A Comparative Investigation of Volatile Organic Compounds of Cattle Rumen Metabolites using HS-SPME and PoraPak-Q Odor Trapping Methods

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Abstract: In the rapidly increasing field of metabolomic research, fast and accurate trapping of volatile odor compounds from biological samples is critical. Here a comparative evaluation of HS-SPME and PoraPak-Q adsorbent odor trapping methods followed by GC-MS analysis were used to determine volatile compounds in cattle rumen. Compared to the PoraPak-Q adsorbent, the HS-SPME method was more effective in trapping diverse metabolites including low molecular weight and highly volatile compounds such as carbon dioxide, acetic and butyric acid. Additionally, using the HS-SPME method, shorter trapping times were achieved (30 minutes) whilst the PoraPak-Q adsorbent required longer time extending beyond 1 hour for effective volatile trapping. In the context of metabolomics analysis from biological samples, the two different methods vary in determination of chemo-diversities, qualitatively and abundance of shared odor, and time required to trap odors, which are critical in such studies.

Keywords: Gas chromatography-Mass spectrometry, Headspace Solid Phase Microextraction, PoraPak-Q adsorbent, Volatile odor compounds.

Introduction
Cattle rumen houses some of the most diverse and complex microbial colonies 1 capable of breaking down fibrous plant materials. Hence both ruminants and humans are able to derive energy and food from the interactions of rumen microbiome and plants 2, popularly called fermentation. However, this process has been attributed to production of numerous metabolites including greenhouse gases such as CO₂ and CH₄ 3. With the growing attention on such volatile organic compounds, little information exists on suitable techniques available for their extraction from biological matrices. Presently, many bioanalytical research like metabolomic studies prefer sample extraction procedures that demand relatively smaller sample volumes, rapid, high throughput, non-depletive, and easy to interphase with existing analytical instrumentation 4,5. Thus, choosing which odor collection method to use is of particular importance.

In the recent years, different odor collection techniques like HS-SPME 6, HS-SPME Arrow 7, stir-bar sorptive (SBSE) extraction 8 dynamic
headspace extraction, PoraPak-Q adsorbent extraction followed by Gas Chromatography have been used to determine volatile odors from various matrices including food products and urine.

In the present study, we compared the performances of HS-SPME and PoraPak-Q adsorbent, both applied in the direct headspace extraction method using the same rumen sample. Here, we show that the two methods demonstrated both qualitative and quantitative differences in relation to chemodiversity, relative abundance and trapping duration required.

Materials and methods
Sample collection
Rumen fluid was collected from five randomly selected freshly slaughtered male boran cattle (Bos indicus) from choice meats abattoir located in Kahawa west, Nairobi County. A minimum of 1 litre of rumen fluid was collected in sterile airtight freeze-resistant glass jars. The samples were kept in a cooler ice box and transported to the chemistry research laboratories at the International Center of Insect Physiology and Ecology (icipe), Duduville campus, Kasarani, Nairobi County where experimental studies were conducted. From the same cattle, rumen fluid was aliquoted into 200 ml each in two jars for odor trapping by the two methods.

Headspace-SPME (HS-SPME) trapping
Volatile rumen metabolites were trapped from 200 mL freshly obtained ruminal fluid samples in airtight 1L glass odor collection jars (Sigma Scientific, USA) by using the HS-SPME technique. A general purpose 65 μm PDMS/DVB (polydimethyl siloxane/divinylbezene) Supelco, Bellefonte, PA, USA, SPME fibers were used for this study (Fig. 1B). This type of SPME fibers are suitable for trapping of low molecular weight volatiles, amines including nitro-aromatic compounds. Additionally, more volatile polar analytes, like alcohols, are adsorbed more efficiently and released faster on this type of fiber. Prior to analysis, the HS-SPME fibers were conditioned by heating at 250°C (injector port temperature) for half an hour on a gas chromatography system GC-HP-7890B, Agilent Technologies, USA and later introduced into the sample headspace for volatile metabolite adsorption (Fig. 1D). The volatiles were adsorbed on a 65 μm PDMS/DVB (polydimethyl siloxane/divinylbezene), stableflex 24Ga, manual holder SPME fiber (Fig. 1B-C, Supelco, Bellefonte, PA, USA). The extractions were carried out at 37°C for 5, 15, 30 minutes and 1 hour, after equilibration of the samples for 15 minutes at the same temperature, ensuring a homogeneous temperature for sample and headspace.

Headspace-PoraPak-Q adsorbent trapping
Prior to extraction, PoraPak™-Q 50-80 mesh 30 mg adsorbents (Fig. 1A, Sigma Scientific, VCT USA) were cleaned and conditioned with 1 mL of GC-MS-grade hexane, followed by same amount of dichloromethane (DCM). Volatiles were collected from 200 mL freshly obtained ruminal fluid sample in an airtight 1L glass odor collection jar (Sigma Scientific, USA) using a dynamic headspace volatile extraction technique comprised of a portable volatile collection pump, PoraPak™-Q adsorbent (Sigma Scientific, USA) 13,14. The PoraPak-Q adsorbent was attached to a Teflon tube connected to a portable odor collection pump and placed onto the sample headspace (Fig. 1E). Clean air was pumped at the rate of 2.5 L/min on the sample to facilitate volatile equilibration at the headspace and a pull set at 2 L/min to draw volatiles through to the adsorbent for adsorption. The experiment was set to run for 5 minutes, 15 minutes, 30 minutes and 1 hour respectively each time using newly acquired clean PoraPak-Q adsorbent. Adsorbed volatiles were desorbed from the PoraPak-Q adsorbent by passing through 300 μl GC-MS-grade hexane and the eluate was collected into a 1.5 mL vial.

GC-MS analysis
HS-SPME adsorbed volatiles and PoraPak-Q adsorbed volatiles were analyzed by Gas chromatography GC-HP-7890A, Agilent technologies, USA) coupled with quadruple mass analyzer Mass spectrometry system (MS-
Figure 1. Photo showing odor extraction materials and methods used in this study: (A) PoraPak-Q adsorbent (B) SPME fibers (C) SPME holder (D) HS-SPME rumen odor collection setup and (E) Dynamic Head space with PoraPak-Q adsorbent set up

5975C, Agilent technologies, USA) with a slight variation in sample injection. Whereas HS-SPME adsorbed volatiles were introduced into the GC-MS instrument by manual injection of the SPME fiber into GC-MS inlet port, 1 μl of PoraPak-Q adsorbed volatile eluate were automatically injected to the GC-MS using an auto sampler (7683B series, Agilent Technologies, USA). Desorption of the HS-SPME volatiles were conducted at the injection port fitted with straight inlet liners without glass wool, 2 mm id (Agilent technologies, USA) at 250°C for 2 minutes in splitless mode 15. Chromatographic separation of the volatiles was achieved by HP-5MSI, 30m X 0.25 mm i.d, 0.25 μm thick capillary column (J & W scientific, USA) immobilized with 5% (phenyl methyl silicone) as the stationary phase. Helium gas (99.99% purity, Air Products & Chemicals, USA, through local supplier Chemigas Ltd Kenya) was used as the carrier gas at a flow rate of 1.2 mL per minute. Initial oven temperature was programmed at 30°C where it was held for 5 minutes followed by a progressive increase at the rate of 10°C/min to 280°C where it was held at an isothermal state for 10.5 minutes. The mass spectrometry (MS) detector was operated in the
scan mode within a mass range of 16 to 550 m/z at 1 scan s⁻¹, with electron energy of 70 eV. The MS ion source was set at 230°C while the mass quad set at 150°C. The total analysis time was 35 minutes.

**Data processing**
After chromatographic analysis, all the data were analyzed with the Agilent MSD productivity chemstation software for GC and GC/MS systems (Agilent technologies, USA). The integration was done with probability based matching algorithm, initial peak width set at 0.034 and initial threshold of 15.7. Individual compounds were identified by computer-aided comparison based on their retention times and respective mass spectral data against the MSD library (NIST, 2005, NIST 05a, and Adams MS HP, USA). The compounds were considered as correctly identified when their spectra showed a minimum probability match factor >80% 16,17. Compounds that appeared in at least 3 out of 5 cattle rumen samples were considered as positively present in cattle rumen.

**Statistical analyses**
To establish the variations in the identified volatile organic compounds extracted by the two extraction techniques, chromatogram profiles were assessed based on the relative abundance of individual compounds and later analyzed using PAST statistical software Version 4.02. Additionally, the number of identified volatiles extracted by both HS-SPME and PoraPak-Q adsorbent including their mean values and standard error at different extraction durations were calculated from the five cattle rumen sample replicates using independent t-test of GraphPad Prism version 9.

**Results and discussion**
In the present study, two methods were used to provide an overview of the chemical diversity exhibited by cattle rumen. Observed GC-MS chromatograms (Fig. 2) shows significant variation in the type and relative abundance of volatile compounds extracted by both HS-SPME and PoraPak-Q adsorbent. The study reveals presence of diverse chemical entities harbored by cattle rumen. Greenhouse gases (carbon dioxide), volatile fatty acids (acetate & butyrate), phenolic compounds (p-cresol), monoterpenes (limonene, camphene), and hydrocarbons (eicosane) which are metabolic products of ruminal fermentation induced by microorganism 18.

Generally, popular volatile odors like p-Cresol, limonene, camphene were the most abundant in both methods. Such volatiles which are mainly analogous to cattle, have been demonstrated to affect the host-vector interaction 13 by acting as chemical cues for host-seeking vectors 13,19. Using the HS-SPME-GC/MS, we show the presence of CO₂, a major greenhouse gas 20 and a chemostimuli for stable flies, mosquitoes, and other vectors 21,22, but was not detected in PoraPak-Q adsorbent (Fig. 2 and supplementary Fig. S1 & Table S1). Furthermore, short chained volatile fatty acids like acetic and butyric acid which are essential in energy metabolism and protein synthesis in livestock 3 were determined exclusively by HS-SPME-GC/MS technique.

The number of extracted volatile compounds increased progressively with increased extraction time, thus more volatile compounds were extracted at 1 hour extraction period than 5 minutes for both HS-SPME and PoraPak-Q adsorbent (Fig. 3B). Moreover, there was notable variation in the number of extracted volatile compounds between the two techniques at different extraction durations (Fig. 3A&B). Whereas trapping time affect efficiency, absorptive affinity of odor compounds on the adsorbent, and subsequently the analysis performance 23, this study reveals that it might also affect the odor diversity. More volatile odors were determined as the extraction period was extended for both methods (Fig. 4).

The optimal trapping period for each method differed based on the compounds’ competitive absorption on the adsorbents. For instance, at 30 minutes of extraction time, the SPME fiber captured most of the metabolites and extending this time further yields no qualitative significance. However, PoraPak-Q adsorbent demands extended trapping period running beyond 1 hour (Fig. 3 & 4). Considering the higher extraction
Figure 2. Representative GC-MS chromatogram of cattle rumen volatile odor profile trapped using HS-SPME and PoraPak-Q extracted volatiles from rumen fluid for 5 minutes (A), 15 minutes (B), 30 minutes (C) and 1hr (D) extraction times, (E) structure of some selected compounds
Figure 3. (A) Heat Map coded matrix showing relative percent contribution of individual volatiles to the total composition of analyzed compounds extracted by each method. (B) Box plot representation of mean number of identified compounds extracted by both HS-SPME and PoraPak-Q adsorbent at different time durations. *, P < 0.001 independent t-test, for 15 minute, Mann Whitney test, P=0.008, n=5

durations for most compounds, 30 minutes was the optimal trapping time duration for the direct insertion HS-SPME-GC/MS method and 15 minutes as the minimum time required for trapping odors from cattle rumen fluid. On the other hand, PoraPak-Q required at least 1 hour for significant odor trapping. 

The simplicity and efficiency in quick sampling of HS-SPME method as demonstrated in the study qualifies the technique for investigation
of odor, including highly volatile odors such as CO\textsubscript{2} profiles in biological matrices. Thus, it is well-suited for the study of biodegradation pathways of metabolites\textsuperscript{5}. However, it demands extreme care especially when handling to avoid fiber breakage. Additionally, complete quantification of compounds is still a bottleneck when using HS-SPME technique, however it is possible to use this approach to correlate the relative abundances of compounds among samples when the same analytical procedure is used. The inability to elute and use the extract for other experiments, which can easily be done when PoraPak-Q used is the other drawback of using HS-SPME. Finally, the HS-SPME-GC/MS technique described here is a promising approach for bioanalytical and metabolomic studies which demand rapid, non-destructive, and efficient analysis methods.

Conclusions
Odors from host are emitted in a spatiotemporal dynamic in a small amount. HS-SPME success in trapping odors including greenhouse gases in a short period of time clearly shows their potential for spatiotemporal odor dynamics study and expand the range of bio-chemical research questions that is possible to address. However, a longer period of time is required for effective odor trapping when using PoraPak-Q adsorbent method.

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Supplementary data
Fig. S1 and Table S1 are given as supplementary information.

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