (Markes International Limited, Llantrisant, UK). A 500 mL breath sample was drawn across the tube using a constant-flow pump, placed in a bag to protect the operator against possible bTB exposure (Fig. 7). The tubes were then removed, sealed, and treated at 80 ºC for 1 h to kill any bacteria that might have been present and were subsequently analyzed by ATD-GC-MS [20]. The effect of incubation on the loss of VOCs from sealed tubes was not reported by the authors. A need for incubation could be eliminated by using an inline fine mesh filter between the sampling bag, and sorbent tube, similar to separating sampled gas phase from infectious aerosol was described elsewhere [33].


Fig. 7 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 protect the

equipment and operators from potential bTB release. (Turner et al. 2012; [20]; Published with publisher's permission 5123290725432).

The designed samplers were first tested on healthy dairy cows who tolerated the samplers very well and were then used to take breath samples from cows experimentally infected with M. bovis (before experimental infection with M. bovis, and then at 2, 3, and 5 weeks post-infection). The sampling from the second group was performed while the animals were temporarily held in a chute for safety.

Bergmann et al. (2015) pre-concentrated VOCs from breath and feces of 42 goats (16 controls and 26 animals inoculated with *Mycobacterium avium ssp. paratuberculosis* (strain JII-1961) using needle trap microextraction (breath) and SPME (feces) and determined them with GC-MS [22]. Breath sampling was done using an automated alveolar sampling device (Fig. 8), previously designed and tested by Trefz et al. for human breath sampling [50]. The breath sampler (A) was connected to the analog reader of a fast-responding capnometer (response time < 60 ms) (B), which constantly measures the partial pressure of $CO_2$ in the breath. Once the $CO_2$ level surpassed a preset threshold, a signal was sent to the valve (D) to open, and the breath sample was drawn through the NTD (C). The flow rate was controlled by a mass flow controller at 20 mL/min. The automated alveolar sampling system was used to eliminate the operator's error in sampling exhaled breath, as discussed earlier in this section. A minimum of two-room samples per day was also collected per stable using the same procedure without using the capnometer [22].

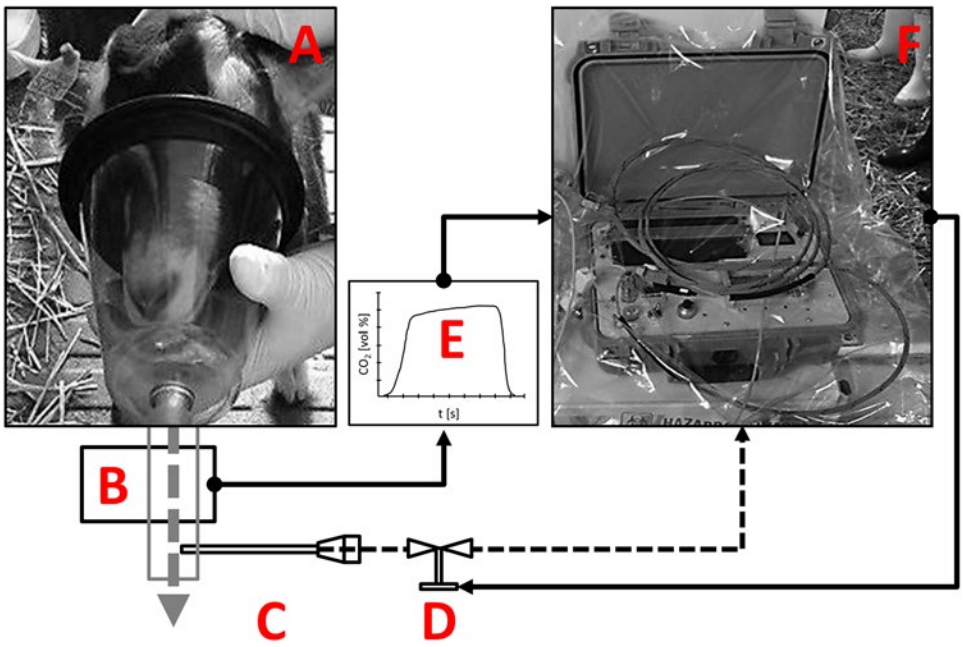


Fig 8. Experimental setup for alveolar breath sampling in goats. A—sampling mask; B—$CO_2$-sensor; C — Needle Trap Device (NTD); D—$CO_2$ triggered flow valve; E—Capnogard for time-resolved $CO_2$-monitoring; F—sampling box; dashed arrows represent air flows; continuous arrows represent electronic signals. (Fig. 1 in Bergmann et al. 2015; [22]; Published under the Creative Commons Attribution (CC BY) license).

## Results

As mentioned in the introduction section, the ultimate goal of studies aiming at the analysis of breath samples for VOCs in bovine subjects is the early diagnosis of cattle disease by either identifying unique biomarkers or by studying the variations in VOC profiles. In this section, the results obtained by each study are summarized.

*Utility of VOC biomarkers in ruminant disease diagnosis*

Dobbelaar et al. [32] used the nostril sampling prototype proposed by Mottram [48] to determine the concentration of acetone in exhaled breath of ketotic cows, using gas chromatography-mass spectrometry (GC-MS) and showed that the values correlated with

concentrations of 3-hydroxybutyric acid in serum (r=0.81), and acetoacetate+acetone in milk (r=0.70), which are known biomarkers of ketosis in serum and milk [32].

Martin et al. [30] designed and used a hand-held version of Mottram's nostril sampling prototype to collect breath samples from healthy and ketotic cows under field conditions for the first time [30]. The collected samples were subsequently analyzed by a gas sensor array, gas chromatograph-mass spectrometer (GC-MS), and Fourier transform infrared spectrophotometer (FTIR), and the concentrations of methane, dimethyl sulfide, butan-2-one, and propanone (acetone) were determined [30]. Principal components analysis of sensor array response to breath samples resulted in an 89% success rate in classifying the cows as healthy or ketotic, which the authors expect to improve in a more controlled trial and by a more improved sampling device [30].

Mottram et al. [34] used the same hand-held nostril sampling device reported by Martin et al. [30] to determine if detection of hyperketonaemia in dairy cows was feasible and showed that the acetone concentration in the breath samples of treatment cows significantly increased after 7 h following food reduction compared to controlled cows/background air, indicating hyperketonaemia [34]. Although preliminary, these results showed promise that breath analysis could be used to diagnose ketosis faster compared to the onset of clinical signs [34]. In this study, the clinical onset of ketosis was not observed until 180 h after feed reduction, even though acetone concentration increased significantly in breath samples in as little as 7 h. This represented a 96% reduction in the amount of time required for the diagnosis of ketosis [34].

Another research group that designed a nostril sampling device was Turner et al. (2012) [20]. However, their study was mainly focused on designing the sampling device and developing the analysis method. Although the authors reported that over 100 compounds were detected in the

breath samples, with the most common compounds being: acetone, dimethyl sulfide, and 2-butanone, a complete list of all detected compounds was not provided [20].

Spinhirne et al. [31] designed and used a face mask-like device for sampling breath from steers. Using SPME, the VOCs were extracted from the breath samples in real-time and were determined using GC-MS. Comparing the chromatograms for breath samples, taken from two steers in a feedlot, with the ambient air, the sampling system blank, and the field sampling trip blank, the authors identified the compounds acetone, methyl ethyl ketone, toluene, tetradecane, pentadecane, nonanal, and decanal in cattle breath [31]. This study was mainly focused on testing the newly designed mask and developing the SPME method for real-time extraction of VOCs. The results showed that the extraction time of 15 min, with DVB/Carboxen/PDMS 50/30 μm coating, and preserving the fiber with Teflon caps, refrigeration, and analysis with GC-MS, worked well, and the sampling device showed potential for rapid, non-invasive, on-site research on animal breath analysis [31].

Spinhirne et al. (2004) [19] improved the device further and used it to determine VOCs in the breath of both healthy and sick steers to identify unique biomarkers of respiratory disease. A visual scoring system was used to classify ten crossbred beef steers as sick (with respiratory tract infections) (n=5) and healthy (n=5), from which three healthy and three sick animals were randomly selected. Breath samples were analyzed seven times in 19 days for 15 min at each sampling, and a total of 21 VOCs were detected in cattle breath: heptane, octanal, acetaldehyde, 2,3-butadione, isovaleric acid, decanal, hexanoic acid, phenol, toluene, propionic acid, acetic acid, acetophenone, hexane, isopropyl alcohol, nonane, octane, dodecane, acetone, styrene, tetradecane, and methyl ethyl ketone. Six of the identified compounds, i.e., toluene, octanal, acetic acid, propionic acid, isovaleric acid, and hexanoic acid, were expected to have originated from the

rumen, based on the findings in a separate study of the analysis of the headspace of ruminal culture [51]. To investigate the possibility of differentiating between the sick and healthy subjects, the frequency of detection of all 21 compounds was compared using the Chi-square statistic approach. The presence of acetaldehyde ($p \leq 0.05$) and decanal ($p \leq 0.10$) was associated uniquely with the clinically sick subjects, and heptane, octanal, 2,3-butadione, hexanoic acid, and phenol were associated with the healthy subjects at $P \leq 0.10$ [19].

Peled et al. [18] also used a face mask-like device in their study and compared the VOCs in breath samples collected from 14 cattle infected with *Mycobacterium bovis* (*M. bovis*), from which 10 cattle were tested positive after necropsy. A control group of 13 cattle was used as well. The authors first used GC-MS to identify the VOC patterns linked to the disease, and then based on the detected VOCs, designed and tested a nanotechnology-based array of sensors, termed Nano Artificial NOSE (NA-NOSE), to detect *M. bovis* infection from the breath samples of cattle [18]. An overall of 16 compounds consisting of two ketones, two aromatic compounds, one methylated alkane, one cycloalkane, one ether compound, one alcohol, one benzene derivative, one amine, two dienes, two aldehydes, and two acids were identified through the GC-MS analysis of the breath sample. However, ten compounds identified in the exhaled breath of most cattle could not be statistically associated (the Wilcoxon tests) with bTB infection. From the remaining six compounds, one cycloalkane and one diene were detected in the breath of the majority of infected subjects, and two aldehydes and two acids were found in the breath of infected animals [18].

Bergmann et al. [22] also used a face mask-like device and compared VOCs in the breath of 42 goats (16 controls and 26 animals inoculated with *Mycobacterium avium ssp. paratuberculosis* (strain JII-1961), using needle trap microextraction gas chromatography-mass spectrometry (NTME-GC/MS). Nine substances as potential biomarkers and among these compounds, 2-

butanone, benzene, and 2-methyl-butanal, were shown to have significant differences in concentration between the MAP-inoculated and a non-inoculated group of animals, using the Mann-Whitney-U-tests ($p < 0.05$), having higher concentrations in breath gas of the non-inoculated group [22].

Oretel et al. (2018) [47] argued that animal breath analysis studies lacked the differentiation between the VOCs originating from ruminal gas and investigated the effect of the physiological eructation of ruminants on VOC exhalation. A face mask-like device that covered the mouth and the nose of the cattle was used, and continuous breath profiles were obtained in real-time by means of PTR-MS. Through real-time monitoring, a distinction of different episodes in the breath profiles of ruminants was observed. Subsequently, an algorithm was established to differentiate between the eructation episodes and alveolar breath [47].

*Utility of diagnosis of cattle disease using VOC profiles*

Besides looking into specific biomarkers for the diagnosis of cattle diseases, the variations in VOC profiles of breath samples in sick/inoculated and healthy animals have also been investigated [18, 35, 36]. Purkhart et al. [35] designed and performed a randomized, negatively controlled study on two groups of six goats, which were orally exposed to two different dosages of MAP (*Mycobacterium avium*), and a control group consisted of six goats fed with pure milk replacer. The analyses of VOCs emitted in exhaled breath of goats were performed ten months after inoculation. The subjects were euthanized and necropsied 2 weeks after the DMS determination for accurate diagnosis of the disease. A total of 408 peaks (compounds) were detected and further clustered, subjected to the Mann-Whitney U test due to the non-normal distribution of tested features. The main focus of the study was the differentiation between chemical patterns detected in analyzed breath (and feces headspace samples). It was shown that three of the selected features

were associated with the disease, two increased and one decreased ($p<0.01$) [35]. They were able to find significant differences in the breath VOC profiles in both types of samples prior to the animals showing clinical signs of the disease [35].

Gardner et al. [36] reported the use of the sampling device, reported by Martin et al. [30] to collect breath samples from seven cows of a herd of dairy cattle (five intervention and two control cows) during a two-week period. Three days after the cows were brought to a tie-stall barn and fed equally, the feed for the intervention cows was reduced, and the number of milking per day was increased compared to the control cows for which the feed and milking periods were not changed. To determine the VOCs in breath samples, an array of six semiconducting oxide gas sensors were used, and the responses were modeled by a time-dependent, linear, second-order system. Four characteristic sensor parameters were estimated using a neural network, which was used to train a predictive multilayer perceptron network. The researchers concluded that either a static response parameter (based on the difference in the signal from zero time) or a single time constant could be used to predict the health of the cow, as judged against blood sample, with an identification rate of unknown samples being approximately 76%. The authors believed that the most significant source of error was in the manual sampling of breath rather than the repeatability of the e-nose sensors [36].

Peled et al. [18] used an NA-Nose made of gold nanoparticle (GNP) sensors designed to discriminate the VOC patterns. Discriminant Factor Analysis (DFA) was performed to identify the cattle infected with *M. bovis* from healthy ones [18]. Using a blind leave-one-out cross-validation procedure, the NA-NOSE system successfully discriminated bTB positive animals from other animals with 100% and 79% sensitivity and specificity, respectively [18].

Santos-Rivera et al. (2022) used a different approach, namely near-infrared aquaphotomics, to detect bovine respiratory syncytial virus (BRSV) infection in exhaled breath condensate (EBC) of dairy calves (n=5) undergoing a controlled infection with BRSV [37]. Rather than focusing directly on the changes in the VOC profiles in the breath samples, this group used aquaphotomics to observe the changes in the spectral patterns of water, which reflects the changes in the composition of EBC, including VOCs, due to infection [37]. Using principle component analysis-linear discriminant analysis (PCA-LDA) models, the chemical profiles of samples collected during healthy and infected stages were discriminated with accuracy, sensitivity, and specificity of >93% in both the calibration and validation [37].

Table 1 summarizes the matrices, animal subjects, sampling protocols, devices, sample preparation, analysis, and statistical approaches provided in this review.

Table 1. Animal subjects, sampling protocols, devices, sample preparation techniques, analysis, and statistical approaches in ruminant breath analysis.

| Ref. No. | Disease/ Condition | Sampling protocol | Sampling device | Sample preparation | Instrumentation/ analysis | Statistics/ Data analysis |
|---|---|---|---|---|---|---|
| [32] | Induced ketosis | Samples were taken on day 12 of restricted feed intake from four cows. | Nostril sampling device | Multiple beds of adsorbent materials (40-60 mesh silica gel and charcoal) | TD-GC-MS | Quantification using calibration graphs |
| [30] | Ketosis | Samples were taken from several cows belonging to four different herds. | Nostril sampling device | Silica and carbon-based adsorbents | Gas sensor array, GC-MS, and FTIR | Chemometric techniques (PCA) |
| [34] | Induced ketosis | Samples were taken from 7 cows each morning as well as a background sample from a point at cow head height 1 m in front of the cows. | Nostril sampling device | Silica and carbon-based adsorbents | GC-MS | N/A |
| [36] | Induced ketosis | Samples were taken from seven cows of a herd of dairy cattle (five intervention and two control cows) during a two-week period. | Nostril sampling device | No sample preparation. Sample was discharged directly into six gas sensors. | e-Nose | Time-dependent, linear, second-order system model, neural network, multilayer perceptron network |

| | | | | | | |
|---|---|---|---|---|---|---|
| [31] | Prototyping breath sampling device in cattle | Samples were taken from two heifers on days 1, 3, and 22. | A face mask-like device, HDPE bucket | On-site bovine breath sample collection with SPME. DVB/Carboxen/PDMS 50/30 µm coating, extraction time 15 min | GC-MS | Visual comparison of chromatograms. |
| [19] | Respiratory tract infections in cattle | 10 cows were classified as sick (n = 5) and healthy (n = 5) with a visual scoring system. Of those animals, three healthy and three sick animals were randomly selected. Samples were taken seven times in 19 days for 15 min at each sampling. | A face mask-like device, Stainless steel bucket | SPME, DVB/Carboxen/PDMS 50/30 µm coating, trip blanks, and ambient air samples | GC-MS | Statistical analyses using Chi-square test on the frequency of detection of each VOC in each group |
| [35] | Induced chronic intestinal infection caused by Mycobacteria | Two groups of six goats were orally exposed to two different dosages of MAP (sick), and a control group of six goats fed with pure milk replacer (healthy). Breath sampling started ten months after inoculation. | The tightly fitting face mask used for drug administration was modified. | Inspired breath entered a spacer, and 30 mL gas samples were directly suctioned into the mass analyzer. | DMS | Peak detection, cluster analysis, selection of discriminating VOC features, Mann–Whitney U test, support vector machine |
| [18] | Mycobacterium bovis (*M. bovis*) infection | 14 cattle were classified as sick, from which 10 were tested positive after necroscopy, and 13 animals were used as a control group. | Modified mask designed to deliver nebulized medication to horses | Tenax sorbent | GC-MS and NA-NOSE. | Wilcoxon rank-sum test for GC-MS data, DFA, pattern recognition algorithm for NA-Nose data |
| [20] | Tuberculosis (TB) | Samples were taken from ten experimentally infected cows before experimental infection and at two, three, and five weeks post-infection. | Two sets of breath samplers, one large sampler to cover the whole nose, and a nostril sampler | 50% Tenax and 50% Carbotrap. | ATD-GC-MS | Not applicable |
| [22] | *Mycobacterium avium ssp. paratuberculosis* (MAP) inoculated | Samples were taken from 16 controls and 26 MAP inoculated goats 18, 29, 33, 41, and 48 weeks after inoculation | A face mask-like device and automated alveolar sampling device | NTD | GC-MS | Mann-Whitney-U-tests and PCA |
| [37] | *Bovine Respiratory Syncytial Virus (BRSV) Infection* | Samples were taken from 5 calves | Nasal sampling bag | Condensation at -80°C | NIR aquaphotomics | PCA-LDA |

Table 2 summarizes VOCs identified in bovine breath samples collected from sick (showing visual symptoms or positive-inoculated, S) and healthy (non-inoculated, H, or negative-inoculated, NI) cattle.

Table 2. VOCs (CAS Reference Numbers) identified in breath samples of sick (S), healthy (H), and negative-inoculated (NI) ruminants, Resp = natural or induced upper respiratory tract infections; Keto = natural and induced ketosis; S = showing visual symptoms or positive-inoculated; H = non-inoculated; NI = negative-inoculated.

| **Condition** | **Induced ketosis** | **Ketosis** | **Induced Ketosis** | **Symptoms of respiratory infection*** | **Respiratory tract infections** | ***Mycobacterium bovis*** | **Tuberculosis** | ***Mycobacterium avium ssp. paratuberculosis*** |
|---|---|---|---|---|---|---|---|---|
| Reference | [32] | [30] | [34] | [31] | [19] | [18] | [20] | [22] |
| Number of animals | 4 | ** | 7 | 2 | 5 | 27 | 10 | 42 |
| 1-Methylethyl benzene (98-82-8) | | | | | | Resp | | |
| 1-Propanol (71-23-8) | | | | | | | | Resp |
| 1,3-Dimethylbutyl cyclohexane (61142-19-6) | | | | | | Resp | | |
| 2-Butyltetrahydrofuran (1004-29-1) | | | | | | Resp | | |
| 2,2-Dimethyl undecane (17312-64-0) | | | | | | Resp | | |
| 2,3-Butadione (431-03-8) | | | | | Resp, H | | | |
| Butanal, 2-methyl- (96-17-3) | | | | | | | | Resp, H>S |
| 2,3-Dimethyl, 1,3-pentadiene (1113-56-0) | | | | | | Resp | | |
| 2,4-Hexadiene (592-46-1) | | | | | | Resp | | |
| Acetaldehyde (75-07-0) | | | | | Resp, S | | | |
| Acetic acid (64-19-7) | | | | | Resp | | | |
| Acetone (67-64-1) | Keto, S | Keto, S | Keto, S | Resp | Resp | | Resp | Resp |
| Acetophenone (98-86-2) | | | | | Resp | Resp | | |
| Benzene (71-43-2) | | | | | Resp | Resp | | Resp, H>S |
| Benzothiazole (95-16-9) | | | | | | Resp | | |
| Benzyl alcohol (100-51-6) | | | | | | Resp | | |
| Butan-2-one (78-93-3) | | Keto, S | | Resp, S | Resp | | Resp | Resp, H>S |
| Cyclohexanone (108-94-1) | | | | | | Resp | | |
| Decanal (112-31-2) | | | | Resp, S | Resp, S | Resp, H | | |
| Dimethyl sulfide (75-18-3) | | Keto, S | | | | | Resp | |
| Dodecane (112-40-3) | | | | | Resp | | | |
| Ethylbenzene (100-41-4) | | | | | | | | Resp |
| Heptane (142-82-5) | | | | | Resp,H | | | |
| Hexadecanoic acid (57-10-3) | | | | | | Resp, H>NI | | |
| Hexanal (66-25-1) | | | | | | | | Resp |
| Hexane (110-54-3) | | | | | Resp | | | |
| Hexanoic acid (142-62-1) | | | | | Resp, H | | | |
| Isopropyl alcohol (67-63-0) | | | | | Resp | | | |
| Isovaleric acid (503-74-2) | | | | | Resp | | | |
| Methane (74-82-8) | | Keto, S | | | | | | |
| Naphthalene (91-20-3) | | | | | | Resp | | |
| Nonanal (124-19-6) | | | | Resp, S | | Resp, H | | Resp |
| Nonane (111-84-2) | | | | | Resp | | | |
| Octanal (124-13-0) | | | | | Resp, H | | | |
| Octadecanoic acid (57-11-4) | | | | | | Resp, H, NI | | |
| Octane (111-65-9) | | | | | Resp | | | |
| Pentadecane (629-62-9) | | | | Resp, S | | | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Phenol (108-95-2) | | | | | Resp, H | | | |
| Propionic acid (79-09-4) | | | | | Resp | | | |
| Styrene (100-42-5) | | | | | Resp | | | Resp |
| Tetradecane (629-59-4) | | | | Resp, S | Resp | | | |
| Toluene (108-88-3) | | | | Resp, S | Resp | | | |
| Triethylamine (121-44-8) | | | | | | Resp | | |

Note: * not tested for any diseases.** several from four different herds.

**Discussion**

To date, a limited number of studies have been conducted, with most being focused on the proof-of-concept. Therefore, the classification of VOCs identified in breath samples as biomarkers for diseases is difficult. The diseases studied can be classified into ketosis, respiratory disease, and mycobacterial infections. Among the identified VOCs, acetone is known to be a biomarker for ketosis; however, it was detected in the breath of cattle with respiratory diseases as well, indicating that the mere presence of acetone is not proof of ketosis. Dobbelaar et al. (1996) [32] found a correlation between the concentration of acetone in breath and the concentration of 6-hydroxybutyrate in serum and acetoacetate & acetone in the milk of two ketotic and one non-ketotic cow. However, more studies need to be performed to find a threshold for acetone in the breath that is indicative of ketosis. Among the compounds that were not expected to have originated from the rumen, tetradecane, styrene, acetaldehyde, acetic acid, decanal, dodecane, hexane, nonane, octane, and isopropyl alcohol, were only reported for breath samples of animals with respiratory disease [19, 31] and not in any other studies. However, only the presence of acetaldehyde and decanal was statistically associated uniquely with subjects clinically ill with BRD in one study [19]. Nevertheless, decanal was reported to be present in the breath samples of healthy animals in another study [18]. Three compounds, namely 2-butanone, benzene, and 2-methyl-butanal, were shown to have statistically different concentrations between the MAP-inoculated and a non-inoculated group of animals [22].

The inconsistency in the results can be explained by differences in the sampling and sampling preparation approach, chemical analysis methods, method detection limits, animal species, disease types, number of animals and representativeness, and possible other confounding factors. Many of these differences are summarized and illustrated in Table 1. For example, SPME fiber is known to have limited capacity, and the absorption of chemicals is competitive, whereas NTD is an exhaustive extraction technique. Therefore, while decanal was not detected using SPME in breath samples of healthy animals in one study [19], it was detected when NTD was used in another study [18].

Clearly, some level of experimental protocol standardization and consistent data analysis is needed. Miekisch et al. (2012) classified the most common data analysis related problems in VOC research into three groups: confounding variables (CVs), which have a real correlation with both the diseased state and a breath marker but lead to the erroneous conclusion that disease and breath are in a causal relationship; voodoo correlations (VCs), which can be understood as statistically true correlations that arise coincidentally in the vast number of measured variables; and statistical misconceptions in the study design (SMSD). To avoid these errors, every effort should be made to implement method validation, data cross-testing, and statistical validation [52]. It is highly recommended to approach breath biomarker studies with a multi-disciplinary team consisting of, but not limited to, chemists, engineers, statisticians, veterinarians, and animal scientists.

*Future Applications of VOC Technology*

The various technologies used to measure VOC emissions have shown a wide array of applications. VOC determination has been used to evaluate organ function in humans, monitor food quality and safety, and determine the presence and level of environmental pollutants [53-55]. As mentioned previously in this review, there has also been considerable effort devoted to the

collection and identification of potential biomarkers to be used to identify cattle suffering from an infectious process or an abnormal metabolic state (Tables 1 and 2). These studies have typically utilized blood, serum, breath, and/or fecal samples from individual animals to identify potential disease markers. This approach, while useful in the identification of the abnormal individual animal, has multiple shortcomings in regards to being adapted to population-based decision-making at the farm level.

Once disease biomarkers are identified, the widespread adoption of this technology will depend on multiple factors. The first will be the potential economic return to the livestock producer. This return will be affected by the cost of the test, ease of sample collection, labor availability, and incidence and cost of the disease of interest. Ideally, testing would be targeted at common diseases or abnormalities that have a high economic cost to the production unit or negatively affect animal welfare or food safety. The ability to collect samples and perform the test on-farm, will most likely exclude the current methods utilized to identify breath VOCs. This is simply due to the cost of the equipment, the expertise needed to operate the equipment, and the length of time needed to sample. Therefore, potential biomarkers found in breath samples need to be present in other easily sampled fluids such as saliva, feces, milk, blood or nasal secretions.

In large beef and dairy production units, cattle must be handled humanely and efficiently in order to maximize animal comfort and optimize input costs. There are instances where individual handling and testing may not be timely or feasible in these production units. For example, running feedlot cattle through the processing facility multiple times in order to collect diagnostic samples for testing is neither humane nor efficient. Increasing the level of handling stress on dairy animals prior to milking can lead to milk loss and increase the incidence of mastitis. Gierschner et al. (2019) used PTR-MS in real-time to determine VOCs in different groups of dairy cows prior to milking

[43]. The changes in VOC emissions were related to differences in average milk yield per group, time of day milking occurred, and the presence of cows that were infected with paratuberculosis. These results indicate that PTR-MS can be used to monitor VOC emissions from groups of cattle in order to evaluate their health status and metabolic state. While not able to individually identify which cows were afflicted with paratuberculosis, this non-invasive monitoring would serve to narrow the population that would be targeted for individual testing [43].

Both the biomarkers and the testing modality must have a high degree of sensitivity and specificity for the disease condition of interest. This will ensure that a high proportion of affected animals are identified, and normal animals are not unnecessarily treated. Enzyme-linked immunosorbent assays (ELISA) are typically easy to perform and can provide a high level of accuracy [57]. Considering the vastly different environments and production systems utilized in animal agriculture around the world, testing modalities will need to accurately identify animals of interest over extremes of temperature, humidity, rainfall, and season of the year. Also, the actual "shelf life" and shipping requirements of the tests will be critical in the development of the widespread adoption of testing. As animal production moves into the era of precision agriculture, the ability to accurately identify abnormal animals in an efficient manner will enhance animal welfare, disease prevention practices, food safety for the consumer, and improve profitability for the owner [58]. A futuristic concept of real-time monitoring for disease biomarkers in barn air is presented in Fig. 9.

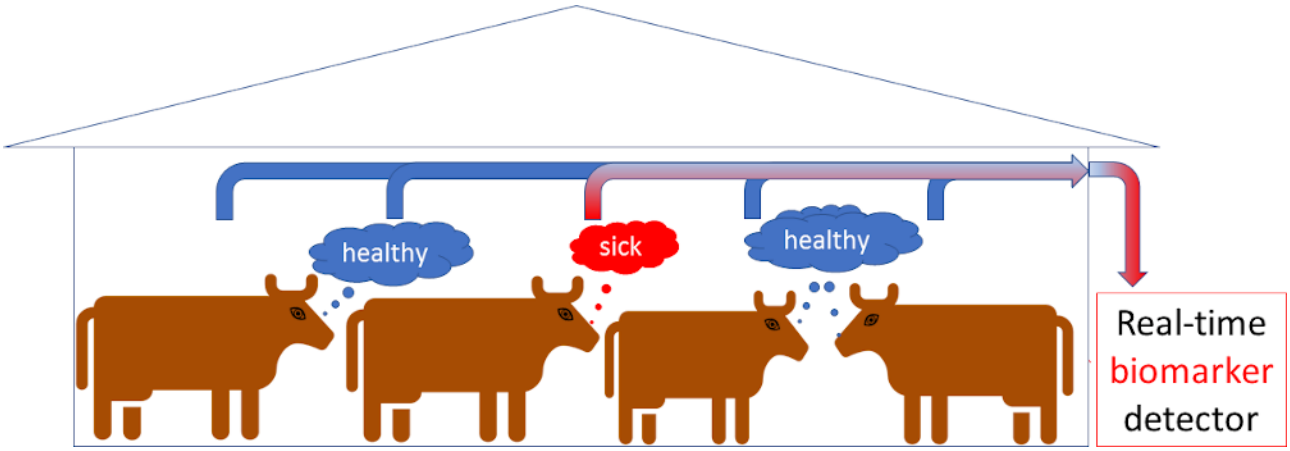


Fig. 9 A concept for real-time monitoring for disease biomarkers in barn air. Biomarker sensing is part of technology-driven precision livestock agriculture.

There can be considerable cost savings associated with improved diagnostic accuracy in cattle feeding operations. Calves identified early in the disease process respond to treatment faster, have fewer relapses, and have better feeding performance. Feedlot studies have clearly shown the substantial negative economic effects of treatment failure on antibiotic costs, average daily gain, carcass traits, and death loss [59, 60]. Groups of calves with an unknown health history, hauled over long distances, and subjected to high-stress levels, are often "mass treated" with an antibiotic at feedlot arrival. This treatment, referred to as metaphylaxis, is utilized because these groups consistently run a high level of morbidity and mortality [61]. While this practice is expensive, it does improve labor allocation and animal welfare as fewer animals need to be re-treated. However, many of these calves do not need treatment, but metaphylaxis is used due to the expected high level of BRD in the group and the inability to individually differentiate healthy calves from those that will break with BRD over the next 3-7 d. In these cases, a "chuteside" diagnostic test that could be used to differentiate "sick" from "well" calves at feedlot arrival could improve antibiotic stewardship and decrease input costs.

**Conclusion**

There is clearly a need in the cattle industry for improved disease diagnostics. With the relatively low accuracy at which the clinical signs (Depression, Appetite, Respiration, Temperature – DART methodology) correctly identify animals with bovine respiratory disease (BRD) comes the need for improved sensitivity and specificity. The current studies have shortcomings in regards to defining useful VOC profiles, the impact on animal welfare, and the practical application at the producer level. Improvements in this area would contribute greatly to the judicious use of antimicrobials and improve antibiotic stewardship by cattle producers and their veterinarians. All major cattle veterinary and producer groups in the US have endorsed policies that call for antibiotics to be utilized only for valid reasons, after considering alternatives, and within the confines of a valid veterinarian-client-patient relationship (VCPR) [62-64]. A key component to all of these policies is obtaining an accurate diagnosis of the disease condition so that the appropriate treatment for the individual and preventative measures for the herd can be instituted. Improved diagnostic methods should lead to more precise antibiotic regimens that will better target a specific organism or complex and decrease overall antimicrobial use. This will lead to less selection pressure for the development of antimicrobial resistance and improved food safety and consumer acceptance.

As summarized in this review, research on the possibility of a rapid and reliable diagnosis of BRD has focused on developing sampling devices, sample preparation, and analytical and statistical methods in breath and barn air (Tables 1 and 2). This rationale was based on the belief that disease will produce specific VOC biomarkers characteristic of the infection or metabolic abnormality. The challenges of these studies include the elimination of background impurities from ambient air, differentiating ruminal gas from exhaled breath, the efficacy of sorption/desorption onto/from sampling devices, and sample loss during storage. Overall, on-site detection and analysis are

favored; however, the limitations appear to be the chemical detection limits, ruggedness, size & portability of equipment, availability of electric power, sample throughput, and the speed of detection consistent with the practice times available to process and handle cattle. Furthermore, since certain diseases (e.g., bTB and rabies) may be transmitted to humans, the sampling and analysis must be designed in a way to minimize human exposure to the possible exiting pathogens in the breath. Differentiation between patterns of VOCs in sick vs. healthy requires sophisticated statistical support. The rapidly developing artificial intelligence field offers help with recognizing patterns, process simplification, and automation.

The results of currently available studies were not indicative of consistent specific biomarkers for BRD, and further investigation is required using the provided methods in better controlled clinical studies. While the desired end-point is an animal-side detection, there is still a need to develop reliable sampling devices and analytical instrumentation capable of relatively fast detection and identification of specific biomarkers. The GC-MS-based identification will likely be still required for the determination of VOCs in controlled clinical trials.

**Acknowledgments**:

Jacek A. Koziel's participation was partially supported by the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa. Project no. IOW05556 (Future Challenges in Animal Production Systems: Seeking Solutions through Focused Facilitation) sponsored by Hatch Act & State of Iowa funds.

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