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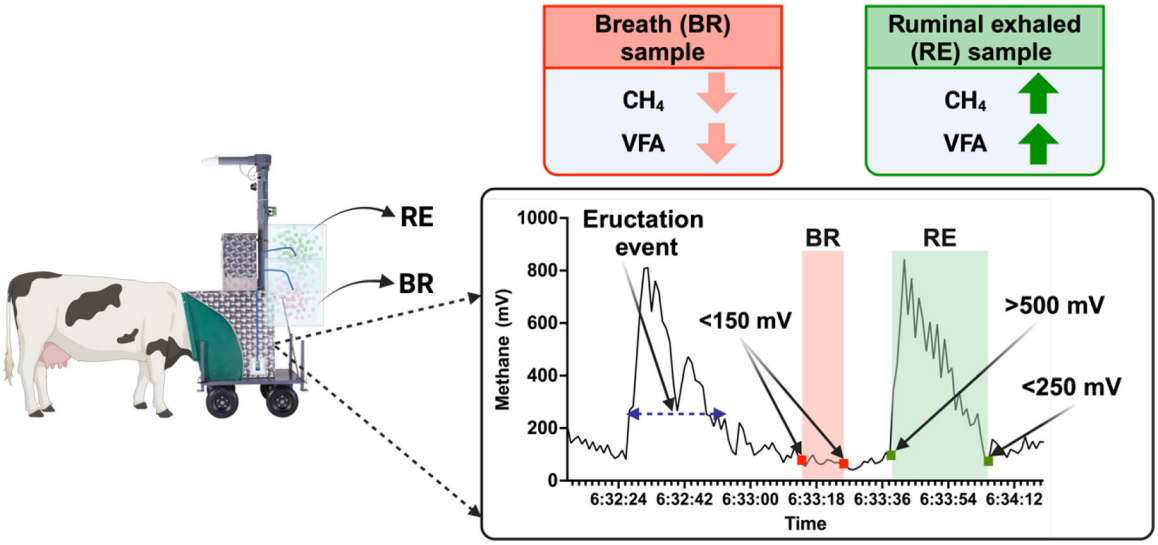
Short Communication
Physiology

A sampling method for differentiating breath and ruminal exhaled volatile organic compounds in dairy cows using methane as a marker

M. A. Barrientos-Blanco,[1] U. Arshad,[1] S. Giannoukos,[2] M. Z. Islam,[1] C. Kunz,[1] R. Peng,[1] S. E. Räisänen,[1] R. Zenobi,[2] and M. Niu[1]*

Graphical Abstract



Summary

Rhythmic eructation in ruminant animals releases rumen head space pressure and results in a blend of exhaled ruminal and breath volatile organic compounds (VOC). The objective of this study was to establish a benchmark sampling method for collecting breath samples from dairy cows. Twelve multiparous mid-lactation Holstein cows were enrolled to collect (1) breath (BR) and (2) ruminal exhaled (RE) samples. Samples were collected using a head chamber system with real-time CH_4 measurements. Implementing CH_4 as a marker to differentiate breath from ruminal eructation gave an 80% lower CH_4 concentration in BR compared with RE. Mole fractions of the 3 volatile fatty acids (VFA), acetate, propionate, and butyrate, were significantly greater in RE than in BR. Lower concentrations of CH_4 in the BR and greater concentrations of VFA in the RE validated the methodology to differentiate breath from RE VOC.

Highlights

- A blend of ruminal eructation and breath could limit the use of breathomics.
- A method was established to sample breath from dairy cows.
- Breath can be differentiated from RE samples by up to 80% lower CH_4.
- Greater VFA levels in RE versus BR samples further validated our method.

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The list of standard abbreviations for JDSC is available at adsa.org/jdsc-abbreviations-25. Nonstandard abbreviations are available in the Notes.

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Abstract: Frequent eructation in ruminant animals results in an exhaled blend of ruminal eructed and breath volatile organic compounds (VOC). The physiological distinction between the gas sources can limit the applicability of breath metabolomics (or breathomics) in describing the metabolic phenotype of cows. The objective of this study was to establish a benchmark sampling method for collecting breath samples in dairy cows while they were not eructating. Twelve multiparous mid-lactation Holstein cows were enrolled to collect (1) breath (BR; bloodborne VOC exchanged at the lungs) and (2) ruminal exhaled (RE; a mixture of VOC from ruminal eructation and breaths during eructations) samples. Gas samples were collected using a head chamber (GreenFeed system) with real-time CH_4 readings. By monitoring eructation events, a threshold of <150 mV CH_4 was set to sample breath, and >250 mV was used to collect BR and RE. Both samples were analyzed using secondary electrospray ionization-high resolution MS (SESI-MS) and GC. Implementing CH_4 as a marker resulted in 80% lower CH_4 concentrations in BR compared with RE. Analysis using SESI-MS revealed a total of 324 and 242 features consistently identified across all periods of the study in $[M\text{-}H]^-$ and $[M+H]^+$ MS ion mode, respectively, for BR and RE. In BR, 18 features exhibited greater concentrations, whereas 8 had a tendency to have greater concentrations compared with RE. In contrast, RE revealed 51 features with greater concentrations, and 13 with a tendency for greater concentrations compared with BR. Ruminal VFA acetate, propionate, and butyrate were 20.9%, 27.4%, and 32.7% greater in RE compared with BR, respectively. Lower CH_4 levels in BR and the greater VFA concentrations in the RE validated the ability of the method to differentiate breath from ruminal eructed VOC. Our study established a method to distinguish and separately collect BR and RE samples in dairy cows. This advance shows the potential to use breathomics as a reliable and noninvasive tool for metabolic assessments in ruminant research.

Exhaled breath analysis is a noninvasive approach that is increasingly used for metabolic assessments and phenotype profiling in humans (Giannoukos et al., 2019). Breath contains blood-borne volatile organic compounds (**VOC**), which serve as fingerprints for metabolic and health profiles in humans and dairy cows (Mottram et al., 1999; Islam et al., 2024; Song et al., 2024). Although breathomics in human medical diagnostics is relatively advanced, it remains understudied in dairy cows. An important caveat that limits the implementation of breathomics analysis in dairy cows is their rhythmic eructation events that release ruminal fermentation gases. Endogenously produced VOC that diffuse from the bloodstream into the alveoli constitute the gases present in breath, which can reflect cellular and lower gastrointestinal tract metabolism (de Lacy Costello et al., 2014; van der Schee et al., 2015; Sharma et al., 2023). However, in ruminants, because of rhythmic eructations, the exhaled gases consist of both breath and ruminal VOC—a blend referred as the exhalome (Dougherty, 1968; Oertel et al., 2018; Reinhold et al., 2020; Islam et al., 2024). Therefore, to be able to investigate breathomics as an approach for primarily characterizing the metabolism of dairy cows and not the ruminal activity, it is essential to develop a method to reduce the confounding effect of eructed VOC on breath.

From ruminal fermentation, CH_4 is one of the major gases produced (~30% of total production), of which approximately 95% is excreted via ruminal eructations (Dougherty et al., 1964; Dougherty, 1968; Murray et al., 1976). The high removal rate of CH_4 from rumen through eructation makes it an optimal marker to differentiate the eructation events from regular respiration. In addition, the measurement of gaseous exchanges with high-resolution sensing devices in the GreenFeed (**GF**) system (C-Lock Technology Inc., Rapid City, SD) allows the recognition of CH_4 peaks originating during eructation events (Hardan et al., 2022; Islam et al., 2023, 2024). Based on this premise, the objective of this study was to establish a benchmark method for collecting breath samples for breathomics in dairy cows while they were not eructating. We hypothesized that using CH_4 as a potential marker of eructation events can be used to differentiate breath from eructed ruminal fermentative VOC.

All experimental procedures involving animals were approved by the Cantonal Veterinary Office of Zürich (ZH042/2023). The experiment was conducted at the AgroVet-Strickhof Dairy Farm (Lindau, Switzerland). Twelve Holstein multiparous lactating cows were enrolled with (mean ± SD) 136 ± 53 DIM, 36.2 ± 3.04 kg/d milk yield, and 750 ± 52.6 kg of BW at the beginning of the

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experiment. The current study was part of a larger experiment, but only data based on the origin of exhaled gases are presented here, with the goal to characterize the method for breath sampling. For this purpose, cows were subjected to collect (1) breath (**BR**; bloodborne VOC exchanged at the lungs) and (2) ruminal exhaled (**RE**; a mixture of VOC from ruminal eructation and the breaths during eructations) samples. The experimental design of the larger study involved randomly assigning cows to 1 of 4 diets following a 4 × 4 Latin square design. The dietary interventions were (1) high energy basal diet plus placebo (NEL = 1.72 Mcal/kg of DM, starch = 26.4%, CP = 13.5%, NDF = 37.9% of DM, and metabolizable His = 45 g/d), (2) high energy plus rumen-protected (**RP**) His (NEL = 1.72 Mcal/kg of DM, starch = 26.4%, CP = 13.5%, NDF = 37.9% of DM, and metabolizable His = 65 g/d), (3) low energy basal diet plus placebo (NEL = 1.69 Mcal/kg of DM, starch = 16.4%, CP = 13.9%, NDF = 44.4% of DM, and metabolizable His = 44 g/d), and (4) low energy plus RP-His (NEL = 1.69 Mcal/kg of DM, starch = 16.4%, CP = 13.9%, NDF = 44.4% of DM, and metabolizable His = 66 g/d). The supplementation of RP-His and placebo (coating material composed of SFA) supplied by King Techina Group (Hangzhou, China), was provided as a top-dress, with the placebo ensuring similar supply of lipids across diets. The supply of metabolizable His and nutrient composition of each diet was estimated with observed mean DMI of the experimental cows and using NASEM (2021). The experimental duration was 21 d, including 14 d of dietary adaptation and 7 d of sample collection. During the first 14 d of the experimental period, the cows were housed in a freestall barn with wheat straw bedding and equipped with feeding troughs (Waagen Döhrn GmbH, Germany) and a Calan-gate system (American Calan Inc., Northwood, NH) for individual feeding. On d 14, the cows were moved to a tiestall barn, where they underwent 4 d of adaptation, followed by 3 d of sampling. Cows were milked twice daily, at 0530 and 1530 h, fed ad libitum at 110% twice daily at 0830 and 1700 h, and had free access to drinking water.

The GF was used to measure gaseous exchanges. During the last 3 d of each experimental period, gas samples were collected every 6 h, resulting in 8 time points representing a 24-h feed cycle. Sampling occurred on d 19 at 1000, 1600, and 2200 h; d 20 at 0400, 1300, and 1900 h; and d 21 at 0100 and 0700 h, according to the procedures described by Hristov et al. (2015) and Islam et al. (2023). The gaseous exchange measurements included CH_4, CO_2, and O_2. The gaseous exchange measurements were recorded using GF over the course of 5 min. Alfalfa pellets were used as bait feed for the gas sample collection. Between measurements from each cow, the GF was allowed 2 min of flushing to remove gas traces before moving on to the next cow. Methane was used as a potential marker to differentiate the breath from eructation events. Real-time CH_4 monitoring was conducted through the mobile application Control Feed (C-Lock Technology Inc., Rapid City, SD).

Parallel to the gaseous exchange measurements, a gas sampling valve attached to the GF allowed for the collection of both BR and RE samples, as detailed by Islam et al. (2023). Gas samples were collected into 1-L Tedlar gas sampling bags with Thermogreen LB-2 septa (Merck, St. Louis, MO). All bags were flushed and emptied under vacuum 5 times with nitrogen gas before each sampling to remove traces from previous samples. Bags containing samples were stored in a dark environment at room temperature and shipped for laboratory analysis within 24 h of collection.

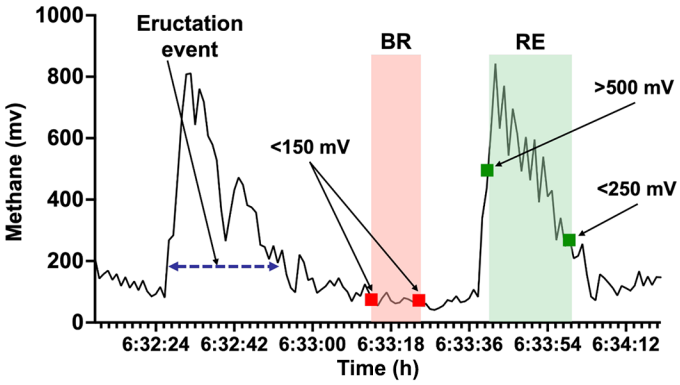

Figure 1. Real-time CH_4 measurements (x-axis: real time of measurement; y-axis: CH_4 sensor voltage, mV) on the "Control Feed" mobile app (version 1.51, C-Lock Technology Inc.), showing an eructation event from one cow during sample collection at 1000 h. Collection of breath (BR) samples was performed when CH_4 sensor voltage was <150 mV, with a duration of 10 s, repeated 3 times (30 s total). Collection of ruminal exhaled (RE) samples was conducted immediately after the appearance of a true eructation event (>500 mV) and stopped when CH_4 levels decreased to 250 mV, repeating it for 3 eructation events.

The methodology used to acquire BR samples, while differentiating them from RE, is presented in Figure 1. Methane levels during eructations exhibit rhythmic patterns with distinctive spikes, though the magnitude is inconsistent. Posteructation, a following period of consistently low levels of CH_4, occurs at regular intervals (Figure 1). Multiple observations of eructation events, using GF sensor voltage measurements (raw values) from a previous pilot study (Barrientos Blanco et al., 2024) allowed us to recognize the visual disappearance of these events. From the pilot study, we identified consistent posteructation CH_4 levels not exceeding 150 mV, represented by a continuous stable line on the real-time CH_4 gaseous exchanges Control Feed mobile application plot. Therefore, a maximum allowable CH_4 level of 150 mV was established as the maximum permissible limit for collecting BR samples. In the case of RE, Islam et al. (2023) reported a true eructation event when CH_4 is greater than 500 mV. The sharp increase in the CH_4 levels facilitated the recognition of the onset of the eructation event. Considering that 150 mV was defined as the threshold for BR, we set 250 mV as the lower limit for RE. Based on these parameters, we collected RE samples when the CH_4 sensor voltage was >500 mV at the spike of the eructation event, and >250 mV as the lower limit as an indicative of the end of the eructation event. Due to the unpredictability of the next eructation event, once the BR threshold was reached after an eructation, a sampling time of 10 s was considered to avoid possible contamination. If an unexpected event occurred during sampling where CH_4 levels exceeded the threshold for BR, the sample was discarded. Sample collection was repeated 3 times for both BR (30 s) and RE (3 eructation events).

Samples collected at 0400, 0700, 1000, 1300, and 1600 h were analyzed using GC for CH_4 concentrations as described by Soliva and Hess (2007). Given that GC is a widely established analytical platform and GF relies on calibrated gas sensors, we selected GC to quantify CH_4 levels in our samples as the method for data measurement. The GC (6890N, Agilent Technologies, Wilmington, DE) was equipped with a flame ionization detector and a 4.5 m long × 3.2

mm o.d. × 2.1 mm i.d. packed GC column (60/80 Carboxen-1000, model 12392-U, Supelco Inc., Bellefonte, PA). To analyze the VOC profile of BR and RE samples, a secondary electro-spray ionization (**SESI-MS**, Fossil Ion Tech, C. de los Cipreses, Madrid, Spain) device was coupled with an Orbitrap mass spectrometer (Q-Exactive Plus, Thermo Fisher Scientific, Freiburg, Germany) was used. Offline sample analyses were performed as described by Islam et al. (2024). Briefly, the sampling line of the SESI source was constantly heated at 130°C, and the ionization chamber was kept at 90°C. An electrospray solution of formic acid at 0.1% was passed through a nanoelectrospray capillary (20 µm i.d. and 365 µm o.d., Fossil Ion Tech, C. de los Cipreses, Madrid, Spain). The electrospray solution voltage was 3.5 kV, and the MS inlet capillary was heated to 250°C. Scans were recorded every 500 ms, with a mass-to-charge ratio (***m/z***) range of 50 to 500 and a mass resolution of 140,000. An acquisition time of 5 s for background baseline and 10 s for sample readings was used to measure each sample on positive (**[M+H$^+$]**) and negative (**[M-H$^-$]**) ion modes for protonated and deprotonated features, respectively.

Acquired MS raw files were processed as described by Islam et al. (2023). Briefly, mass spectrometric scans were converted into .mzXML files using MSConvert (ProteoWizard, version 3) according to Chambers et al. (2012) and preprocessed in Matlab version 2023a (The MathWorks Inc., Natick, MA). The data preprocessing included interpolation, baseline adjustment, peak signal integration, and peak picking. Spectra cubic interpolation was defined with peak range of *m/z* 50 to 500 and a mass resolution of 140,000. Time trace intensities for each *m/z* were averaged every 5 scans to retrieve a 2-dimensional matrix [*m/z* vs. counts per second (**CPS**)]. For each *m/z*, CPS were normalized by sample total ion count, as suggested by Streckenbach et al. (2023). The VFA acetic acid, propionic acid, and butyric acid were tentatively annotated based on previously reported masses (Islam et al., 2023). Putative VFA annotation was performed with a mass tolerance of 5 ppm, using [M-H$^-$] readouts. All other *m/z* were treated as features as no additional annotation steps were taken for further identification (Bruderer et al., 2019). Mass drift caused by different SESI batches was corrected by grouping each *m/z* with a mass tolerance of 5 ppm, assigning a unique *m/z* value for each repeated measurement.

For the data processing, all *m/z* with a prevalence lower than 50% were removed from the dataset. Raw readings from SESI-MS and GC more than 3 SD from the mean were considered outliers and removed from the dataset. All time points were analyzed to compare VOC concentration between BR and RE (data not shown). The data shown include only 1000 h sampling time because it corresponds to the time of maximum CH_4 concentration difference observed between RE and BR. In addition, the 3 VFA were found consistently greater in RE versus BR ($P < 0.05$), except for propionate, which tended to be greater at 0400 h ($P = 0.06$). To describe the concentration differences of VFA between BR and RE, a volcano plot was created for the VOC dataset, with fold change calculated using a logarithmic transformation of the LSM ratio, as described below:

$$\text{fold change} = \log_2\left(\frac{\text{LSM of breath}}{\text{LSM of ruminal exhaled}}\right).$$

The experiment followed a Latin square design, and the cow was the experimental unit. Data on concentrations of CH_4 were analyzed using a linear mixed model with repeated measures, employing the *lmer* procedure (Bates et al., 2015) in R statistical software (R Core Team, 2024, version 4.4.1) with the following model:

$$Y_{ijmknz} = \mu + S_i + C_j(S_i) + D_m + P_k + O_n + T_z + O_n \times T_z + e_{ijmknz},$$

where Y_{ijmknz} is the variable of interest; μ denotes the overall mean; S_i is the random effect of dietary supplementation sequence (i = 1 to 4), $C_j(S_i)$ is the random effect of cow nested within the sequence (j = 1 to 4); D_m is the random effect of diet (m = 1 to 4); P_k is the fixed effect of the period (k = 1 to 4); O_n is the fixed effect of the sample origin (n = BR or RE); T_z is the fixed effect of the time of day (n = 1 to 5); $O_n \times T_z$ is the interaction of origin and time of day the measurements occurred; and e_{ijmknz} is the residual error. Because effect of diet was not the primary focus of the current dataset, we treated it as a random effect to account for possible variability due to difference in diets between experimental periods. This ensured generalizable inference without emphasizing specific diet effects.

Data on VOC from SESI-MS measurements were analyzed using the following model:

$$Y_{ijmnk} = \mu + S_i + C_j(S_i) + D_m + O_n + P_k + e_{ijmnk},$$

where Y_{ijmnk} is the variable of interest, e_{ijmrk} is the residual error, and the remaining model components are analogous to previous model. Statistical significance for main effects and their interaction was considered at $P \leq 0.05$, and a tendency was considered at $0.05 < P \leq 0.10$.

Overall, concentrations of CH_4 were 147 ± 9.8 and 610 ± 9.4 ppm in BR and RE, respectively. An interaction ($P < 0.01$) was observed between the origin of the gas and the time of day that measurements occurred for CH_4 concentration (Figure 2). The greatest differentiation was observed at 1000 h, with CH_4 levels in BR being 80% lower compared with RE (Figure 2). The SESI-MS analysis indicated that a total of 324 and 242 features were consistently identified across all periods of the study in [M-H]$^-$ and [M+H]$^+$ MS mode, respectively, in BR and RE. Analysis from SESI-MS in [M-H]$^-$ mode detected a total of 69 features different ($P \leq 0.05$) and 21 with a tendency ($0.05 < P \leq 0.10$), between BR and RE, respectively (Figure 3A). In BR, 18 features had greater concentrations ($P \leq 0.05$), and 8 had a tendency ($0.05 < P \leq 0.10$) for greater concentrations compared with RE (Figure 3A). In the case of RE, 51 features were greater ($P \leq 0.05$), and 13 had a tendency ($0.05 < P \leq 0.10$) for greater concentrations compared with BR (Figure 3A). Along the features measured in [M-H]$^-$, the VFA acetic acid, propionic acid, and butyric acid were tentatively annotated with the *m/z* 59.0139, 73.0297, and 87.0452, respectively, as previously reported by Islam et al. (2023). The concentrations of acetic acid, propionic acid, and butyric acid were 20.9%, 27.4%, and 32.7% greater in RE compared with BR, respectively (Figure 3A). The highest differentiation in the [M-H]$^-$ mode was measured from *m/z* 87.0719, with 64.9% greater concentration in RE compared with BR ($P < 0.01$). From the SESI-MS analysis on [M+H]$^+$ mode, a total of 16 features were different ($P \leq 0.05$) and

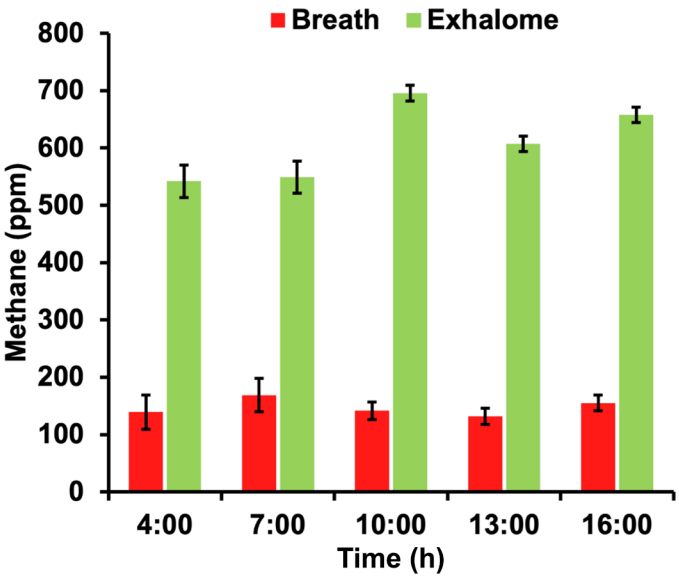

Figure 2. Concentrations of CH_4 in breath (BR) and ruminal exhaled (RE) samples from dairy cows at different hours across the day in dairy cows. Effect of origin: $P < 0.01$; effect of time: $P < 0.01$; and interaction between origin and time: $P < 0.01$. Error bars indicate SEM.

19 had a tendency to differ ($0.05 < P \leq 0.10$) between BR and RE, respectively (Figure 3B). In breath, 8 features had a greater ($P \leq 0.05$) concentration, and 11 had a tendency ($0.05 < P \leq 0.10$) for greater concentration compared with RE (Figure 3B). In the case of RE, 8 features were greater ($P \leq 0.05$) and 8 had a tendency ($0.05 < P \leq 0.10$) for greater concentration compared with BR (Figure 3B). The highest differentiation in the $[M+H]^+$ mode was found for the *m/z* 196.1137, with 41.1% greater concentration in the BR compared with RE ($P < 0.01$).

By implementing our sampling method, we were able to differentiate CH_4 concentration by up to 80% between BR and RE. It is essential to highlight that 100% separation of the VOC originating from the lungs and rumen is likely not feasible in practical setting. This can be explained by the uniqueness of the blending effect of the eructation, and the hindgut CH_4 synthesis and absorption. After an eructation event, more than 50% of the eructated gases are forced into the lungs, allowing the uptake of some eructated VOC through the lungs (Dougherty et al., 1964; Dougherty, 1968). In addition, in ruminants, around 90% of the CH_4 produced in the hindgut is excreted through the lungs (Murray et al., 1976). Additionally, the presence of CH_4 in the cows' breath is expected, as they are constantly inhaling from the environment in dairy barns (Rodrigues et al., 2024). Therefore, for the particular case of CH_4 as a marker, for the distinction between ruminal and breath VOC, it is expected to still be present to a smaller extent in the ruminant breath.

The recognition of eructation events, using real-time CH_4 as a marker, enabled us to differentiate VOC profiles originating from ruminal fermentation and breath. Through the SESI-MS untargeted analysis, we annotated acetate, propionate, and butyrate, as previously reported by Islam et al. (2023), to quantify their differential concentrations between BR and RE. As expected, these VFA products from the ruminal fermentation were found at greater concentrations in RE, as it contains the eructated VOC. However, the 80% proportional difference in CH_4 between BR and RE was not translated to other detected features. In addition to this, the relative differences among the VFA varied. In order of magnitude, acetate, propionate, and butyrate were 20.9%, 27.4%, and 32.7% greater in RE than BR, respectively. These results could be explained by the rate at which VOC are exhaled, which is determined by the retention rate of the molecules that remain in the respiratory tract after inhalation and exhalation (Haick et al., 2014). The primary property affecting the retention rate is the solubility of the compounds (Jakubowski and Czerczak, 2009; van der Schee et al.,

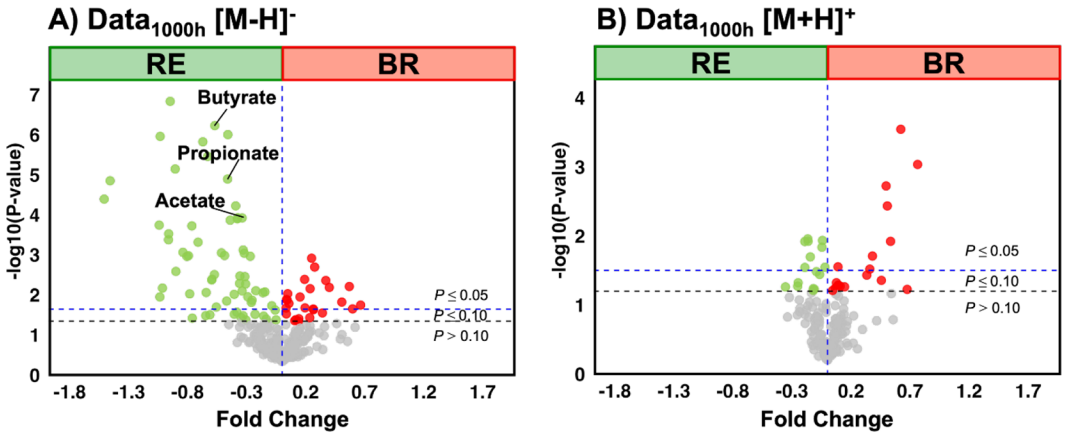

Figure 3. A volcano plot to differentiate the concentration of volatile organic compounds from secondary electrospray ionization-mass spectrometer analysis for negative A ($[M-H]^-$) and positive B ($[M+H]^+$) ion mode on breath (BR) and ruminal exhaled (RE) samples at 1000 h in dairy cows. The green and red circles indicate an increase in the abundance of features in RE and BR, respectively. Data shown include 1000 h time, which corresponds to the time of maximum CH_4 concentration difference between RE and BR. The vertical blue dotted line divides positive from negative fold change. The horizontal blue dotted line indicates the threshold for *P*-value ≤ 0.05. The black horizontal dotted line indicates the threshold for *P*-value ≤ 0.10.

2015; Oertel et al., 2018). The $\log_{10}$ of the partition coefficient of a solute (**logP**) metric is used to assess the compound's solubility under organic and aqueous conditions (Berger et al., 2012). The logP are −0.17, 0.33, and 0.79 for acetic acid, propionic acid, and butyric acid, respectively (Wishart et al., 2022). Considering the lower retention rate of butyric acid (which showed greater differentiation) compared with acetic or propionic acid in this study, it is reasonable to suggest that the solubility of the VOC played a role in the magnitude of the differentiation of the features measured between RE and BR. Nevertheless, further exploratory research is needed to determine the magnitude at which the VOC are differentiated between BR and RE.

Collectively, the implementation of CH_4 as a marker to track real-time eructation events offered a platform to construct a sampling method that revealed up to 80% lower CH_4 concentration in BR compared with RE. The greater concentrations of VFA observed in RE validate the ability of the proposed sampling method to differentiate between ruminal fermented products and breath. However, future research needs to be conducted to verify the capacity of the method to differentiate metabolic conditions under controlled experimentation. Breathomics is a promising tool for noninvasive assessment of dairy cows' performance and health, and our developed sampling method enables differentiation of VOC origins, allowing its implementation in dairy cattle research.

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Nonstandard abbreviations used: BR = breath; CPS = counts per second; GF = GreenFeed; logP = $\log_{10}$ of the partition coefficient of a solute; $[M+H]^+$ = protonated molecules (in positive ion mode); $[M-H]^-$ = deprotonated molecules (in negative ion mode); *m/z* = mass to charge ratio; RE = ruminal exhaled; RP = rumen protected; SESI-MS = secondary electrospray ionization mass spectrometer; VOC = volatile organic compounds.