Characterization of volatile organic compounds and odors by *in vivo* sampling of beef cattle rumen gas using solid phase microextraction and gas chromatography-mass spectrometry-olfactometry

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Abstract Volatile organic compounds (VOCs) and odors in cattle rumen gas were characterized using *in vivo* headspace sampling with solid phase microextraction (SPME) coupled with gas chromatography-mass spectrometer-olfactometry (GC-MS-O) analysis. A novel device allowing for headspace SPME (HS-SPME) sampling through the cannula was designed, refined, and used to collect rumen gas samples from steers. Carboxen/polydimethylsiloxane (PDMS) fiber (85 μm) was used in the SPME sampling. Fifty VOCs belonging to 10 chemical functional groups were identified in the rumen headspace. The identified VOCs had a wide range of molecular weight (MW) (34 to 184), boiling point (-63.3 to 292 °C), vapor pressure (1.05×10^{-5} to 1.17×10^{2} Pa), and water solubility (0.66 to 1×10^{6} mg/L). Twenty two compounds have a published odor detection threshold (ODT) of less than 1 ppm. More than half of the identified compounds are reactive and have an estimated atmospheric lifetime of < 24 hr. The amounts of VFAs, sulfide compounds, phenolics and skatole, and odor intensity of VFAs and sulfide compounds in the rumen gas were all higher after feeding than those before feeding. These results indicate that rumen gases can be an important potential source of aerial emissions of reactive VOCs and odor. *In vivo* sampling via SPME coupled with GC-MS-O analysis can be a useful tool for qualitative characterization of rumen gases, digestion, and its relation to odor and VOC formation.

Keywords Rumen gas · Odor · *in vivo* sampling · SPME · GC-MS-O

Introduction

Rumen headspace is saturated with compounds produced during digestion. Degradation of feed in the rumen is the processes of fermentation resulting from the physical and microbiological activities that digest feed under anaerobic conditions. These products could be useful (VFAs, microbial proteins, B-vitamins), useless (CH_4, CO_2), or even harmful (ammonia, nitrates) for the host animal [1]. Composition of ruminal fluid has implications on the digestion processes. Thus, chemical composition of ruminal fluid is important for nutritional studies. Feed utilization and feed additives can have an impact on odor and gas emissions from manure. Cattle production is associated with aerial emissions of odor, VOCs and other gases originating mainly from manure and the animals themselves. Chemical compositions of rumen liquid and gas can have implications on air quality. Rumen gas can be released to atmosphere via eructations and exhaled breath. Digested products from rumen can be also released with manure and therefore be a source of aerial emissions of VOCs and odor.

Numerous methods are available for the collection of rumen fluid for analysis. Rumen fluid can easily be collected through a cannula that is surgically placed in the rumen [2]. Other approaches with animals without rumen cannula either involved the use of stomach tubes [3] or percutaneous needle aspiration (rumenocentesis) [4]. Both techniques are stressful to the animals. Samples taken by stomach tube are often contaminated with saliva [3] and rumenocentesis has led to infections in some cases [4].

Characterization of fermentation products is used in assessing the extent and nature of the microbial fermentations [5]. Several methods are used for the quantification of these products. High-performance liquid chromatography was used to quantify ethanol, n-butanol and VFAs in the early 1980s [6]. Gas chromatography has been commonly employed to quantify VFAs and alcohols in rumen fluid [7, 8, 9, 10, 11, 12] since the 1960s. Most of these methods involve time-consuming sample preparation procedures. Solvent extraction with ether [12] or methylene chloride [10] and pre-injection derivatizations of acids [7, 13, 14] are often used. Comparison of sampling and analytical method between the present and previous studies to determine the rumen fermentation products in rumen fluid is presented in Table 1 [2, 9, 10, 11, 15, 16, 17].

To date, nearly all studies focused on the characterization of ruminal fluid itself. Relatively little is known about the composition of rumen gas and its implication for gaseous emissions. Sampling of gas instead of liquid is more challenging from the analytical standpoint. However, one benefit is a minimization of multiphase liquid-solid sample matrix which requires extensive sample preparation. Measurement of gases produced by rumen microbes could be very useful in evaluating diets, animal health status, feed additives, dietary amendments, and rumen fermentation [15].

Only one study reported sampling and analysis of rumen gas to gain information about rumen processes [15]. Dewhurst et al. [15] investigated certain gases in rumen headspace using active gas collection to 2L food-grade polyethylene terephthalate (PET, also referred to as Melinex) bottles with rubber stoppers for on-site and next day analyses in laboratory using selected-ion-flow-tube mass spectrometer. A total of 14 gases, including several alcohols, ammonia, five VFAs (from acetic to hexanoic), acetone, acetaldehyde, and H_2S and other sulfides were reported [15]. However, potential sample recovery problems and uncertainties associated with quantitative analysis of gas samples in rumen still exist with this approach and similar conventional sampling methods. This is due to the porous nature of polymeric materials used for sampling containers, adsorption to walls, condensation and partitioning to water, reactivity of gases and reactions between gases inside the sample container, and false positives caused by gases emitted by sampling containers.

Poor sample recoveries for VFAs were reported when PET sampling bags were used [18, 19, 20]. Mean gas sample recoveries were 66.1% for 7 VFAs from acetic to hexanoic acid, respectively, after 24 hr storage time at room temperature in PET bags [18]. These recoveries were 27.6%, 61.4%, 73.9, 51.1%, and 38.2% for acetic, propanoic, butanoic, pentanoic, and hexanoic acids, respectively [18]. The PET bags were also not recommended for the collection and 24 hr storage of H_2S or ammonia [20]. Alcohols, VFAs, ammonia can also readily partition to water in air samples. Acetaldehyde is a reactive gas and is typically sampled via derivatization [21].

No olfactometry analyses were reported on rumen liquid or gas in previous studies. Odor analysis could provide additional insight to the specific makeup of gas, particularly in some cases where human nose is more sensitive than conventional

analytical detectors. Many compounds well known to be in rumen liquid are also known to be offensive odorants and are emitted from manure. Thus, the link between specific diet, rumen gases and livestock odor warrants research.

Solid phase microextraction eliminates the use of sampling containers and it combines sampling and sampling preparation into one step. Air sampling with SPME presents many advantages over conventional sampling methods [22, 23, 24, 25] due to its simplicity, reusability, very good sample recovery [18] and hydrophobic behavior of SPME coatings. Koziel et al [18] reported average 105% (±11.4%) recoveries of gaseous VFAs (from acetic to hexanoic acid) at room temperature and 24 hrs storage time from the 75 µm Carboxen/PDMS SPME fiber coatings [18]. The variability (measured as standard deviation) for recoveries of VFAs were as low as 2.0%, 3.6%, 9.7%, and 5.6% for propanoic, butanoic, pentanoic, and hexanoic acids, respectively. Spinhirne and Koziel used SPME to sample the headspace gases of closed *in vitro* cultures to evaluate ruminal fluid and ruminal fluid with feed containing a feed additive using GC-MS [16]. Spinhirne et al. [26] reported the use of SPME for on-site breath sampling of steers and characterization of 21 VOCs [26].

This study was conducted to characterize volatile organic compounds (VOCs) and odors in cattle rumen gas through *in vivo* sampling of the rumen gas. In this research, a novel device allowing for headspace SPME (HS-SPME) sampling through the cannula was designed, refined, and used to collect rumen gas samples from steers. Rumen gas samples were analyzed using a GC-MS-Olfactometry system allowing for simultaneous VOCs/odor qualitative characterization [27, 28, 29]. To our knowledge, this is the first of this kind of investigation to conduct *in vivo* SPME and evaluating rumen gas odor.

Experimental

Rumen Gas Sampling Device

A novel device (Figure 1a,b,c) allowing for headspace (HS) SPME sampling through the cannula was designed, refined and used to collect rumen gas samples from three steers for three days. This device uses a cannula stopper modified with a sealed septum port for insertion of SPME fibers. The objective was to (1) modify a typical cannula plug

with common, low cost materials, (2) make the modified plug easily removable for replacement with a regular plug, (3) make the modified plug safe to animal while sampling, (4) make the separation of rumen gas and fluid possible, (5) to provide a means of SPME insertion into the sealed rumen headspace, and (6) to protect the fragile SPME fiber assembly from possible damage by floating undigested and partially digested feed and rumen fluid. A PVC 'snorkel' was constructed internally to protect the SPME fibers from contact with the rumen fluid/forage mixture while allowing the fibers to interact with the headspace gases. The main tubing and screen for the device were made from 6 cm dia PVC tubing and bushings purchased at a local hardware store (Lowes, Ames, IA). The 'snorkel' was fixed to the cannula plugs with bushings. A 3 mm dia (1/8") bulkhead fitting (Swagelok, Kansas City, KS) was mounted in the center of the plug. A thermogreen half-hole septum (Supelco, Bellefonte, PA) was inserted into the bulkhead fitting and held tight with the Swagelok nut to seal and to guide the SPME needle into the rumen headspace. All dimensions are provided in Figure 1c.

Sampling of Rumen Headspace Gas with SPME

Carboxen/PDMS SPME fiber (85 µm) (Supelco, Bellefonte, PA, USA) was used for rumen headspace gas sampling. Carboxen/PDMS fiber coating has proven to be very effective in extracting VFAs and sulfides [30, 31], i.e., the types of compounds known to be in rumen gas [15]. Carboxen has small diameter (10A on average) pores which are suitable to adsorb molecules in the C2-C12 range [24]. Fibers were conditioned according to the manufacturer's instructions. Fiber assemblies had their tensioning spring removed and samples were collected manually, i.e. without SPME holder. Before sampling, fiber was desorbed for 5 min at 260 ºC, then wrapped in clean aluminum foil. Tight wrapping of SPME assemblies in aluminum foil sealed the fibers from the ambient environment. The operator wore nitrile gloves and avoided direct contact with the SPME needle to minimize interferences. SPME fibers were transported to and from the laboratory enfolded in aluminum foil, placed inside a clean jar with tight cover and placed in an ice cooler immediately after sampling.

Three rumen cannulated (101 mm I.D.) Angus steers (868 ± 49 kg body weight) were fed 27.2 kg rations of Fescue grass hay twice daily (8:00 hr and 16:00 hr) in individual (3.7 m × 12.6 m) pens. The feed were weighed before each feeding. Water was available *ad libitum*. All sampling was conducted on October 9th – 11th, 2005 at the Iowa State University Beef Nutrition Center, Ames, Iowa. The steers were individually restrained in a hydraulic chute during the SPME sampling.

Rumen gas samples were collected before morning feeding (9:00 am) and 2 hours after feeding (1.00 pm). For each animal, the cannula stopper was replaced with the modified sampling device. For each cattle, sampling device was fitted in rumen cannula for 5 min before SPME sampling to allow rumen gases to reach equilibrium inside the headspace of sampling device. During SPME extraction the septum fitted in the sampler was pierced using the SPME needle and exposed the SPME fiber to the headspace for 5 min. These sampling times were selected based on animal wellbeing considerations and on previous experience with restraining steers in hydraulic chutes [26]. After SPME sampling was complete, SPME fiber was enfolded in aluminum foil to be transferred to the Atmospheric Air Quality Laboratory at Iowa State University to be analyzed. The desorption time of SPME fiber was 40 min at 260 ºC. The same sampling device, i.e., the modified cannula plug was used for all 3 steers. Thorough rinsing with hot water and air drying was used to clean the device between applications. HS-SPME extraction of blank device did not result in significant amounts of target analytes selected for analyses.

Analysis of Rumen Gases

Multidimensional GC-MS-O (Microanalytics, Round Rock, TX, USA) was used for all analyses [27, 28, 29]. The system integrates GC-O with conventional GC-MS (Agilent 6890N GC / 5973 MS, Wilmington, DE, USA) as the base platform with the addition of an olfactory port and flame ionization detector (FID). The system was equipped with a non-polar precolumn and polar analytical column in series as well as system automation and data acquisition software (MultiTrax™ V. 6.00 and AromaTrax™ V. 6.61, Microanalytics and ChemStation™, Agilent). The general run parameters used were as follows: injector, 260 °C; FID, 280 °C, column, 40 °C initial, 3 min hold, 7 °C

/min, 220 °C final, 10 min hold; carrier gas, He. Mass to charge ratio (m/z) range was set between 33 and 280. Spectra were collected at 6 scans/sec and electron multiplier voltage was set to 1200 V. The MS detector was auto-tuned weekly.

The identity of compounds was verified using (a) reference standards (Sigma-Aldrich, Fisher, Fluka) and matching their retention time on multidimensional GC capillary column and mass spectrums; (b) matching mass spectrums of unknown compounds with BenchTop/PBM (Palisade Mass Spectrometry, Ithaca, NY, USA) MS library search system and spectrums of pure compounds, and (c) by matching the description of odor character. Rumen gas was analyzed only qualitatively. Rumen gas abundance was measured using area counts under peaks of characteristic single ions for separated gases. The peak area counts were reported for comparisons only. One human panelist was used to sniff separated compounds simultaneously with chemical analyses. Odor caused by separated gases was evaluated with a 64-descriptor panel and intensity scale in Aromatrax software [27, 28, 29]. Odor evaluations consisted of comparisons of (a) the number of odor events and (b) the total odor measured as the product of odor intensity and odor event time length recorded in an aromagram. Aromagrams were recorded by panelists utilizing the human nose as a detector. Odor events resulting from separated analytes eluting from the column were characterized for odor descriptor and odor intensity.

Results and Discussion

Effects of SPME extraction time on VOCs and odor of rumen Gas

The effects of SPME extraction time 1 min, 5 min (triplicate) and 10 min, respectively, at the fixed temperature of 39 ºC inside the headspace of rumen for selected compounds is presented in Figure 2. These selected compounds were the main rumen fermentation products and also compounds which significantly contributed to the offensive odor of rumen gas. Odorous gases included most of the well known gases emitted from cattle and swine operations [28], e.g., VFAs, volatile sulfur compounds (VSCs), phenolics and indolics. The amount of each selected compound extracted by

SPME fiber increased with the sampling time except for H_2S. This could be due to the Carboxen-PDMS coating's limited number of adsorption sites and possible competitive adsorption and displacement. Higher MW compounds, e.g., semi-VOCs, can displace lower MW compounds as a consequence of competition for active sites on the fiber [25], particularly for complex matrices. This can be minimized when shorter extraction times are used [23, 32]. In this study, 5 min was selected for all target compounds. This sampling time was selected due to the feasibility of restraining the steer for a limited time of sampling.

The repeatability of *in vivo* rumen gas sampling using the modified cannula/SPME port sampler was evaluated by comparing 3 replicate rumen gas samples. Average RSD of selected compounds except propanoic acid was 26 %. The RSDs for octene isomers, phenolics, indole, alcohols were less than 20%. Both the VSCs and VFAs had much greater RSD ($\geq$ 30%). Compounds, H_2S, 3-methyl thiophene, 4- methyl phenol and indole had RSDs of 19%, 13%, 18% and 16%, respectively. This is likely because of the relatively short sampling time and the dynamic nature of the rumen headspace. The dynamic nature of rumen gas was also implicated as a possible source of uncertainties by Dewhurst et al. [15].

Effect of SPME extraction time on total odor and the total number of odor events for the series of aromagrams of rumen gas is shown in Figure 3. The total odor was estimated as the sum of products of odor duration and odor intensity for all odor events in all time series samples of rumen headspace. As can be seen in Figure 3, longer extraction time resulted in a significant increase in the total odor and total number of odor events.

Sixteen characteristic odors that were most frequently present in rumen gas were selected for further evaluation of the effects of SPME sampling time on odor (Figure 4). These characteristic odors were correlated with corresponding compounds (Table 2). Data presented in Figure 4 indicates that the odor intensity of most of those characteristic odors increased with longer sampling time. Particularly noteworthy were 'mushroom/moldy (1-octen-3-one)' and 'taco shell (2'-aminoacetophenone)'. The presence of these odor-causing compounds could be easily overlooked in a conventional analysis of MS chromatograms. This is because they were present in rumen gas at very low concentrations and the resulting MS detector responses were not within the

background signal. Only the use of more sensitive detector (i.e., human nose) and matching of aromagrams with total ion chromatogram (TIC), that is possible with the GC-MS-O approach, allowed us to identify those compounds. We were able to identify them only because of their significant odor intensity and the characteristic odor perceived by olfactometry panelist. Both 1-octen-3-one and 2'-aminoacetophenone are very potent odorants. The odor detection threshold in air (ODT_{air}) for 1-octen-3-one is 0.03-1.12 ng/L [33]. The ODT_{air} of 2-aminoacetophenone is not published. However, its ODT in water (ODT_{water}) is 0.2 µg/L [33].

Identification of VOCs in rumen headspace

Rumen gas samples were analyzed on a multidimensional GC-MS-O system allowing for the simultaneous identification and analysis of chemicals and corresponding odors and the collection of a chromatogram and aromagram. Comparison of a typical chromatogram (lower, red line) and aromagram (upper, black line) of rumen gas after feeding is shown in Figure 5. A variety of compounds with wide range of odor characteristics were found. Total ion chromatogram typically showed a complex peak/compound pattern. The 50 compounds with most prominent peaks are listed in Table 2. These compounds are typical rumen fermentation products such as VFAs and VSCs have been reported in the literature [15]. The aromagram (upper, black line in Figure 2) recorded as many as 38 distinct odors in rumen gas. The majority of odors detected in rumen gas were perceived as offensive (Table 2).

Fifty VOCs belonging to 10 chemical function groups were identified in rumen gas, i.e., sulfides and thiols (8), VFAs (7), ketones (4), alkanes (17), alcohols (2), phenolics (4), benzenes (3), nitrogen heterocycles (3), aldehydes (1), and monoterpenes (4), of which 37 have never been reported in previous studies of rumen fluid and gases [9, 10, 11,12,15,16,17, 33] (Table 2). It is interesting to mention that chemical compound groups identified in this study were similar to those found previously in ambient air at a dairy farm [34] except for VSCs. One new chemical group found in rumen gas in this study was monoterpenes. Four monoterpenes including α-pinene, camphene, sabinene and limonene were identified in rumen gas. Sunesson et al. [34] reported that

monoterpenes were found in ambient air in dairy farm and attributed the sawdust used for bedding as being the main source. Wood is well known source of monoterpenes. In this study, other sources (e.g., *Fescue* grass in the feed) could be the source of monoterpenes [35]. Thus, eructated rumen gas could be another potential source of monoterpenes in ambient air in and around cattle feedlots and possibly dairies. However, more research is needed to confirm this hypothesis. Rabaud et al. [36] reported that the vast majority of compounds emitted from a commercial dairy such as VFAs, alcohols, aldehydes and ketones resulted from carbohydrate oxidation and fermentation during and after digestion.

Thirty four out of 50 compounds identified in this study were confirmed with the retention time and spectrums of authentic standard compounds (Table 2). The remaining 15 were identified with BenchTop/PBM mass spectrometry library search system (match above 70%) and by matching their known odor character [37]. Identified VOCs had a wide range of molecular weight (MW) (34 to 184), boiling point (-63.3 to 292 °C), vapor pressure (1.05×10^{-5} to 1.17×10^{2} Pa) and water solubility (0.66 to 1×10^{6} mg/L). As many as 22 compounds had a published ODT less than 1 ppm [33]. Four compounds including 2-butanone, toluene, phenol and p-cresol are classified as HAPs [38]. As many as 54% of compounds had the estimated atmospheric lifetime < 24 hours based on the reaction with OH radicals. Estimating actual emissions of reactive organic compounds from rumen gases could be useful in emission inventories of in protected airsheds with large cattle population.

Comparison of rumen gases between before and after feeding

The feasibility of rapid testing of rumen gas with SPME to elucidate useful information related to digestion was demonstrated by sampling before and after feeding. Twelve odorous gases were then selected for further comparisons. Odorous gases included the well known gases emitted from manure, e.g., VFAs, VSCs, phenolics and skatole. A qualitative comparison of the 12 rumen gas compounds before and after feeding for three animals and 3 days is shown in Figure 6. Carbon 2-C6 short-chain fatty acids were identified, including acetic acid, propanoic acid, butanoic acid, 3-methyl butanoic acid, pentanoic acid and hexanoic acid. Volatile fatty acids are the main products of bacterial fermentation in the rumen and are absorbed through the rumen wall

into blood stream and form the primary energy source for the host animal [39]. Low molecular weight VFAs had been used to determine the energetic efficiency of microbial fermentation in the ruminant [16]. Many researchers believe that C2 to C9 VFAs are the most important odor indicators compared to all other volatile organic compounds (VOCs) found in agricultural air [40, 41]. All VFAs had higher relative abundance in rumen gas after feeding compared with their abundance before feeding. This could be due to the pH of the rumen fluid which is very responsive to meals and chewing behavior; ruminal pH decreases rapidly following meals and increases rapidly during rumination [42]. At lower ruminal pH after feeding, a greater fraction of the VFAs was previously observed in the associated form in rumen fluid, so the concentration of VFAs in rumen gas would likely increase. Dewhurst et al. [15] observed that the relative concentrations of VFAs in rumen gas and rumen fluid decreased with increasing chain length. In addition, the overall molar proportions of VFAs were very similar between rumen fluid and rumen gas [15].

The apparent amounts of dimethyl sulfide, dimethyl disulfide and dimethyl trisulfide in rumen gas after feeding were much higher than those before feeding. Dimethyl sulfide after feeding was more than 10-fold higher than that before feeding. These observations were consistent with the significant levels of production of dimethyl sulfide by the rumen reported previously [43, 44] and consistent with the higher levels of dimethyl sulfide production instantly after feeding [45]. It is noteworthy that dimethyl sulfide (DMS) is an inverse ‘greenhouse effect’ gas [45, 46] and may be released to atmosphere by cattle eructation. Besides VFAs and VSCs, phenol, 4-ethyl phenol and skatole was also present in higher amounts in after feeding rumen gas. While these preliminary observations are generally consistent with literature had only limited statistical significance due to relatively small number of replications (3 steers and 3 days). Only phenol showed significant difference between before and after feeding (p-value=0.0391).

Comparison of the difference of total odor and characteristic odors associated with the rumen gas samples collected before and after feeding of three animals and 3 days is shown in Figure 7. There were no significant differences in the total odor measured with GC-O approach of rumen gas between before and after feeding (p-value=0.9293). However, the comparison of the characteristic odor was consistent with

the specific compounds discussed above. The odor intensity in rumen gas caused by VFAs and VSCs after feeding was higher than that before feeding. One of the characteristic odors, i.e., 'onion, foul' (caused by dimethyl sulfide) showed significant difference (p-value=0.0049) which is consistent the increase of dimethyl sulfide in the rumen headspace (p-value=0.0867).

More research is needed to quantify gases in the rumens headspace and to determine the rumen liquid-gas correlations related to digestion. Rumen gases reflect the processes of fermentation within the entire reticulo-rumen system. Thus, *in vivo* sampling of rumen gases may overcome some of the sampling challenges related to compartmentalization and variation across the rumen [3]. The information about rumen gases can be potentially used to assess the extent of digestion of feed in the rumen [2, 15] and to diagnose disease or rumen disfunction.

Conclusions

Several conclusions can be made from this study:

1. New device proved useful for *in vivo* rumen gas collection with SPME in field conditions. Sampling times as long as 10 min were practical. Longer extraction times may be possible with free-ranging steers, if the septum port is protected.
2. SPME-GC-MS-O can be a useful technique to monitor feed digestion *in vivo* and to observe the relation between feed and odor/VOC emissions from beef cattle operations.
3. Rumen gas contains at least 50 VOCs belonging to 10 chemical functional groups. In this research, 34 were confirmed with pure standards. Identified compounds had a wide range of MW, boiling point, vapor pressure and water solubility. New chemical group found in rumen gas were monoterpenes.
4. Many of the most offensive and characteristic odorants associated with livestock production were found in rumen gas. Odorous gases included those emitted from manure such as VFAs, VSCs, phenolics, and indolics. As many as 22 compounds had an ODT < 1 ppm. These results indicate that rumen gases could be a source of aerial emissions and odor. Amendments to the rumen environment could potentially have implication to odor control.

5. More than half the rumen gas compounds identified in this research are reactive and have an estimated atmospheric lifetime of < 24 hours. At least one gas (dimethyl sulfide) is suggested as an inverse 'greenhouse effect' gas. More research is warranted to determine actual concentrations and emissions of these rumen gases to atmosphere as they may be important odor sources in areas with large cattle populations.

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FIGURE CAPTIONS

Figure 1. Schematic of device for *in vivo* sampling rumen gas with SPME. Part A: cattle rumen with modified cannula and SPME sampling device. Part B: cross section of rumen. Part C: Modified cannula with SPME in rumen headspace.

Figure 2. Effect of time on HS-SPME extraction of 16 compounds from rumen gases before feeding at fixed temperature 39 ºC inside cattle rumen with 85 µm Carboxen/PDMS fiber. Extraction time = 1 min, 5 min (n = 3), and 10 min. Error bars signify the standard deviation of the mean. Number in parentheses is the single ion of each compound used for peak area count integration.

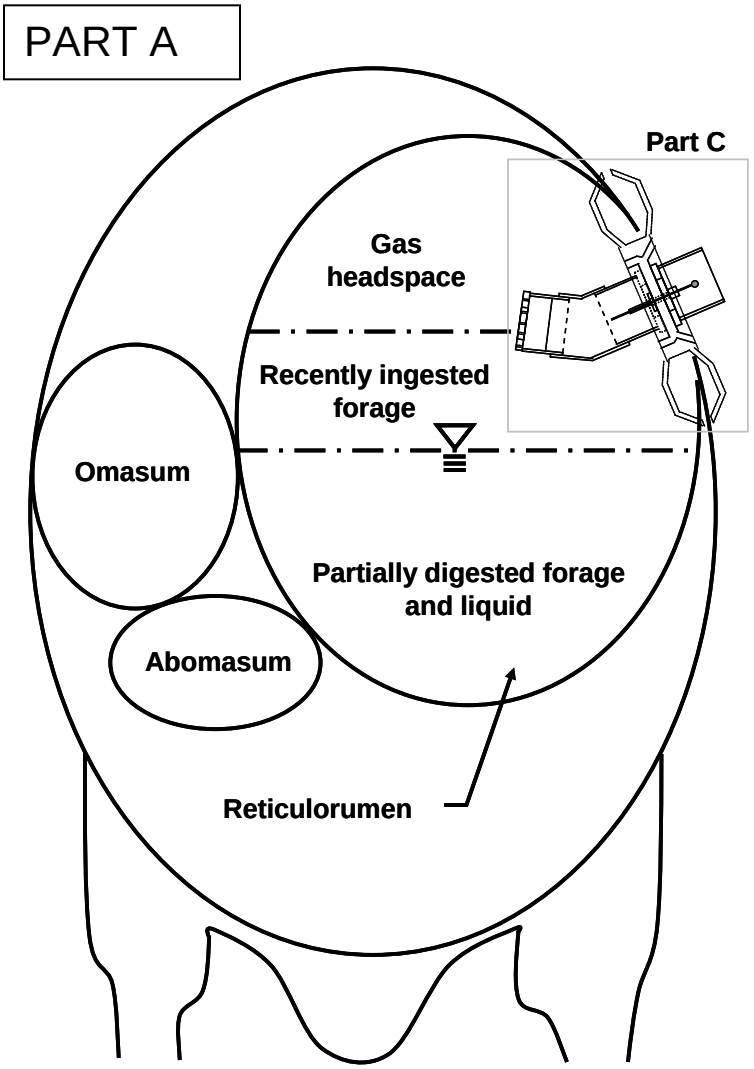
Figure 3. Effect of time on HS-SPME extraction of total odor from rumen gases before feeding at fixed temperature 39 ºC inside cattle rumen with 85 µm Carboxen/PDMS fiber. Extraction time = 1 min, 5 min (n = 3), and 10 min. Error bars signify the standard deviation of the mean. Number signifies total odor events in a sample.

Figure 4. Effect of time on HS-SPME extraction of 19 characteristic odors from rumen gases before feeding at fixed temperature 39 ºC inside cattle rumen with 85 µm Carboxen/PDMS fiber. Extraction time = 1 min, 5 min (n = 3), and 10 min. Error bars signify the standard deviation above the mean.

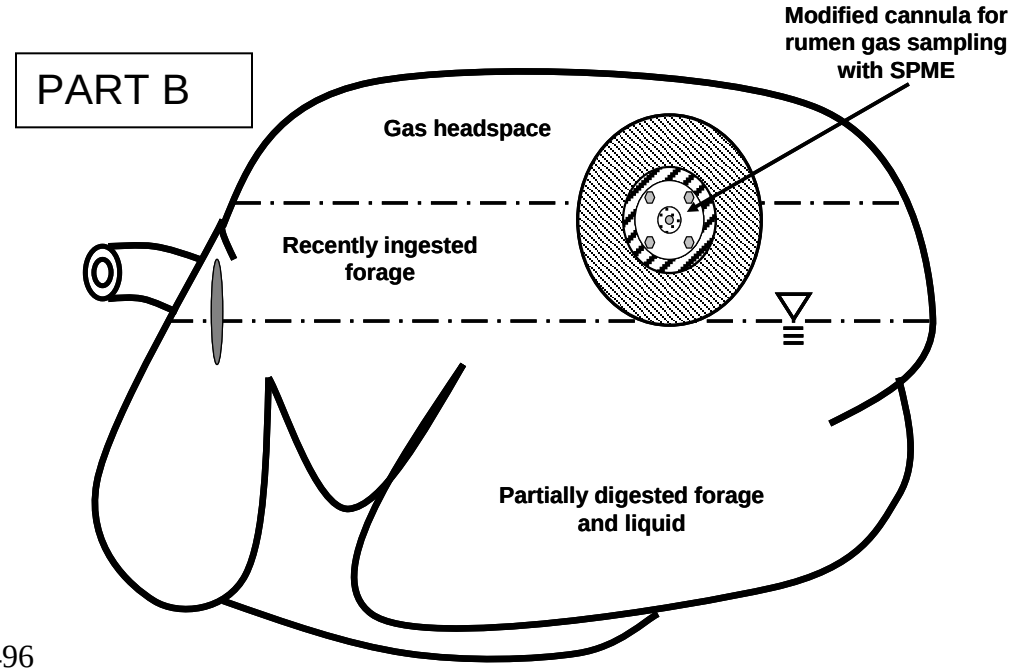
Figure 5. Total ion chromatogram (TIC) (lower, red line) and aromagram (upper, black line) of rumen gas after feeding. Samples were collected using Carboxen/PDMS 85 µm SPME fiber and 5 min *in vivo* rumen sampling time. Numbers signify odor events (Table 2).

Figure 6. Comparison of peak area count of 12 characteristic compounds in rumen gas for before and after feeding of three steers with 85 µm Carboxen/PDMS SPME fiber. Extraction time = 5 min. Error bars signify the standard deviation above the mean. Number in parentheses is the single ion of each compound used for peak area count integration. * = significant difference between before and after feeding.

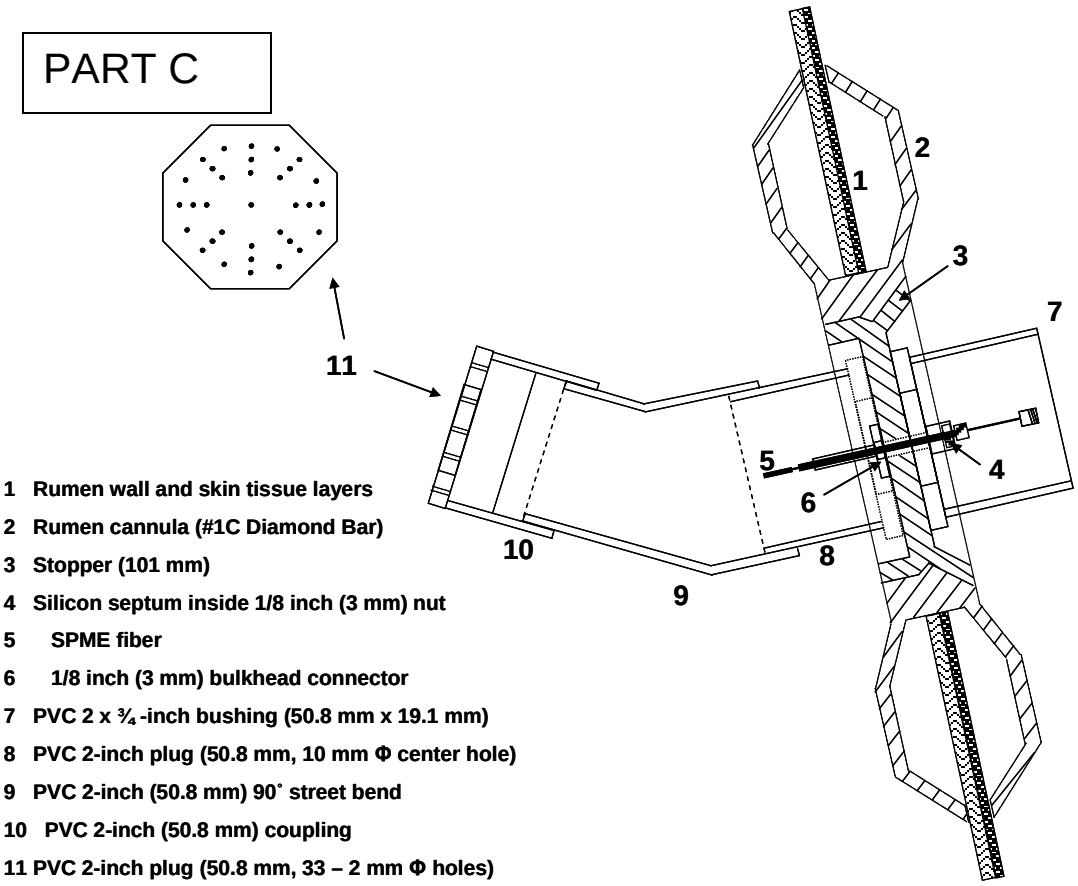
Figure 7. Comparison of the difference of total odor and characteristic odors between rumen gases before and after feeding of three steers. Error bars signify the plus standard deviation of the mean.


PART A
Part C
Gas
headspace
Recently ingested
forage
Omasum
Partially digested forage
and liquid
Abomasum
Reticulorumen

495


Modified cannula for
rumen gas sampling
with SPME
PART B
Gas headspace
Recently ingested
forage
Partially digested forage
and liquid

496



1 Rumen wall and skin tissue layers
2 Rumen cannula (#1C Diamond Bar)
3 Stopper (101 mm)
4 Silicon septum inside 1/8 inch (3 mm) nut
5 SPME fiber
6 1/8 inch (3 mm) bulkhead connector
7 PVC 2 x ¾-inch bushing (50.8 mm x 19.1 mm)
8 PVC 2-inch plug (50.8 mm, 10 mm Φ center hole)
9 PVC 2-inch (50.8 mm) 90˚ street bend
10 PVC 2-inch (50.8 mm) coupling
11 PVC 2-inch plug (50.8 mm, 33 – 2 mm Φ holes)

Figure 1 (Parts A, B and C)

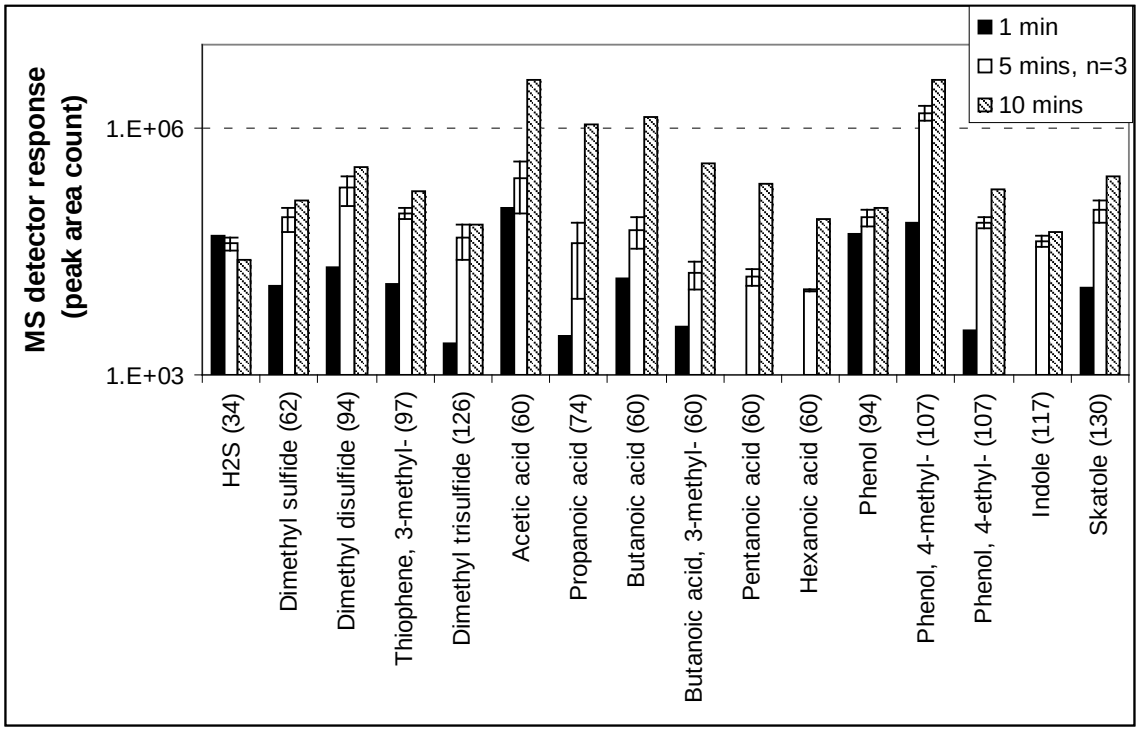
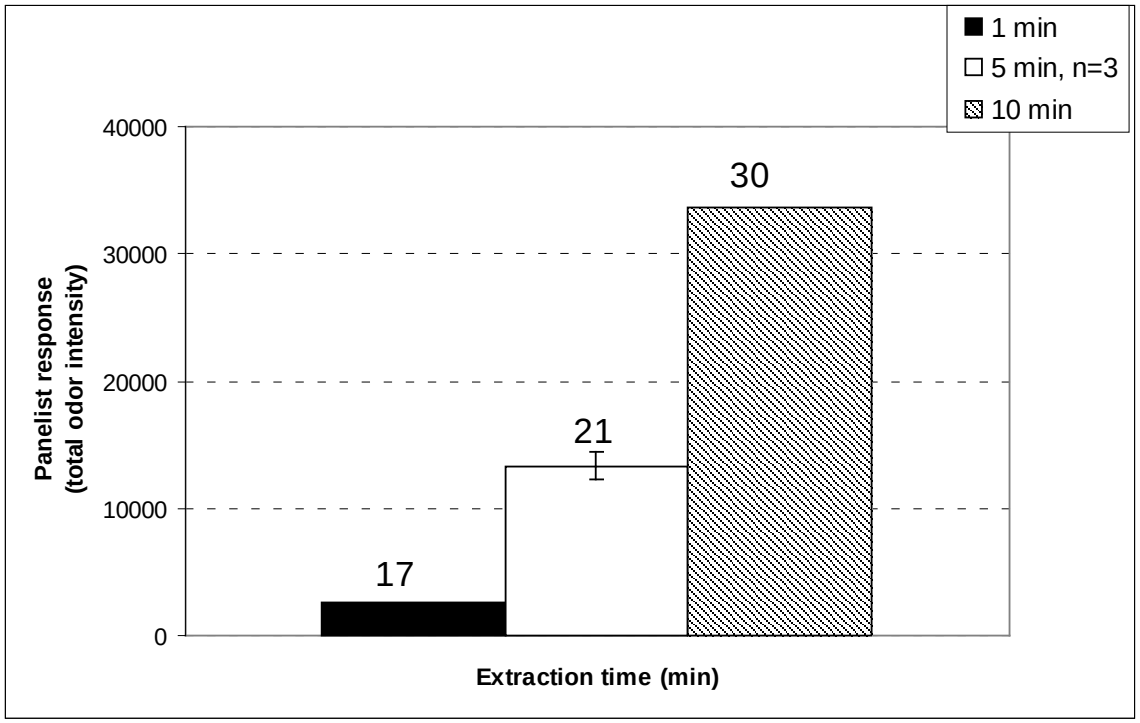

MS detector response
(peak area count)
1.E+06
1.E+03
1 min
5 mins, n=3
10 mins
H2S (34)
Dimethyl sulfide (62)
Dimethyl disulfide (94)
Thiophene, 3-methyl- (97)
Dimethyl trisulfide (126)
Acetic acid (60)
Propanoic acid (74)
Butanoic acid (60)
Butanoic acid, 3-methyl- (60)
Pentanoic acid (60)
Hexanoic acid (60)
Phenol (94)
Phenol, 4-methyl- (107)
Phenol, 4-ethyl- (107)
Indole (117)
Skatole (130)

Figure 2

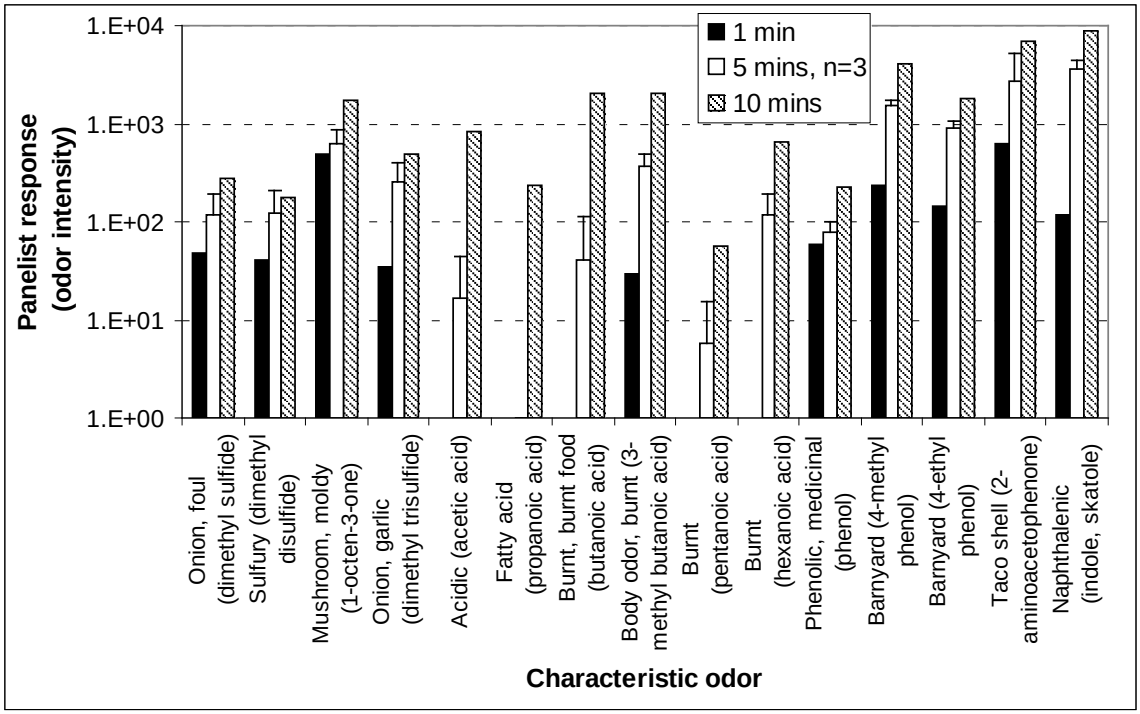
503



504

505 **Figure 3**

506



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509 **Figure 4**

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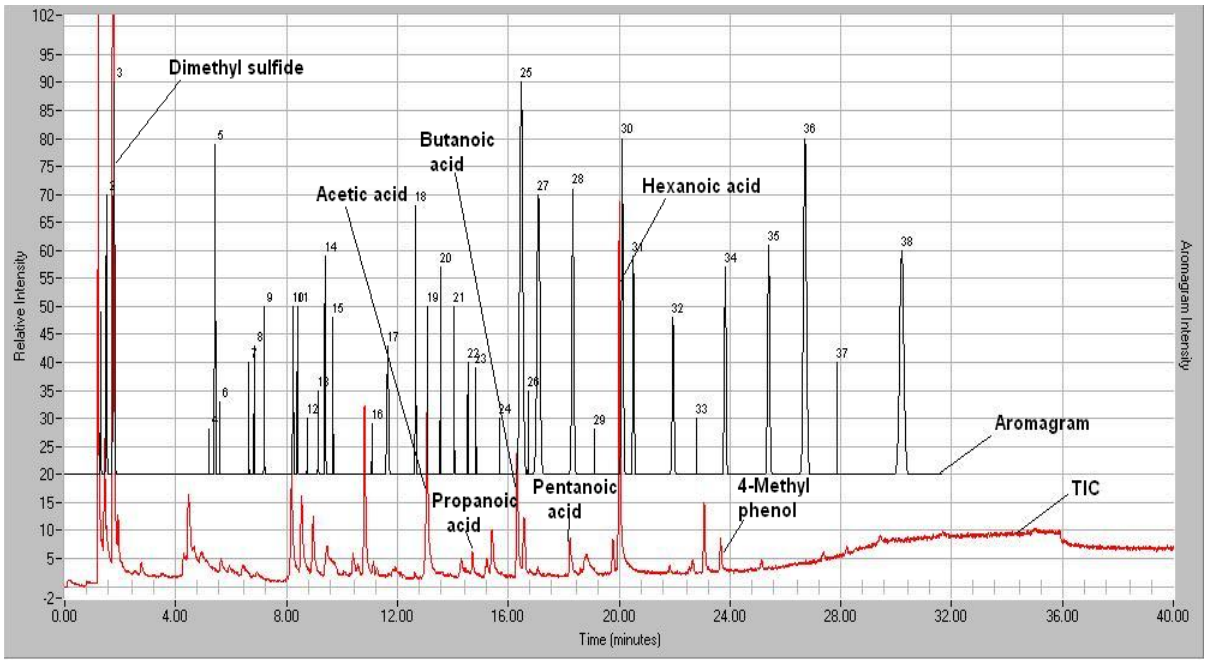
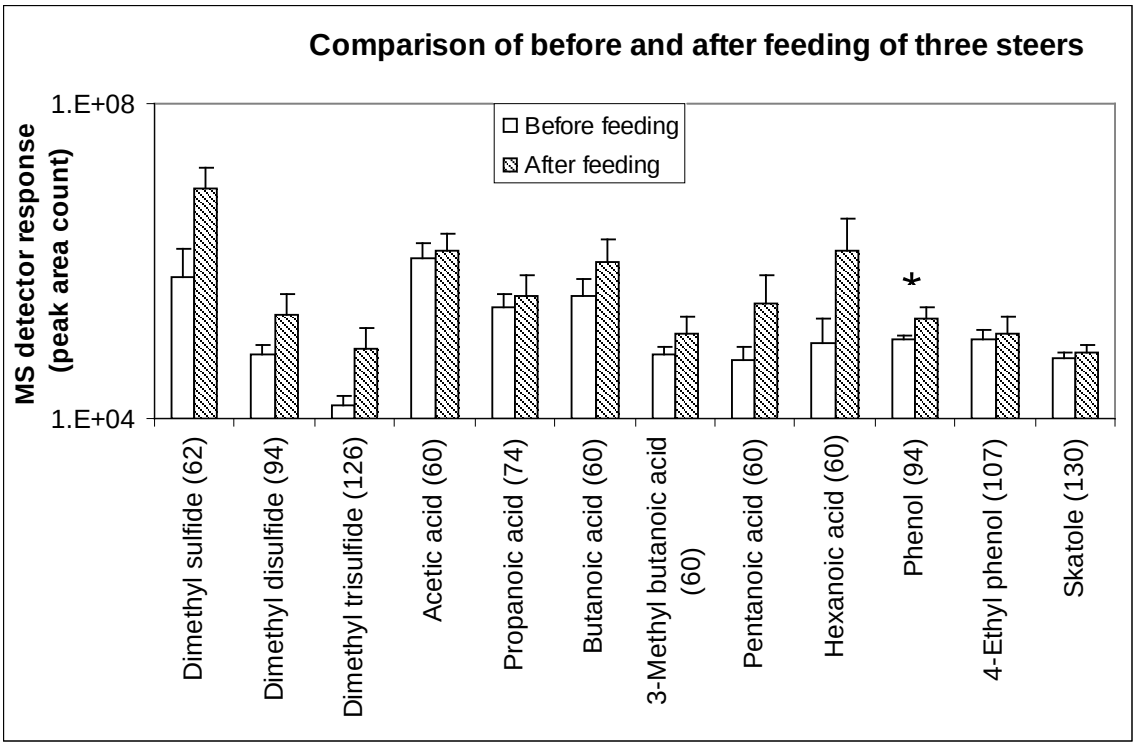

Dimethyl sulfide
Butanoic acid
Acetic acid
Hexanoic acid
Propanoic acid
Pentanoic acid
4-Methyl phenol
Aromagram
TIC
Relative Intensity
Aromagram Intensity
Time (minutes)

Figure 5



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519

520 **Figure 6**

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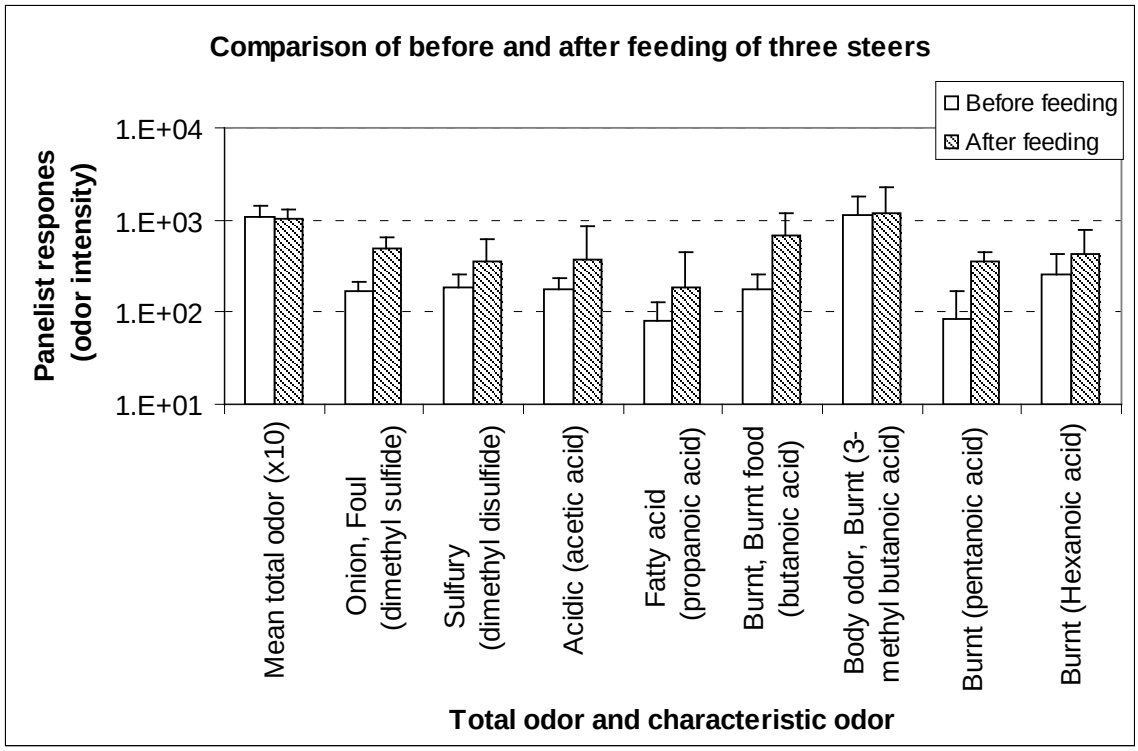

522

523 **Figure 7**

524 Table 1. Comparison of sampling and analytical methods used to characterize VOCs in
525 rumen gas and rumen fluid.

Reference #	Sampling	Sampling Preparation	Analyses	Odor Analysis	Identified compounds
This work	SPME, cattle with rumen cannula, *in vivo* sampling	SPME (Carboxen/PDMS) Extraction condition: 39 ºC, 5 min	GC-MS-O	Sniff port on GC-MS-O	Cattle rumen headspace 50 compounds
Dewhurst, et al. [15]	cattle with rumen cannula, rumen gas was pumped into evacuated plastic bottles (2L) *in vivo*	The caps of the bottles containing the rumen gas were punctured with a needle connected directly to the inlet port of the SIFT-MS	Selected-ion-flow-tube Mass (SIFT-MS)	None	Dairy cows rumen headspace 14 compounds
Spinhirne, et al. [16]	Ruminal fluid from cannulated heifer	SPME (DVB/Carboxen/PDMS) Extraction condition: 39 ºC, 1 min	GC-MS	None	Heifer rumen fluid headspace 12 compounds
Schneider, et al. [17]	Fistulated cow	Centrifuged and filtered, reacted with NaOH, then derivated with trifluoroacetic acid and extracted by chloroform	GC-FID	None	Cow rumen fluid 20 compounds
Teunissen, et al. [9]	Fistulated sheep	Filtered on a Whatman GF/C glass microfiber filter and centrifuged. The supernatants were pipetted into 1.5 ml Eppendorf reaction vessels, and then stored at 4-10 ºC up to 48 h	GC-FID	None	Sheep rumen liquor 22 compounds
Faichney, et al. [10]	Fistulated sheep and cattle	Distilled and made alkaline with sodium hydroxide, then evaporated on a hot plate and dried. Then dissolved in acetone	GC-FID	None	Sheep and cattle rumen fluid 6 compounds
Williams, et al. [11]	–	Centrifuged and extracted by methylene chloride	GC-FID	None	Goat rumen liquor, plasma, and tissue of ruminants 2 compounds
Calabro, et al. [2]	Rumen cannula.	Centrifuged and diluted with oxalic acid	GC-FID	None	Buffalo and sheep rumen fluid 4 compounds

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527

528 Table 2. Summary of compounds identified in rumen gas.

529

No	Retention time	Compound	CAS	MW	Odor threshold[h] (ppm)	Odor character
1	* 1.20	H_2S	7783-06-4	34.08	0.01778	Sewer
2	1.41	cis-1,2-Dimethyl cyclopropane	930-18-7	70.14	n/a	Sweet
3	1.53	2-methyl-1-butene	563-46-2	70.14	n/a	
4	* 1.68	Ethanethiol	75-08-1	62.13	0.001072	Foul, fecal
5	* 1.70	Dimethyl sulfide[a]	75-18-3	62.13	0.002239	Onion, garlic
6	* 1.80	1-Propanethiol	107-03-9	76.16	0.001259	
7	*1.93	2-Propanone[a]	67-64-1	58.08	14.45	
8	2.15	3-Hexyne	928-49-4	82.15	n/a	
9	*2.71	2-Butanone	78-93-3	72.11	7.762	
10	3.50	2-Nitro pyridine	15009-91-3	124.1	n/a	
11	3.96	2,4-Hexadienal	142-83-6	96.13	0.0005495	
12	* 4.11	2-Pentanone	107-87-9	86.14	1.549	Ketone
13	* 4.36	Octane	111-65-9	114.2	5.754	
14	* 4.76	4-Octene	7642-15-1	112.2	n/a	
15	* 4.56	3-Octene	14919-01-8	112.2	n/a	
16	* 4.48	2-Octene	111-67-1	112.2	0.07586	
17	* 5.66	Methyl benzene[b]	108-88-3	92.14	1.549	Ketone
18	* 5.88	Dimethyl disulfide[b]	624-92-0	94.2	0.0123	Sulfury
19	*6.41	2-Pentanol	6032-29-7	88.15	n/a	
20	* 6.50	3-methyl thiophene	616-44-4	98.17	n/a	Sulfury, skunky
21	* 7.03	Nonane	111-84-2	128.3	1.259	
22	8.08	2,6-dimethyl-1,7-Octadiene	6874-35-7	138.1	n/a	
23	8.21	3-Nonyne	20184-89-8	124.2	n/a	
24	*8.25	Alpha-pinene	80-56-8	136.2	0.6918	Ketone
25	8.63	3,7-Dimethyl-octa-1,6-diene	-	138.1	n/a	Moldy
26	*8.88	Camphene	79-92-5	136.2	n/a	
27	9.03	2,6-Dimethyl-2-octene	4057-42-5	140.3	n/a	Sweet
28	9.71	Sabinene	3387-41-5	136.2	n/a	
29	10.68	3-Ethyl-2,5-dimethyl-1,3-Hexadiene	62338-07-2	138.1	n/a	
30	10.91	1-Methyl- 4-[1-methylethyl] cyclohexene	1195-31-9	138.3	n/a	
31	*11.33	Limonene	138-86-3	136.2	0.4365	

32	11.88	1-Methyl-4-[1-methylethyl] benzene	99-87-6	134.2	n/a	
33	12.01	[2Z]-8-Methyl-2,7-nonadien-4-one	89780-46-1	152.1	n/a	
34	* 12.56	Dimethyl trisulfide	3658-80-8	126	0.00166	Onion, garlic
35	* 13.03	Acetic acid[a,b,c,d,e,g]	64-19-7	60.05	0.1445	Acidic
36	13.68	2-Butyl naphthalene	1134-62-9	184.3	n/a	
37	* 14.48	2-Ethyl-1-hexanol	104-76-7	130.2	0.2455	
38	* 14.65	Propanoic acid[a,b,c,d,e,g]	79-09-4	74.08	0.03548	Burnt, burnt food
39	* 15.18	Dimethyl propanedioic acid	595-46-0	132.1	n/a	Burnt
40	* 16.28	Butanoic acid[a,b,c,d,e,g]	107-92-6	88.11	0.00389	Burnt, body odor
41	* 17.00	3-Methyl butanoic acid[b,c,d,e,g]	503-74-2	102.1	0.002455	Burnt, body odor
42	* 18.18	Pentanoic acid[a,b,c,d,e]	109-52-4	102.1	0.03715	Burnt, body odor
43	* 19.98	Hexanoic acid[a,b,c,d]	142-62-1	116.2	0.01259	Fatty acid
44	* 20.95	Dimethyl sulfone	67-71-0	94.1	n/a	Burnt
45	* 22.51	Phenol	108-95-2	94.11	0.1096	Medicinal, phenolic
46	* 23.63	4-Methyl phenol	106-44-5	108.1	0.1096	Barnyard, urious
47	* 25.01	4-Ethyl phenol	620-17-7	122.2	0.001862	Barnyard, phenolic
48	* 26.28	3-Propyl phenol	621-27-2	136.2	n/a	Phenolic
49	* 28.65	Indole[f]	120-72-9	117.2	0.000032	Barnyard
50	* 29.26	Skatole[f]	83-34-1	131.2	0.000562	Naphthalenic

[a]Dewhurst, et al. [15]; [b]Spinhirne, et al. [16]; [c]Schneider, et al. [17]; [d]Teunissen, et al. [9]; [e]Faichney, et al. [10]; [f]Williams, et al. [11]; [g]Calabro, et al. [2].

[h]Devos, et al. [33].

*Confirmed with authentic standards.

n/a=not available