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Comparison of the suitability of different sampling techniques for exhaled volatile organic compounds in dairy cows

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Abstract

Currently, there are no standardized procedures for sampling exhaled volatile organic compounds (VOCs) from dairy cows. Therefore, this study aimed to compare exhaled VOCs captured on solid-phase extraction (SPE) cartridges using five variants of three breath collection devices (face mask and GreenFeed system [C-Lock, South Dakota, US] collecting unfiltered [$GreenFeed_U$] and filtered [$GreenFeed_F$] air). The variants were:

- a tight-fitting face mask ($Mask_N$),
- the $Mask_N$ with the openings sealed using activated carbon filters ($Mask_F$),
- the $Mask_N$ covered with an over-mask ventilated with synthetic air for cow breathing ($Mask_V$),
- the $GreenFeed_U$, and
- the $GreenFeed_F$.

The variants were compared in two experiments (trial registration number (2023-30-FR) regarding possible VOC carryover over the samples (Experiment 1) and their suitability for sampling exhaled VOC from cows (Experiment 2). In both experiments, the SPE cartridges were connected to capture VOCs from collected air before GC-MS-based analysis. In Experiment 1, our data showed evidence for VOC deposits and potential VOC carryover, particularly for $GreenFeed_U$ (16.3%). In exhaled breath samples from Experiment 2, we detected 1217 ± 197 peaks. After subtracting the background air peaks, the exhaled VOCs consisted mostly of esters (20.9%), ketones (13.2%), and alkanes (13.0%). $Mask_V$ detected the highest number of aldehydes, ketones, alcohols, alkanes, and alkenes, and $GreenFeed_U$ the highest number of esters. The highest relative concentrations of most individual exhaled VOC were detected using $Mask_V$. The tested variants, except $Mask_F$ due to low acceptance of the animals, seemed suitable for exhaled VOC sampling, with $Mask_V$ seemed to be most suitable due to the detection of the highest VOC number and the lowest VOC carryover.

1 Introduction

Exhaled breath from animals and humans are used for the low-invasive sampling of volatile metabolic end products, namely volatile organic compounds (VOCs). VOCs are a heterogeneous group of organic substances, including carboxylic acids, alcohols, aldehydes, ketones, and terpenes, with molecular weights ranging between 50–400 Da and boiling points ranging from 50°C to 250°C [1]. The methodology for routine sampling of VOCs which was originally designed for the detection of environmental exposures [2], is already well established in human exhaled breath analysis. These methods are now widely applied in diagnostics, for instance, in disease biomarker monitoring such as for diagnosing various types of sugar malabsorption [3], for “illicit drug consumption” or for testing breath alcohol [4]. Humans are instructed to exhale into a mouthpiece, which facilitates the collection of exhaled breath and reduces contamination from environmental VOCs. A wide range of detection methods is employed in human breath analysis, including GC-MS [5], quadrupole systems [6], Fourier Transform Infrared spectroscopy [7], and other high-resolution mass spectrometry techniques, reflecting the high degree of methodological development in this field.

In contrast to humans, the optimal device and workflow for exhaled VOC sampling and analysis from animals - particularly cattle - remain to be determined, as bovine breath research is now gaining increasing attention and application. In cattle, exhaled VOCs and gases such as methane and carbon dioxide provide information about ingested feed [8], rumen fermentation, digestive efficiency [9], and metabolic status [9, 10]. In contrast to humans, the optimal device for exhaled VOC sampling from animals, particularly cattle, remains to be determined.

However, exhaled VOC sampling from cattle presents specific challenges. Unlike humans, cattle cannot be instructed to follow breathing commands, which makes breath collection technically demanding. Additionally, there are high levels of environmental VOC contamination to consider, and a balance must be struck between manual handling and the automation of sampling procedures. This highlights the need to evaluate not only the technical performance but also the practicality, consistency, and animal acceptance of different sampling approaches. In the literature, exhaled breath sampling from cattle has already been performed using whole-animal chambers, the GreenFeed (C-Lock, Concourse Drive Rapid City

South Dakota, US) system [9], ventilated hoods, whole-face masks, face attachments [11], with face masks being the mostly applied method [5, 12].

Given the challenging barn environment, with its high load of concentrated VOCs, exhaled breath collected using the mentioned devices could be subject to contamination [13]. Furthermore, deposited VOCs, or VOCs adsorbed onto the sampling device or equipment have not been studied yet. In this study, we aimed therefore to compare five variants of two sampling devices – the GreenFeed system and the face mask - for exhaled VOC collection from dairy cows. We selected these two devices for this comparison as they do not collect the total volatilome but instead focus on exhaled VOCs. As the GreenFeed system conventionally used for automatic methane and CO_2 analysis, it has been applied only rarely to VOC sampling [8]. However, it offers a promising, minimal invasive option, as cows voluntarily access it to receive bait feed, thus allowing sample collection without manual intervention.

These five variants for collecting exhaled VOCs from dairy cows were connected to polymer-based solid-phase extraction (SPE) cartridges for further untargeted VOC analysis. The latter method was established in our previous research for sampling exhaled VOC from dairy cows [5]. Specifically, the breath collection variants should be compared regarding (1) possible VOC carryover between cows and (2) their suitability for sampling exhaled VOC from dairy cows evaluating the number, chemical compound groups, and concentrations of detected VOCs.

2 Materials and Methods

2.1 Animals and housing

The experimental protocol complied with Swiss animal welfare legislation and was approved by the Animal Care Committee of the Canton Fribourg, Fribourg, Switzerland (license no. 2023-30-FR). The experiment was conducted as part of a larger feeding study at the experimental farm of Agroscope (Posieux, Switzerland). This experiment was divided into two sub-experiments (Exp1 and Exp2).

In Exp1, four healthy, multiparous (2nd and 3rd lactation), lactating (33.92 ± 4.69 kg milk per day) Holstein Friesian cows (94.25 ± 35.5 DIM) were used. The cows were fed a silage-based diet *ad libitum* (mainly 38.06% corn silage, 32.30% sorghum silage, 11.73% hay, and 10.87%

potato) and concentrates containing a mineral-vitamin premix to meet the requirements of a dairy cow with a production potential of 30 kg d^{-1}.

In Exp2, six healthy, multiparous (2nd and 3rd lactation), dried-off Holstein Friesian dairy cows were included, which were at the time of sampling at day 46.5 ± 8.6 before calving. Two cows were fed a partial mixed ration (20.55% corn silage, 21.78% grass silage, 26.48% hay, and 31.19% straw) ad libitum and concentrates according to recommendations for transition cows. The other four cows were fed an energy-richer diet consisting of a partial mixed ration (29.93% corn silage, 31.58% grass silage, and 38.50% hay) and concentrates. During both experiments, the cows were housed in a tie-stall, with only every second place occupied, and free access to fresh water.

2.2 Breath collection variants

For sampling exhaled breath and barn air, five different breath collection variants were used (Figure 1; Supplementary Figure S1):

(i) a tight-fitting face mask originally produced for horses (**$Mask_N$**; Figure 1; Supplementary Figure S1, II; Air One, Hippomed/Neu-Tec GmbH, Steinhagen, Germany);

(ii) **$Mask_N$** with the openings sealed using activated carbon filters (**$Mask_F$**; Figure 1; Supplementary Figure S1, III) aimed to filter and reduce barn VOCs [11];

(iii) **$Mask_N$** covered with a self-sewn over-mask (Supplementary Figure S2; Backpack fabric, marine, No. 1274, polyester waterproof, 210 g m^{-2}, Alja, Bern, Switzerland), ventilated with synthetic air for cow breathing (PanGas AG, Dagmersellen, Switzerland; air flow of 40 L min^{-1} determined by a flow meter [SFAH 50 U, Festo, Lupfig, Switzerland]) to reduce the inhalation of barn VOC **($Mask_V$**; Figure 1; Supplementary Figure S1, IV);

The GreenFeed system (C-Lock, Concourse Drive Rapid City South Dakota, US), which collects air using an internal airflow mechanism that extracts a mixture of exhaled breath and barn air with a dilution factor of approximately 1:40 for exhaled breath with barn air. The surrounding air is sucked into the GreenFeed system and then directed through a dust filter. For air sampling, we used two locations within the GreenFeed system:

(iv) before the dust filter to collect unfiltered air (**GreenFeed$_U$**; Figure 1; Supplementary Figure S1, V, red circle), and

(v) the GreenFeed system with VOC sampling conducted after the dust filter to collect potentially filtered air (**GreenFeed$_F$**; Figure 1; Supplementary Figure S1, VI, red circle; [8]).

These five breath collection variants were connected sequentially to our developed sampling system (Supplementary Figure S1, I), as previously described by Eichinger et al. [5], which was further optimized. In brief, the collected air (irrespective of the breath collection variant) was pumped by a vacuum pump (V-300 coupled with an interface I-300, Büchi, Flawil, Switzerland) from the breath collection variant through the collection devices, an internal standard bottle (100 ppb dimethylsulfide-d6, 10 ppb dimethylsulfoxide-d6 in acetonitrile), and simultaneously through two SPE cartridges. The pump reached approximately 180 mbar during sampling, with an enrichment flow of approximately 4.0 L min^{-1}. To minimize VOC contamination from the tube material, we used polytetrafluoroethylene tubes connected by tube connectors in polytetrafluoroethylene material (both Festo, Lupfig, Switzerland). The SPE cartridges used were the Chromabond HR-XAW cartridges (XAW; Macherey-Nagel, Oensingen, Switzerland), which contain polystyrene-divinylbenzene copolymer with an additional secondary weak anion exchanger favoring the broad coverage of cow-derived exhaled VOCs [5]. After each air collection, the internal standard bottle and the two SPE cartridges were replaced with new ones.

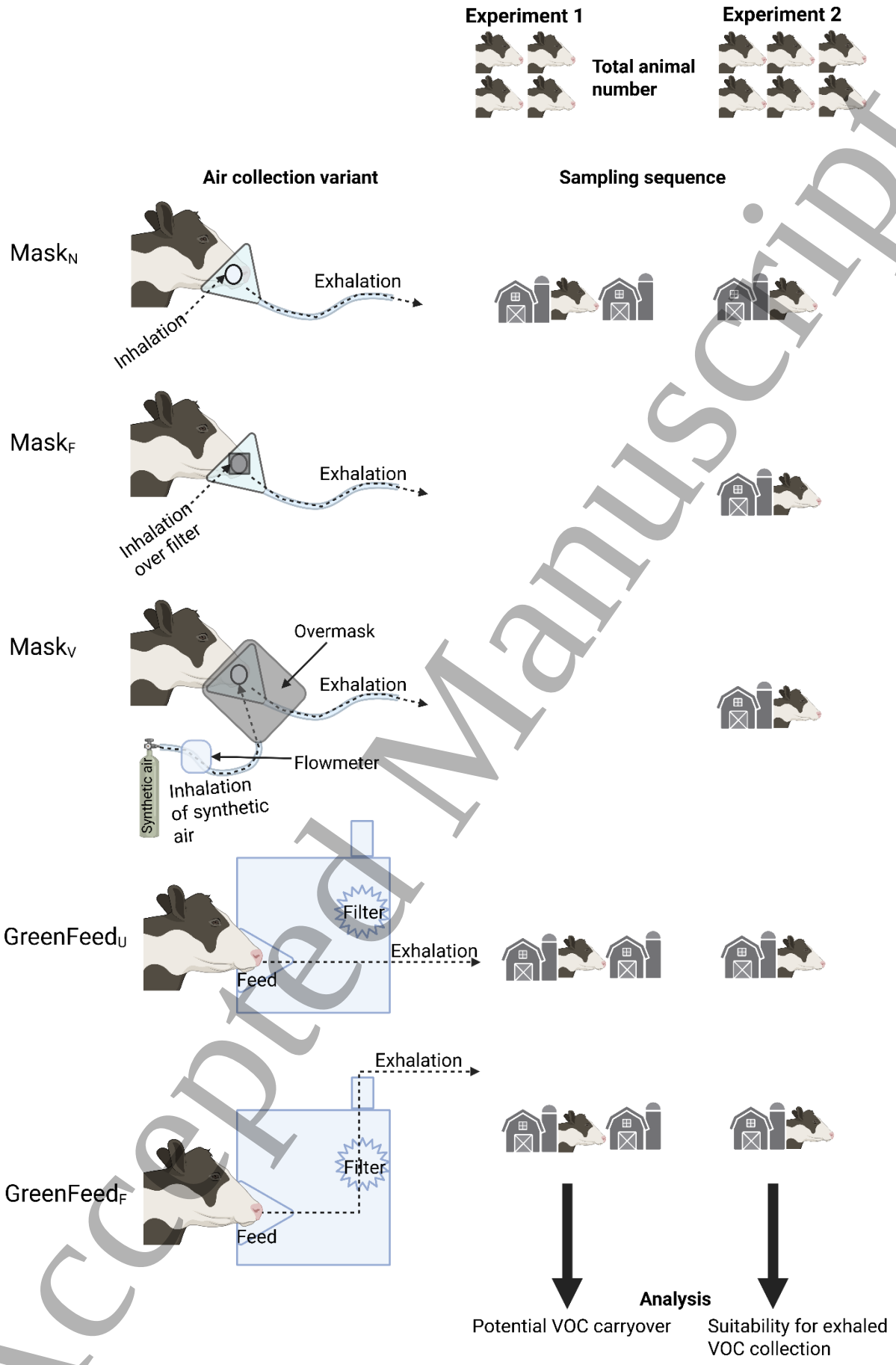

Figure 1: Sampling exhaled breath from dairy cows and barn air in experiment one (analyzing potential VOC carryover) and experiment two (analyzing the suitability for exhaled VOC collection). The sampling system [5] (consisting of an internal standard bottle [100 ppb dimethylsulfide-d6, 10 ppb dimethylsulfoxide-d6 in acetonitrile], two SPE cartridges and a vacuum pump [180 mbar] connected with polytetrafluoroethylene tubes) was connected to different breath collection variants: a tight-fitting face mask ($Mask_N$), the face mask with the openings sealed using activated carbon filters ($Mask_F$), the face mask covered with an over-mask ventilated with synthetic air for cow breathing ($Mask_V$), and the GreenFeed system with air sampling conducted before the filter ($GreenFeed_U$) or after the filter ($GreenFeed_F$). The figure was created using BioRender.com.

2.3 Sample collection

2.3.1 Sampling materials

To analyze VOCs originating from the sampling materials, an unused segment of each carbon filter, the over-mask, the Teflon tube, and the XAW polymer material (232 ± 5.68 mg) were collected. The sampling materials were transferred separately to a 20 mL headspace vial together with 100 µL of Milli-Q water, hermetically sealed with a silicone/Teflon septum (Macherey-Nagel AG, Switzerland), and stored until VOC analysis.

2.3.2 Exhaled breath and background air

Air collection took place at the tie-stall. The tie-stall was chosen to assess the effectiveness of our breath sampling methods in minimizing environmental VOC contamination under conditions that are representative of typical housing and research settings for dairy cows. The feed for each cow was removed 1 h prior to sampling. During collection, the cows remained in their positions in the tie-stall. The mask was held by the experimenter, and the GreenFeed system was positioned in front of the cow.

Experiment 1 (Exp1). Exhaled breath and barn air samples were collected on December 19, 2023 from 09.00 h to 14.00 h (outdoor air temperature: 6.4°C; relative humidity: 95.8%; data from [forecast meteo.ch]). At the beginning of the day (09.00 h), background air was collected twice using the three different breath collection devices in the following order: $Mask_N$, $GreenFeed_U$, and $GreenFeed_F$. Subsequently, exhaled breath was collected from three dairy cows. For breath collection, the devices ($Mask_N$, $GreenFeed_U$, $GreenFeed_F$) were used in a randomized order, except for $Mask_N$, which was used as the first device for each cow. This was done to prevent VOC contamination from bait feed from the GreenFeed system into the $Mask_N$-collected breath samples. After every breath sample, a post-breath-collection air sample (background air after exhaled breath sampling of each cow) was collected (Table S1). The data from the fourth cow were excluded from this experiment and are not discussed

further, as she was not optimally sampled due to nervousness. After each exhaled breath collection, the $Mask_N$ was rinsed with water and dried with paper towels.

Experiment 2 (Exp2). Background air and exhaled breath samples were collected on three days in January 2024 between 07.00 h and 10.00 h (three cows on 12/01/2024, two cows on 22/01/2024 and one cow on 29/01/2024) (outdoor air temperature: -3.4, 3.4, 3.7°C, respectively; relative air humidity: 99.3, 84.7, 87.4%, respectively [forecast meteo.ch]). Five background air samples were collected sequentially using all five breath collection variants ($Mask_F$, $Mask_N$, $Mask_V$, $GreenFeed_F$, $GreenFeed_U$; Figure 1). Afterwards, five exhaled breath samples were collected from each cow using the same order of collection variants. After each sample was collected with a variant of $Mask_N$, the $Mask_N$ and over-mask were rinsed with water and dried with paper towels.

2.5 Sample preparation

A 24-position SPE vacuum manifold (Chromabond, Machery-Nagel, Oensingen, Switzerland) was used for the pre-conditioning, drying, elution, and cleaning of the SPE cartridges. Prior to sampling, the SPE cartridges were conditioned with 3 × 3 mL Milli-Q water, 3 × 3 mL methanol, 3 × 3 mL acetone, and 3 × 3 mL acetonitrile (all purchased by Merck, Buchs, Switzerland) as described in Eichinger et al [5]. All 24 cartridges were dried at the same time for 15 min under 10 L min^{-1} N_2 flow (~416 mL min^{-1} N_2 for each single cartridge). The SPE cartridges were processed within 2 h after air sample collection. In the laboratory, the SPE cartridges were dried under N_2 flow for 3 min. Then, to elute the captured VOCs from the SPE polymer, 600 µL of acetonitrile were added for 5 min on the SPE polymer. Subsequently, the VOCs dissolved in acetonitrile were flushed with a slight vacuum at 800 mbar using a SPE vacuum chamber (Chromabond, Machery-Nagel, Reinach, Switzerland) into 2 mL amber glass vials, which were stored at -40°C until VOC analyses. No chemical differences were found between samples measured directly after elution with acetonitrile and eluted samples stored for a period of 21 d at −40°C, as tested within pretests in our laboratory.

2.6 Analysis of volatile organic compounds using DHS-V-ITEX-GC-MS

In this study, we were sampling and analyzing VOCs with retention times between those of n-hexane (C6) and n-hexadecane (C16), which corresponds to the conventional VOC range targeted by many GC-MS-based methods [14]. For VOC analysis, the glass vials were thawed at room temperature for 2 h, and 100 µL of the VOC eluate were pipetted into 20 mL headspace vials. The latter and the vials containing the sampling material segments were placed on a tray cooler at 4°C and were analyzed immediately using Dynamic Headspace Vacuum In-Tube Extraction GC-MS (DHS-V-ITEX-GC-MS), as described by Fuchsmann et al. [15]. The V-ITEX system comprised a MPS PAL autosampler (Gerstel, Sursee, Switzerland), ITEX-Tool, ITEX syringe, ITEX trap filled with Tenax TA/Carbosieve SIII, 80/100 mesh sorbent material (all by CTC Analytics, Zwingen, Switzerland), a vacuum pump operating at 1500 Pa (V-300 coupled with interface I-300, Büchi, Flawil, Switzerland) and a V-ITEX valves controller (for details see Fuchsmann et al. [15]). The samples were incubated for 10 min at 60°C and 500 rpm shaking prior to the extraction process. The extraction was conducted for 10 min at 60°C with 800 rpm shaking. The extracted VOCs were desorbed with helium (Carbagas, Gümligen, Switzerland) at a flow of 406 mL min^{-1} for 2 min at 300°C into Cooled Injection System 4 (CAS4) at 10°C equipped with a Tenax TA-filled liner (Gerstel, Sursee, Switzerland). GC 7890 B and MS 5977 B equipped with a high efficiency source (HES) were purchased from Agilent (Santa Clara, USA). The CAS was operated in solvent vent mode with a purge flow to split vent of 130 mL min^{-1} at 2 min and a vent flow of 10 mL min^{-1}. The transfer line was maintained at a temperature of 280°C, the ion source of 230°C, and the quadrupole at 150°C. For the separation of the analytes, an Optima 5 MS capillary column (30 m × 250 µm × 0.25 µm, Machery-Nagel, Reinach, Switzerland) was operated with a column flow of 0.95 mL min^{-1} using helium as carrier gas and the following oven program: the temperature was held for 6 min at 40°C and then it was increased with a rate of 10°C min^{-1} until it reached 280°C. During the analysis, two laboratory blanks (laboratory air) were analyzed per batch to determine VOC contamination from the analysis procedure.

2.7 Data processing and identification of volatile organic compounds

The MS signals were deconvoluted using Masshunter Profinder software in recursive mode (version 10.0, Agilent Technologies, Santa Clara, CA, USA). Following automatic deconvolution,

any missing values resulting from signals below the detection limit (calculated in the deconvolution process) were replaced with zero values, following Xia et al. [16]. The mean peak areas of the two laboratory blanks per batch were subtracted from the peak areas of the sample VOCs of the same batch and are not discussed further, as they were considered artifacts originating from the laboratory air, vials, vial caps, ITEX trap, or the GC column. Manual peak integration was conducted using MassHunter Quantitative Analysis software (version 12.1; Agilent Technologies, Santa Clara, CA, USA). The peak area was calculated for the two sampling duplicates, and their averages were used for further data analysis. VOCs from exhaled breath samples were corrected for background VOCs by subtracting the peak area of background air VOCs from the peak area of exhaled breath VOCs. VOCs from exhaled breath exceeding the VOC concentrations in background air by at least 50% were considered exhaled VOCs [17]. The retention index (RI) was calculated using the temperature-programmed Kovats retention index [18] with alkanes for the method RI references. The VOCs were identified following the standard criteria for identification levels (Levels 1–4), as recommended by the Metabolomics Standards Initiative [19]. At the first identification level, a metabolite is identified by comparing its spectrum with a database (minimum match factor of 90%) and the calculated RI with the reference RI (maximum relative difference of ± 15). A metabolite identified at Level 2 presents a spectrum with a match factor greater than 80% and a maximum relative difference in the calculated RI of ± 15 of the reference RI. At Level 3, metabolites are assigned to their respective compound classes based on their similarities with the compounds in a reference library. Level 4 corresponds to unknown compounds with a calculated RI that differs by > ± 15 from the reference RI [19, 20]. The following peak identification strategies were performed using the National Institute of Standards and Technology NIST/EPA/NIH mass spectral library (NIST17, Gaithersburg, MD, USA):

i) To determine the number and chemical compound groups of VOCs captured by the SPE cartridges, VOCs identified at least at Level 3 (hereafter referred to as Level 3 VOCs) were included.
ii) For the determination of individual exhaled VOCs captured by the SPE cartridges, both Level 1 and Level 2 identified VOCs were considered (hereafter referred to as individual exhaled VOCs).

The VOC analysis is semiquantitative; thus, the reported VOC concentrations in the text refer to relative concentrations (relative to the maximal detected peak area) determined from the peak area of the VOCs (arbitrary unit). Only descriptive analysis was performed in this study, and no statistical tests were conducted.

3 Results and discussion

The objective of this study was to evaluate the suitability of five breath collection variants for onsite exhaled breath sampling from dairy cows connected to SPE cartridges to capture the VOCs from the collected exhaled breath.

3.1 Volatile organic compound contamination from sampling material, background, and deposits

The number of detected GC-MS peaks from the unused, cleaned sampling materials were as follows (mean ± standard deviation): carbon filter: 14 ± 2.83, over-mask: 28 ± 4.24, Teflon tube: 3 ± 0.7, and HR-XAW polymer: 4 ± 2.1. These results indicate that the materials used, particularly the over-mask, released some VOCs. Therefore, it was important to correct the measured VOCs using the corresponding background VOCs. In addition to VOC emissions from the sampling materials themselves, it is also crucial to assess possible VOC deposits within the sampling devices, which could lead to VOC carryover from one sample to another. To investigate this, data from Experiment 1 were used to compare the number of detected GC-MS peaks in barn air samples collected either before (background air) or after (post-breath-collection air) sampling exhaled breath from an animal (Table 1). The difference—i.e., the subtraction of background air peaks from those in post-breath-collection air—provides an estimate of VOCs possibly originating from deposits in the sampling system (potential VOC-deposits), representing a potential risk of VOC carryover between samples.

Table 1: The total number of GC-MS peaks in the barn air before (background air) and after collecting exhaled breath from three individual cows (post-breath-collection air) as well as the resulting calculated potential VOC deposits within the different breath collection devices (mean ± coefficient of variation).

	Breath collection devices		
	$Mask_N$	$GreenFeed_U$	$GreenFeed_F$
Background air	1194 ± 0.01	1230 ± 0.01	1232 ± 0.01
Post-breath-collection air	1183 ± 0.01	1240 ± 0.03	1270 ± 0.03
Potential VOC deposits (Δ Post-breath-collection air – background air)	118 ± 0.17	200 ± 0.53	149 ± 0.17

$Mask_N$: tight-fitting face mask, $GreenFeed_U$: the GreenFeed system with air sampling conducted before the filter, $GreenFeed_F$: GreenFeed system with air sampling conducted after the filter.

Thes GC-MS peak counts differed between the three sampling devices. A high number of peaks in the background air was detected using all three breath collection devices with the highest number of peaks (mean ± standard deviation) was detected using the variants of the GreenFeed system ($GreenFeed_F$: 1232 ± 5.6; $GreenFeed_U$: 1230 ± 2.5), followed by $Mask_N$ (1194 ± 10.2). The high number of peaks detected using GreenFeed can be attributed to the GreenFeed being a more open system (ratio exhaled air:surrounding air 1:40). This may possibly result in sampling more background VOCs compared to $Mask_N$, which provides less contaminated sampling of VOCs by the accumulation of exhaled breath in $Mask_N$. An alternative reason might be that the GreenFeed system involves offering bait feed to the animals, which possibly contaminates exhaled breath samples with feed VOCs.

The post-breath collection air sample contained similar numbers of peaks to the background air samples, but many of its peaks were not present in the background air (Table 1). Those potential VOC-deposits (Δ post-breath-collection air – background air) consisted mainly of esters (21.3%), alcohols (12.2%), alkanes (11.2%), alkenes (10.7%), ketones (10.2%), ethers (8.24%), amines (5.57%), azoles (5.33%), and carboxylic acids (2.55%). This was particularly pronounced for $GreenFeed_U$ samples, in which 16.3% of the detected peaks were not present in background samples, followed by $GreenFeed_F$ (12.1%) and $Mask_N$ (9.88%) samples. These potential VOC deposits on the material of the sampling devices could lead to VOC carryover between cows. Particularly susceptible to VOC deposits are porous materials, filters, plastics, untreated metals, materials with large surface areas, and areas with the presence of dust [21]. A potentially more pronounced VOC carryover using the GreenFeed system may be related to

its larger surface area compared to the $Mask_N$ system. This includes the feed trough from which air is drawn, which is comparable to $Mask_N$ samples in terms of surface exposure. The internal components of the GreenFeed system, including the air filter through which the air is subsequently transported, present potential surface areas for more VOC adsorption. Additionally, a significant proportion of GreenFeed system surfaces are inaccessible for cleaning with water and drying.

As mentioned earlier, VOCs released from bait feed could contribute to an increased number and concentration of VOCs potentially depositing in the GreenFeed filter and being released again later. Such VOC deposition or release could alter the VOC profile in the airflow downstream of the filter, potentially changing the composition of the sampled VOCs before ($GreenFeed_U$) and after filtration ($GreenFeed_F$). We did not identify the 467 (118 + 200 + 149) peaks, which can likely be considered deposited VOCs. However, all the post-breath-collection VOCs were present in exhaled breath samples after correcting for background air peaks [17], but in the latter samples in at least 1.5 times greater concentrations. Despite their relatively low concentrations, VOC deposition on the sampling material may have increased the concentrations of these VOCs in the subsequent sample.

3.3 Exhaled volatile organic compounds

To compare the suitability of the five sampling variants for the collection of exhaled VOCs from cows, the data from Experiment 2 were used. The mean number of GC-MS peaks detected in exhaled breath samples from Exp2 was around 1218. The numbers are comparable with the number of peaks detected in our previous study [5] using $Mask_N$ to sample exhaled VOCs from dairy cows. The number of GC-MS peaks varied by breath collection variant and among the cows sampled (Table 2). Breath samples collected with $GreenFeed_U$ contained the highest mean peak number (+ 11–15% compared to the other variants), followed by $Mask_N$, $Mask_F$, $GreenFeed_F$, and $Mask_V$ samples. The greater number of peaks in $GreenFeed_U$ (+13.93 ± 0.32%) compared to $GreenFeed_F$ samples suggests that exhaled VOCs may either remain attached to the GreenFeed filter or undergo a reduction in concentration, which, unsurprisingly, aligns with the intended function of a filter. As an alternative to air filtering, background VOCs may be reduced by supplying synthetic air as inhaled air for the animal, as demonstrated by the

reduced number of peaks observed using $Mask_V$. To some extent, supplying synthetic air permits the separation of the barn environment from the exhaled breath and the sampling process. The number of GC-MS peaks in exhaled breath samples was corrected using the respective background air samples to determine the exhaled VOCs. Specifically, VOCs were considered exhaled VOCs if their peak areas exceeded those of the background air by at least 50% [17] (Table 2 and Table 3).

Table 2: The total number of GC-MS peaks detected in the exhaled breath and background air samples from six cows using the five breath collection variants as well as exhaled breath peaks exceeding background air peaks by at least 50% as considered exhaled VOCs (mean ± coefficient of variation).

	Breath collection variants				
Number of peaks detected	$Mask_N$	$Mask_F$	$Mask_V$	$GreenFeed_U$	$GreenFeed_F$
Exhaled breath samples	1209 ± 0.08	1197 ± 0.06	1165 ± 0.07	1341 ± 0.31	1177 ± 0.08
Background air samples	1210 ± 0.01	1220 ± 0.03	1161 ± 0.01	1231 ± 0.01	1232 ± 0.01
Exhaled VOCs	512 ± 0.29	539 ± 0.36	596 ± 0.37	541 ± 0.30	516 ± 0.28

$Mask_N$: tight-fitting face mask, $Mask_F$: the face mask with the openings sealed using activated carbon filters, $Mask_V$: the face mask covered with an over-mask ventilated with synthetic air supply for cow breathing, $GreenFeed_U$: the GreenFeed system with air sampling conducted before the filter, $GreenFeed_F$: GreenFeed system with air sampling conducted after the filter, exhaled VOCs: VOCs from exhaled breath samples were considered exhaled VOCs when they exceeded background air peaks by at least 50% [17].

The exhaled VOCs were identified at Level 3 (Table 3), meaning they were assigned to their respective compound classes based on mass spectral similarity [19, 20]. Overall, about 567 Level 3 exhaled VOCs of 15 chemical compound groups were identified, accounting for about 45.1% of all detected VOCs in exhaled breath samples. Esters (20.9%) were the most prevalent, followed by ketones (13.2%), alkanes (13.0%), alkenes (10.2%), alcohols (7.23%), amides (4.70%), amines (4.61%), ethers (3.94%), azoles (2.90%), carboxylic acids (2.39%), aldehydes (2.09%), nitriles (1.76%), pyridines (1.37%), and alkynes (0.39%) (Table 3). The proportions of the most detected chemical compound groups were comparable to those reported by Eichinger et al., who used $Mask_N$ to sample exhaled VOCs from dairy cows [5]. However, in the present study, a higher number of amides (+81.6%), amines (+68.4%), carboxylic acids (+68.6%), esters (+72.6%), and ketones (+66.7%) were identified. These differences may be attributed to the use of XAW cartridges in the present study, which have a higher sensitivity

to capture ketones. An alternative reason might be variations in metabolism, potentially influenced by differences in lactation stages, as in the present study cows in the dry period shortly before calving were used. After calving, energy expenditure is elevated due to the initiation of high milk production. However, the energy uptake through feed is incapable of meeting the energy demands, which consequently leads to catabolism of adipose tissue and elevated ketone body production [22].

All chemical compound groups were detectable in the exhaled breath samples from all six cows, regardless of the breath collection variant. However, the number of VOCs per chemical compound group showed large variations between animals (up to at least 50%). The number of Level 3 VOCs within a particular chemical compound group differed between the breath collection variants. Aldehydes varied most among the breath collection variant, with the highest number of VOCs collected by $Mask_V$ (+ 61-69% compared to the other sampling variants) and $GreenFeed_U$ (+ 27-39% compared to $Mask_N$, $Mask_F$ and $GreenFeed_F$). Furthermore, $Mask_V$ samples contained the highest number of ketones (+19-38%), alcohols (+15-26%), and alkanes (+11-33%) compared to the other sampling variants. $GreenFeed_U$ exhaled breath samples exhibited the highest number of esters (+4-15%) compared to the other sampling variants. In contrast, alkenes were primarily detected using $Mask_V$ (+11-26%) and $Mask_F$ (+10 to 25%) compared to the other breath sampling variants.

Table 3: Number of exhaled volatile organic compounds (VOCs) from six dairy cows, collected using five different breath sampling variants, that exceeded background air peak levels by at least 50%. (mean ± coefficient of variation).

	Breath collection variants				
Chemical compound group	$Mask_N$	$Mask_F$	$Mask_V$	$GreenFeed_U$	$GreenFeed_F$
Aldehydes	9.0 ± 0.47	9.3 ± 0.65	15.8 ± 0.48	12.5 ± 0.48	9.8 ± 0.80
Alcohols	39.8 ± 0.51	36.3 ± 0.30	45.7 ± 0.39	37.3 ± 0.22	36.5 ± 0.32
Alkanes	68.8 ± 0.34	71.3 ± 0.39	79.0 ± 0.55	61.3 ± 0.38	59.3 ± 0.37
Alkenes	55.3 ± 0.53	60.7 ± 0.52	61.5 ± 0.59	48.7 ± 0.54	49.0 ± 0.37
Alkynes	1.8 ± 1.17	2.0 ± 1.00	3.2 ± 0.72	2.0 ± 0.70	1.5 ± 0.73
Azoles	13.3 ± 0.53	16.0 ± 0.34	17.8 ± 0.49	16.5 ± 0.21	14.8 ± 0.45
Amides	27.5 ± 0.61	23.8 ± 0.46	27.7 ± 0.46	27.2 ± 0.48	21.0 ± 0.59
Amines	28.5 ± 0.52	22.2 ± 0.34	26.2 ± 0.40	25.0 ± 0.35	23.0 ± 0.55
Carboxylic acids	13.7 ± 0.34	10.7 ± 0.35	12.7 ± 0.26	12.8 ± 0.55	14.8 ± 0.39
Esters	110.2 ± 0.27	104.8 ± 0.23	116.7 ± 0.28	121.0 ± 0.22	112.2 ± 0.22
Ethers	21.7 ± 0.24	18.7 ± 0.26	22.5 ± 0.44	22.0 ± 0.39	21.8 ± 0.27
Ketones	69.0 ± 0.32	62.7 ± 0.29	86.7 ± 0.35	72.3 ± 0.37	67.7 ± 0.28
Nitriles	8.2 ± 0.28	9.3 ± 0.48	9.0 ± 0.41	10.7 ± 0.24	10.3 ± 0.28
Pyridines	6.8 ± 0.54	8.3 ± 0.41	7.7 ± 0.57	7.0 ± 0.46	7.2 ± 0.57
others	61.5 ± 0.30	53.5 ± 0.30	59.5 ± 0.37	61.8 ± 0.21	62.0 ± 0.30

Exhaled VOCs were identified at Level 3 (assigned to their respective compound classes based on mass spectral similarity [19, 20]), $Mask_N$: tight-fitting face mask, $Mask_F$: the face mask with the openings sealed using activated carbon filters, $Mask_V$: the face mask covered with an over-mask ventilated with synthetic air supply for cow breathing, $GreenFeed_U$: the GreenFeed system with air sampling conducted before the filter, $GreenFeed_F$: GreenFeed system with air sampling conducted after the filter.

A total of 75 individual VOCs were detected and identified at Level 2 (match factor > 80% and difference in the calculated RI of in maximum ± 15 of the reference RI [19, 20]) from the compound groups of alcohols, aldehydes, esters, ketones, phenols, pyridines, and terpenes (Table 4; Supplementary Table S2) in exhaled breath samples. All 75 VOCs were present in all breath samples from all cows using all five breath collection variants. The concentrations of the exhaled VOCs exhibited considerable variation among cows (Table 4; Supplementary Table S2). This may be explained by differences in metabolism and feeding [8, 9]: in Exp2, two cows were fed according to recommendations, and four cows had an energy-richer diet. The concentrations of the exhaled VOCs also differed between the breath collection variants, with concentrations varying up to 95% (e.g., for propyl propionate) between one variant and another. Across the 75 detected VOCs, the highest mean VOC concentrations were observed in the samples collected using $Mask_V$, followed by $GreenFeed_U$ (concentrations around 11% lower than in $Mask_V$ samples), $Mask_F$ (-12%), $GreenFeed_F$ (-25%), and $Mask_N$ (-30%).

Some of the exhaled VOCs originate from the animal's metabolism (endogenous VOCs) [10], while others may derive from ingested feed [8] or from microbial fermentation [9] or were inhaled before (exogenous VOCs). Inhaled VOCs from the environment enter the body primarily through the respiratory tract, where they diffuse across the alveolar membrane into the bloodstream. VOCs ingested with feed, first enter the rumen, from where VOCs can be directly released by the ructus, or they may be absorbed through the ruminal and intestinal mucosa into the bloodstream. Once in the bloodstream, VOCs can undergo further metabolization, particularly in the liver, being transported further by the bloodstream into other body compartments or being excreted for example by the lungs. Before exhalation, the VOC profile may again be modified within the lungs through biotransformation processes – such as those mediated by P450 enzymes, epoxide hydrolases, or due to several barriers like the pulmonary alveolar membrane and the airway epithelium [23].

As both endogenous and exogenous VOCs can be exhaled via the respiratory tract and the upper gastrointestinal tract, exhaled breath is defined as a mixture exhaled from these two compartments. Accordingly, most breath sampling methods collect this combined mixture, rather than distinguishing between its individual sources [8, 9, 12]. However, a possible

approach to differentiate between VOCs originating from the lungs and those from the rumen is the use of methane concentration as a marker [24].

The literature provides evidence of the physiological relevance of some of the identified exhaled VOCs. For example, the alcohols 3-hexen-1-ol, 1-decanol, 1-undecanol, and 1-dodecanol are fatty alcohols, and octanal and decanal are fatty aldehydes. Both fatty alcohols and fatty aldehydes have been found in exhaled breath samples from cancer patients and have been linked to fat metabolism, namely lipid peroxidation and lipid catabolism [25-27]. Esters of propionic acid, such as propyl propionate, ethyl 2-hydroxypropionate, and butyl propionate, have been shown to be produced in the human gut [28] and the developing rumen of calves [29]. Therefore, it can be assumed that these VOC are produced by the gastrointestinal microbiome.

Similarly, esters of butanoic acid, such as ethyl butanoate, n-propyl butyrate, sec-butyl butyrate, n-butyl butanoate, and 3-methyl-1-butyl butanoate, were indicated by de Lacy Costello et al. (2014) [30] as endogenously produced in humans, presumably in the gastrointestinal tract [30]. Therefore, these exhaled VOCs could originate from the rumen and/or the bloodstream after crossing the blood–lung barrier. Ketones are well-known products of fatty acid catabolism. For example, ketone 3-octanone, one of the ketones identified in the present study, was found to be increased in the urine of overweight children compared to normal-weight children, possibly synthesized by the gut microbiota [31]. 3-Heptanone is a naturally occurring endogenous VOC present in the breaths of male Holstein calves [32]. Methyl ketones, including 3-heptanone and 2-heptanone, detected in the present study may be products of lipid oxidation and contribute to the flavor of dairy products [33].

Increased phenol levels in urine and milk have been attributed to increased protein metabolism and bacterial activity in the gastrointestinal tract [34], acetophenone to phenylalanine metabolism [35] and pyrrole-2-carboxaldehyde detected in the urine of humans and rats is associated with collagen metabolism [36]. Therefore, the detection of phenols in breath in the present study may result from exhalation directly from the rumen or after their transfer into the bloodstream and subsequent release from the lungs for expiration. The terpene isoborneol formed by bacterial metabolism or by the host pathogen interaction

has been detected in the breath samples of mice [37] and may therefore originate from gut content.

Benzaldehydes show large inter-individual differences in the exhaled breath of humans [38]. Due to their ubiquitous presence in great concentrations in air samples (often greater than in human breath), benzaldehydes were hypothesized to be of environmental origin [38, 39], but they have also been described as endogenously produced compounds in the breath samples of humans [20]. For example, they may act as alarm pheromones and defense compounds in insects, as pollinator attractants, and as flavor and antifungal compounds in plants [40]. In the present study, we hypothesize that exhaled benzaldehydes were released either from the sampling material by the warm and humid conditions of the breath sample [41] or from the gastrointestinal tract after the ingestion of herbage. Furthermore, other exhaled VOCs may originate from the gastrointestinal tract content of the cows after herbage ingestion. For example, benzyl alcohol [42], fenchone [43], 6,6-dimethyl-, (1R)-bicyclo[3.1.1]heptan-2-one, also called nopinone [44], (+)-2-bornanone [45], α,α-4-trimethyl-cyclohexanemethanol [46], citronellal [47], methyl salicylate [48] propyl benzoate [49] and bornyl acetate [50] can be produced by many plants. It is likely that these VOCs were ingested by cows with herbage and then exhaled, as shown earlier for dairy cows or humans [30].

Table 4: Exhaled volatile organic compounds (VOCs) from the as physiologically relevant considered chemical groups (aldehydes, alcohols, azoles, amides, amines, carboxylic acids, esters, ethers, ketones, nitriles, pyridines, sulfur containing compounds and terpenes) after subtraction of VOCs considered as of barn origin (< 1.5 * blank peak area; [17]) detected in all six dairy cows of experiment 2 using five different collection variants.

	Collection variant (GC-MS peak area)									
	$Mask_N$		$Mask_F$		$Mask_V$		$GreenFeed_U$		$GreenFeed_F$	
Volatile organic compounds	mean	CV (%)	mean	CV (%)	mean	CV (%)	mean	CV (%)	mean	CV (%)
αα-4-Trimethyl-cyclohexanemethanol	4,095,466	144	554,937	71	2,960,303	68	162,238	70	273,726	150
Benzyl alcohol	215,677	183	891,200	116	694,633	210	291,952	104	308,686	95
3,5,5-Trimethyl-1-hexanol	59,453	110	85,720	128	119,894	153	115,739	83	50,101	104
2,6-Dimethyl-7-octen-2-ol	785,551	73	941,985	132	344,196	112	345,757	44	196,822	201
2,6-Dimethyl-2-octanol	4,665,148	113	951,503	71	3,525,890	105	1,904,332	84	1,955,940	125
2,5-Bis(1,1-dimethylethyl)-1,4-benzenediol	92,576	69	110,099	49	109,563	76	82,120	69	143,852	63
2-Ethyl-1-hexanol	64,090	272	516,326	100	476,221	306	82,874	85	71,662	95
1-Undecanol	94,272	83	146,786	71	140,707	129	130,297	71	120,353	84
1-Dodecanol	30,193	71	37,363	59	33,333	123	34,177	63	27,943	80
1-Decanol	34,135	165	93,552	78	105,437	204	67,863	82	50,410	113
1-(2-Methoxy-1-methylethoxy)-2-propanol	134,805	135	161,968	68	238,787	135	151,833	75	123,849	79
(E)-3-Hexen-1-ol	275,317	108	280,291	85	197,356	88	604,189	107	363,193	93
Octanal	163,703	71	178,350	96	208,319	97	250,139	65	211,393	79
Decanal	133,173	174	31,579	159	91,316	89	173,252	105	49,992	218

4-Methyl-benzaldehyde	127,054	97	94,742	100	121,495	111	133,272	107	103,083	160
4-Ethyl-benzaldehyde	7,917,107	61	8,934,549	44	9,340,122	69	8,430,345	46	8,790,349	41
3-Ethyl-benzaldehyde	7,917,118	61	8,933,933	44	9,340,960	69	8,431,196	46	8,790,394	41
2,5-Dimethylbenzaldehyde	4,248,027	99	7,818,764	95	6,952,761	80	4,287,066	73	7,210,844	85
2,4-Dimethyl-benzaldehyde	9,299,362	59	10,214,384	43	10,672,266	63	9,874,552	45	10,212,864	41
2-Methyl-benzaldehyde	805,629	57	829,992	67	1,212,274	78	1,158,376	56	963,391	59
2-Methyl-3-phenyl-2-propenal	15,254	260	77,565	87	112,001	295	22,810	92	17,986	106
2-Ethyl-benzaldehyde	7,917,139	61	8,934,906	44	9,340,101	69	8,431,336	46	8,790,349	41
1H-Pyrrole-2-carboxaldehyde	638,154	106	1,136,345	60	1,051,576	145	543,138	78	557,940	43
(E,E)-2,4-Hexadienal	363,957	133	188,146	94	707,376	106	323,382	110	525,179	105
sec-Butyl Butyrate	168,277	93	173,171	105	263,373	149	162,231	110	154,754	129
Propyl propionate	131,100	305	2,355,518	119	2,551,846	318	297,774	110	201,020	78
n-Propyl butyrate	158,157	93	143,650	72	107,636	105	278,474	90	316,198	104
n-Propyl benzoate	40,199	80	21,951	91	40,218	102	30,921	52	31,477	86
n-Hexyl acetate	97,603	170	143,736	69	165,178	171	139,079	65	88,444	97
n-Butyl butanoate	90,342	266	207,352	79	128,686	225	29,403	97	30,052	264
Methyl salicylate	22,529	137	40,523	91	37,854	178	51,784	92	47,081	74
Isopropyl myristate	36,921	87	29,946	127	51,568	77	38,127	92	30,977	107
Isobornyl acetate	90,433	77	140,607	98	166,150	92	113,542	90	112,370	60
Ethyl butanoate	1,154,337	175	2,707,164	149	2,719,985	187	2,950,710	184	3,178,443	129
Ethyl benzoate	80,197	64	60,858	47	57,351	73	121,748	49	98,444	114

Ethyl 2-hydroxypropionate	6,534,141	62	7,991,283	43	6,017,204	96	5,961,149	40	4,354,650	70
Dimethyl butanedioate	338,974	126	535,227	97	621,562	192	400,493	98	321,881	125
Butyl propionate	126,543	176	194,982	144	225,261	224	148,819	145	93,162	153
Benzyl acetate	30,340	93	39,218	38	46,084	113	24,284	40	23,915	37
3-Methyl-1-butyl butanoate	50,477	69	12,102	139	15,588	89	27,852	186	32,344	108
3-Hydroxy-2,2,4-trimethylpentyl isobutyrate	165,963	110	101,627	104	216,882	160	243,579	108	174,167	176
2-Ethoxyethyl acetate	37,344	130	25,669	263	49,069	173	489,245	130	46,058	225
2-Acetoxy-1-propanol	206,879	99	263,006	110	341,835	114	280,574	120	282,432	81
1-Methoxy-2-propyl acetate	163,140	160	526,284	92	541,571	223	341,622	101	282,810	104
α-Isomethyl ionone	21,521	79	66,017	192	30,973	221	131,513	224	60,039	43
Cyclohexanone	73,519	160	198,487	78	186,763	199	87,001	66	81,377	60
Benzophenone	10,144	193	26,077	41	27,015	212	10,950	33	11,031	39
Acetophenone	423,046	150	1,084,216	81	1,207,895	207	724,362	85	643,905	74
6,6-Dimethyl-, (1R)-bicyclo[3.1.1]heptan-2-one	95,474	113	134,652	138	202,078	132	233,348	103	116,409	141
6,10-Dimethyl-5,9-undecadien-2-one	75,046	77	98,392	69	118,173	72	83,386	57	88,758	76
6,10-Dimethyl-(Z)-5,9-undecadien-2-one	75,046	77	98,392	69	118,173	72	83,386	57	88,758	76
6,10-Dimethyl-(E)-5,9-undecadien-2-one	75,046	77	98,392	69	118,173	72	83,386	57	88,758	76
6-Methyl-5-hepten-2-one	232,683	89	313,892	30	361,747	121	194,888	30	203,885	36
6-Methyl-3,5-heptadiene-2-one	5,679,700	92	8,082,403	105	10,954,734	111	11,587,622	100	7,612,839	124

5-Methyl-2-(1-methylethyl)- (trans)-cyclohexanone	18,377	79	21,705	87	18,076	95	24,723	85	15,386	90
5-Ethyldihydro-2(3H)-furanone	159,791	197	388,063	115	436,963	225	415,022	51	148,122	70
5-(1-Methylethyl)-bicyclo[3.1.0]hexan-2-one	119,106	98	136,295	138	246,225	129	234,437	97	121,233	106
4,7,7-Trimethylbicyclo[4.1.0]hept-3-en-2-one	19,153	108	34,193	142	50,664	142	43,137	135	31,836	103
3-Octanone	258,997	53	140,943	44	109,252	77	141,385	60	138,264	200
3-Heptanone	9,900,835	167	6,380,342	160	10,127,211	223	9,146,264	230	4,968,322	139
2,5-Hexanedione	146,008	128	431,852	123	450,509	180	329,487	117	280,664	93
2,5-Dihydro-3,5-dimethyl-2-furanone	177,027	84	206,916	97	294,463	123	274,390	96	212,979	120
2-Heptanone	123,502	216	446,019	42	581,141	247	170,653	49	174,792	37
1,1'-(1,4-Phenylene)bis-ethanone	451,857	158	460,010	88	758,564	100	1,538,590	81	2,101,431	54
1-(4-Methylphenyl)-ethanone	7,918,294	61	8,935,863	44	9,340,165	69	8,431,842	46	8,790,364	41
1-(4-Ethylphenyl)-ethanone	33,716	81	45,937	95	51,240	94	103,105	101	127,211	97
(E)-3-Hepten-2-one	402,788	38	447,619	47	560,299	33	504,276	45	451,852	25
(+)-2-Bornanone	85,967	68	104,709	62	95,396	81	120,939	74	112,813	57
3-Ethyl-phenol	244,724	71	191,076	51	181,320	58	221,312	109	382,736	81
2,4-Dimethyl-phenol	183,242	163	371,682	139	349,717	121	633,828	105	393,840	91
2-(1,1-Dimethylethyl)-5-methyl-phenol	14,751	103	16,981	131	28,335	128	28,317	116	14,330	137
2-Dimethylaminopyridine	46,597	57	30,375	62	41,719	100	57,081	73	45,863	68
L-Fenchone	20,095	72	25,852	66	17,855	118	30,941	80	27,682	81

Isoborneol	9,040	68	11,965	73	11,559	35	7,289	57	10,690	92
Citronellal	12,788	67	11,816	64	10,216	74	11,958	61	8,508	118

Identified using National Institute of Standards and Technology NIST/EPA/NIH mass spectral library (NIST17) (match factor >80%) after subtraction of barn VOCs (< 1.5 * blank peak area; [17]) and manual peak integration using MassHunter Quantitative Analysis software; RT: retention time (min); CAS: chemical abstracts service registry number; Level: identification level; RI: Retention-Index; RI ref: reference RI after comparison from the NIST chemistry web book (non-polar column 5ms, ramp temperature); RI calc: calculated RI; $Mask_N$: tight-fitting face mask, $Mask_F$: the face mask with the openings sealed using activated carbon filters, $Mask_V$: the face mask covered with an overmask (Supplementary Figure S2; Backpack fabric, marine, No. 1274, polyester waterproof, 210 g/m2, Alja, Bern, Switzerland), ventilated with synthetic air supply for cow breathing, $GreenFeed_U$: the GreenFeed system with VOC sampling conducted before the filter, $GreenFeed_F$: GreenFeed system with exhaled VOC sampling conducted after the filter, CV: Coefficient of variation

3.4 Limitations of exhaled volatile organic compound sampling in dairy cows

The high variation in abundance and concentration of VOCs in exhaled breath among individual cows and in the barn air over time observed in the present study, and reported in several other experiments in dairy cows [8, 9, 12, 22], reflects the dynamic nature of VOCs under *in vivo* on-farm conditions [51]. Multiple factors — including inter-individual differences [5, 52], eructation events [24, 53], the physiological status [10], metabolic activity [8], and environmental influences such as feed and cow excrements [8, 54, 55] — can contribute to the observed fluctuations in breath VOC profiles as well as to changes in barn air VOC profiles [51]. This complexity challenges the direct transfer of a fixed-threshold approach — such as the subtraction of background air concentrations multiplied by a defined factor to estimate physiologically exhaled VOCs, as commonly applied in human breath analysis [17] — to an animal setting. Küntzel et al. (2018) using a sampling system similar to the $Mask_N$ variant of the present study, subtracted inhaled from exhaled VOC concentrations and classified negative values as contamination to be excluded. While this strategy reduces background interference, it may carry the risk of overestimating true exhaled VOCs.

Therefore, in the present study, we applied an operational definition in which a VOC was classified as exhaled when its concentration exceeded that of the immediately preceding background air sample by at least 50 % [17]. For punctual sampling designs, this approach offers a strict and directly comparable framework and thus represents a valuable practical starting point for identifying candidate exhaled VOC markers under controlled experimental conditions. At the same time, it must be acknowledged that such a high threshold may exclude also some relevant VOCs and may not capture all biologically relevant exhaled VOCs.

Longitudinal studies by Küntzel et al. (2018) [12], Islam et al. (2023) [9] and Oertel et al. (2018) [53], which collected exhaled breath repeatedly over the day, further illustrate that both the exhaled and inhaled VOC profiles can vary markedly within a single day. These diurnal changes are driven not only by feeding events but also by physiological rhythms, as well as the dynamic composition of barn air [9, 12, 52, 54]. In addition to daily patterns, longer-term dynamics across lactation [8] and metabolic stage [10, 22] have been documented. Eructation events add another layer of complexity by intermittently releasing rumen gases that alter exhaled VOC composition [53]; in most previous studies these events were excluded manually [12, 22] or identified using methane concentration as a marker [24].

Taken together, these findings emphasize that while a punctual and strictly threshold-based approach using barn air collected directly before exhaled breath sampling is suited for standardized method comparison and device benchmarking, future applications aimed at practical on-farm diagnostics should integrate repeated or temporally resolved measurements in larger animal cohorts. Such strategies will be necessary to capture short-term physiological events, validate or dismiss candidate exhaled VOC markers, and ensure the robustness and reproducibility of breath-based detection in ruminants.

3.5 Feasibility and acceptance of breath collection variants

An ideal breath collection method should, among other criteria, be non-invasive, user-friendly, cost-effective, and require little labor and overall effort. The GreenFeed system is notable for its animal-friendly design, as it does not require restraining cows but allows them to voluntarily approach the system, motivated by bait feed. Depending on its use, the GreenFeed system may still have limitations in terms of user-friendliness and precision. In this study, the system was manually positioned in front of each cow, which is labor-intensive and not ideal for practical application. This approach is primarily used in research settings where breath samples are required from individual cows at specific time points. Further optimization is needed to enhance its usability and efficiency in terms of time, physical work effort, and maintenance. Another limitation is the great and likely non-standardizable dilution factor of exhaled breath in the surrounding air from the system; the sampled exhaled breath is diluted by approximately a factor of 40 with barn air. This leads to increased concentrations of barn VOCs, as well as imprecisely quantifiable absolute concentrations of sampled exhaled VOCs. VOC sampling using SPE cartridges is not yet automated either. Developing an automated system is essential for routine applications.

Sampling exhaled breath using a mask requires restraining the animal, although painless, thus close human contact and handling. In the present experiment, most of the cows tolerated the manipulation well. Using $Mask_N$ and $Mask_V$, the cows were able to breathe comfortably and remain relatively calm during the 3 minutes of sampling, rendering these methods relatively animal-friendly. However, single animals may not tolerate it well, exhibiting defensive movements, heavy breathing, and experiencing stress, fear, and aversion. Attaching the carbon filters we used in these experiments to $Mask_F$ was not well tolerated by the cows, as

respiration became difficult for the animals. Therefore, we advise against this breath collection variant—at least with the filters employed in this study. It is possible that alternative thinner filters or membranes could be used instead for improved compatibility and tolerance by the animals.

$Mask_V$ requires great logistical and technical demands; for example, the synthetic air supply requires additional equipment, such as gas cylinders and careful monitoring of air flow. However, the great advantage of $Mask_V$ is its potential accuracy in measuring absolute exhaled VOC concentrations. To fully realize this potential, further optimization is required, such as incorporating additional flow meters to measure exhaled breath volume and determining the washout time with synthetic air needed to clear the lung volume of background VOCs originating from barn air [56]. Furthermore, it is imperative to establish standardized protocols for cleaning the breath collection devices before each sampling event to minimize VOC deposits and prevent carryover between cows. Alternative methods for breath sampling, not used in this experiment, are common in greenhouse gas research, the advantages and limitations of which have been well-documented. Metabolic chambers enable the measurement of not only exhaled VOCs but the entire volatilome encompassing VOCs excreted through the skin, urine and feces [57]. However, they are costly, require significant maintenance, and involve social isolation, making them less animal-friendly.

Another method is a nostril sampler connected to a plastic bag, which is held over one nostril while the other nostril is covered [58]. This approach is labor-intensive and may cause discomfort due to restraint and blocking of one nostril. Backpack systems, where the cow wears a backpack on its back connected to a tube extending to the nostrils, can also be used in breath sampling. While chest straps and halters are generally well accepted by animals, this method remains highly labor-intensive [59]. Another alternative are sniffers, which allow for "passive," non-invasive sampling of exhaled breath in close proximity to the animal's head, for example, at the feeding trough or milking parlor [60]. However, sniffers usually detect a limited range of known VOCs, thus questioning their suitability for untargeted analysis [61]. Additionally, the measured concentrations can vary depending on the distance between the sensor and the animal, thereby introducing uncertainty and imprecision. These alternative methods for VOC sampling should be further explored, particularly in combination with SPE

cartridges. Automating SPE processes would be a critical step toward improving the efficiency and practicality of VOC sampling for routine applications.

4 Conclusion

The results of our investigation demonstrate a general suitability of the breath collection variants—except $Mask_F$, due to low animal acceptance—for sampling exhaled VOCs from dairy cows. Our findings indicate that the exhaled VOC collection variant has an impact on the VOC chemical compound groups that can be detected and identified, on their relative concentrations, and on possible VOC deposits within the sampling devices. $Mask_V$ was the most suitable collection variant due to the detection of most VOCs in the highest concentrations while reducing the influence of environmental VOCs. This variant seems promising for research purposes and untargeted VOC analysis. The GreenFeed system, although the most animal-friendly and mobile option, demonstrated the highest potential for VOC deposits in the sampling system, sample contamination with background VOCs, and lower VOC concentrations due to the great dilution by background VOCs. Several exhaled VOCs identified in this study could serve as candidates for future biomarker research in animals to describe their metabolism or ruminal fermentation or to characterize the feed they have ingested. Follow-up studies should focus on targeted quantitative analysis of these VOCs and their associations with different feeding interventions, lactation stages, or physiological states in dairy cows. Investigating larger and more diverse animal populations may further help to identify outlier VOC profiles and their concentrations linked to specific metabolic conditions or health statuses.

Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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