Characterizing Volatile Fatty Acids and Other Gases in a Rumen Closed In Vitro Fermentation System using Solid Phase Microextraction

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CHARACTERIZING VOLATILE FATTY ACIDS AND OTHER GASES IN A RUMEN CLOSED IN VITRO FERMENTATION SYSTEM USING SOLID PHASE MICROEXTRACTION

J. P. Spinhirne, J. A. Koziel, N. K. Chirase

ABSTRACT. A new method for rapid sampling and qualitative characterization of the headspace gases of closed in vitro cultures using solid phase microextraction (SPME) was evaluated for ruminal fluid and ruminal fluid with feed containing a feed additive. Gas sample collection was achieved by exposing a DVB/Carboxen/PDMS 50/30 μm SPME fiber to the headspace of cultures for 1 min every hour. This was followed by immediate analysis on a GC–MS and then reuse of the SPME fiber. Acetic, propionic, isobutyric, butyric, isovaleric, valeric, and hexanoic acids and toluene, dimethyl disulfide, and pentadecane were identified in the headspace. SPME technology facilitated rapid sampling and immediate analysis with a GC–MS to identify specific end products of microbial digestion. A SPME–based approach could serve as a new method for the characterization of ruminal fermentation end products and testing effects of feed additives on the formation of end products.

Keywords. Bioreactor, Detection, Fermentation, Gas chromatography, Mass spectrometry, Metabolism, Ruminal gases, Solid phase microextraction, Volatile fatty acids, Volatile organic compounds.

Ruminal fermentation is the physical and microbiological activity that converts components of the diet, under strictly anaerobic conditions, into useful (volatile fatty acids (VFAs), microbial proteins, B–vitamins), useless (methane, carbon dioxide), or even harmful (ammonia, nitrates) products for the host animal (Owens and Goetsch, 1988). Standard procedures for the characterization of these products involve rigorous sample collection, preservation, processing, and use of reagents, and often take days before obtaining results. Low molecular weight VFAs have been used to determine the energetic efficiency of microbial fermentation in the rumen. Several approaches have been used to characterize VFAs produced in the rumen: (1) closed in vitro fermentation systems (Hungate, 1966, pp. 245–280), (2) in vitro continuous culture fermentation systems (Hoover et al., 1976), (3) a suction pump with tubing for obtaining ruminal fluid through the esophagus, (4) rumenocentesis (aspiration of ruminal fluid through the ruminal wall using needle), and (5) gnotobiotic systems (Hungate, 1966, pp. 245–280). These methods typically involve extensive and invasive sampling, sample preservation, and preparation procedures.

The objective of this research was to test the feasibility of sampling gases that are produced and released to the headspace of closed in vitro ruminal fluid fermentation systems with solid phase microextraction (SPME). To our knowledge, this is the first of this kind of test conducted on ruminal fluid fermentation. To date, such analyses were only conducted on the liquid phase. It is reasonable to assume that many of the analytes of interest, particularly the VFAs, volatilize from the liquid phase and saturate the headspace. One advantage of headspace analysis is that it is typically not affected by impurities in the liquid sample matrix and requires less sample preparation. In contrast to SPME, conventional sampling of liquid phase from cultures requires multiple samples (i.e., typically one culture tube per one data point) to obtain trends in metabolic products over time. Detection of headspace VFAs and other gases produced by rumen microbes could be very useful in evaluating diets, feed additives, dietary amendments, and increasing general knowledge related to rumen metabolism.

Specific objectives included rapid and frequent sampling of headspace gases produced by closed in vitro microbial fermentation systems containing rumen fluid only and rumen fluid with feed and small fraction of Promax, a feed additive. Solid phase microextraction was selected as a sampling/sample preparation approach because it is fast and sensitive for the determination of VOCs (including VFAs) in air or in an enclosed headspace (Koziel and Pawliszyn, 2001; Pawliszyn, 1997).

MATERIALS AND METHODS

SPME FIBER SELECTION

It was assumed that VFAs are present in the headspace of in vitro fermentation systems based on their documented
presence in ruminal fluid (Church, 1988). Therefore, we selected SPME fiber coatings that would be suitable to extract VFAs. Initially, six types of SPME fiber coatings were evaluated for their ability to extract VFAs from standard gas using 1 min sampling time (Spinhirne et al., 2002). Based on these experiments, the DVB/Carboxen/PDMS 50/30 μm coating (Supelco, Bellefonte, Pa.) was selected for this study. This coating was very efficient in extracting VFAs from acetic to hexanoic acids. A very short sampling time of 1 min was selected because it preconcentrated a sufficient amount of VFAs to be detected by the MS. This sampling time was selected arbitrarily. The SPME fiber assembly was conditioned by holding it in a gas chromatograph (GC) injector at 270°C for 4 h.

**Sampling System**

To characterize the gas–phase environment of the rumen, the headspace of ruminal fluid and ruminal fluid with feed was sampled in a closed *in vitro* system. Two 250 mL Erlenmeyer flasks equipped with stoppers, septa, and pressure release valves were washed, assembled, and heated in a 110°C oven overnight to purge impurities that could originate from the apparatus (fig. 1). The gas relief vents consisted of a rubber policeman with a small lateral cut to permit the escape of excess gases produced while maintaining an anaerobic environment. Ruminal fluid was obtained from a cannulated heifer within 1 h after the morning feeding at the Texas Agriculture Experiment Station feedlot in Bushland, Texas. The ruminal fluid was strained through two layers of cheesecloth to remove large feed particles. Carbon dioxide was used to purge the headspace of the ruminal fluid in the transportation thermos to maintain anaerobic conditions.

At time t = 0 h, 100 mL of ruminal fluid was placed in each of the two Erlenmeyer flasks (#1 and #2) followed by a second purging with CO₂. Ten grams of feed containing a 1.6% Promax feed additive (Huma Tech, Inc., Houston, Texas) was added to the ruminal fluid in flask #2. The remaining feed consisted of 90% grain concentrate and 8.4% cottonseed hulls, which is a standard diet for finishing beef cattle. The feed additive served only for demonstration of the feasibility of headspace gas detection and no comparisons or applications were planned. Flask #1 served as a control. The two flasks were then placed in a 39°C water bath (fig. 2) and were agitated for 2 s after each SPME sampling to simulate the rumen environment.

**SPME Sampling**

The headspace of each flask was sequentially sampled every hour with the same SPME fiber. Each flask was sampled in cyclic order for 13 h. Sampling was discontinued overnight and resumed the next morning to end the experiment 26 h after it began. Samples of the headspace gases were collected by inserting the SPME needle through the septum installed in the synthetic stopper of the flask for 1 min (figs. 1 and 2). Solid phase microextraction extracts only relatively small masses of analytes from the sampling matrix compared with conventional extraction methods. Thus, it is reasonable to assume that short and repetitive SPME extractions do not disturb the sample matrix in cases when the headspace is saturated and continuously replenished by gases produced in the liquid phase.

Sampling was immediately followed by sample transfer to the GC. The SPME fiber was introduced into the GC and held in the injector from 19 to 24 min for thermal desorption of the fiber. Typically, only minutes are needed to complete this process. However, in this research, the SPME fiber was kept inside the injector for the duration of the GC run to protect it from impurities in the ambient air. The SPME fiber was removed from the injector only for the time needed for sample collection. The sampling/analysis turnaround time was limited by the length of the GC oven program, which required 15 min of run time and 5 min cool down. Temperatures of the *in vitro* cultures were measured with a K-type thermocouple and were equal to 39°C at the SPME sample height in the headspace of the flasks. Column blanks and fiber blanks were used intermittently to prevent carry-over of analytes between runs.

**GC–MS Conditions**

SPME samples were analyzed using a Varian 3800 GC coupled to a Saturn 2000 MS. The injector was held isothermally at 250°C and in the splitless mode for the entire run. The column oven initial temperature was 60°C, followed by a first ramp of 60°C/min to 110°C, a second ramp of 10°C/min to 210°C, and finally a third ramp of
60 °C/min to 250 °C with a final hold time of 3 min. The transfer line, manifold, and trap were set at 200 °C, 40 °C, and 150 °C, respectively. The SPME fibers were desorbed in a model 1079 GC injector. The 99.9995% pure helium carrier gas (Airgas Southwest, Amarillo, Texas) was held at a constant flow of 1 mL/min throughout the run with electronic flow control. The injector was equipped with a 0.8 mm diameter glass liner and an 11.5 mm LB–2 Thermogreen septum (Supelco, Bellefonte, Pa.). The analytes were separated on a 30 m × 0.25 mm × 1.5 μm film CP–Wax capillary column (Varian BV, Middelburg, The Netherlands).

Analytes exiting the column were detected between 35 and 200 atomic mass units (amu) with a Saturn 2000 MS. The electron multiplier voltage was set to 1340 eV. The electron ionization emission current was 10 μA. Analytes were initially identified with the National Institute of Standards and Technology (NIST) MS spectral library (Varian Instruments, 1998). A separate Saturn user library was built with spectra from very short extractions of the headspace of pure compounds. Column retention time and the match to the Saturn user library were used to positively identify VFAs and other gases detected in the rumen headspace. Quantification of headspace gases was not performed in this study.

RESULTS AND DISCUSSION

Typical chromatograms of gas samples collected in the headspace over ruminal fluid and ruminal fluid with feed/feed additive are presented in figure 3. The ruminal fluid and feed/feed additive culture had a chromatographic profile distinct from the ruminal fluid samples for all samples that were collected. Acetic, propionic, butyric, isobutyric, isovaleric, valeric, and hexanoic acids, toluene, and dimethyl disulfide were identified in the headspace of the ruminal fluid (#1) and the ruminal fluid with feed/feed additive (#2) culture. Pentadecane was found exclusively in culture #2. Retention times and qualifier (matching) ions for all positively identified compounds are summarized in table 1. Finding of low molecular weight VFAs is consistent with the assumption that these VFAs volatilize from the liquid phase into the headspace. The presence of VFAs in the headspace gases is also consistent with the well–documented presence of these VFAs in ruminal fluid (Hungate, 1966, pp. 245–280; Hoover et al., 1976). Extracted masses of VFAs and other identified gases varied in each SPME sample. As a result, it can be reasonably assumed that the headspace gas concentrations also varied because all SPME sampling conditions remained constant. Thus, in addition to identifying and characterizing specific end products of digestion via headspace SPME sampling, it may also be feasible to quantify these products in the headspace.

<table>
<thead>
<tr>
<th>Peak</th>
<th>Compound</th>
<th>GC Column Retention Time (min)</th>
<th>CAS No.</th>
<th>Formula</th>
<th>Qualifier Ions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Toluene</td>
<td>2.70</td>
<td>108–88–3</td>
<td>C₆H₅CH₃</td>
<td>91, 92, 65</td>
</tr>
<tr>
<td>2</td>
<td>Dimethyl disulfide</td>
<td>2.90</td>
<td>624–92–0</td>
<td>CH₃S₂CH₃</td>
<td>94, 45, 95</td>
</tr>
<tr>
<td>3</td>
<td>Pentadecane</td>
<td>6.12</td>
<td>629–62–9</td>
<td>CH₃(CH₂)₁₃CH₃</td>
<td>57, 71, 85</td>
</tr>
<tr>
<td>4</td>
<td>Acetic acid</td>
<td>6.57</td>
<td>64–19–7</td>
<td>CH₃COOH</td>
<td>43, 45, 60</td>
</tr>
<tr>
<td>5</td>
<td>Propionic acid</td>
<td>7.53</td>
<td>79–09–4</td>
<td>CH₃CH₂COOH</td>
<td>73, 74, 45</td>
</tr>
<tr>
<td>6</td>
<td>Isobutyric acid</td>
<td>7.84</td>
<td>79–31–2</td>
<td>CH₃(CH₂)₂COOH</td>
<td>73, 43, 41</td>
</tr>
<tr>
<td>7</td>
<td>Butyric acid</td>
<td>8.55</td>
<td>107–92–6</td>
<td>CH₃(CH₂)₂COOH</td>
<td>73, 60, 42</td>
</tr>
<tr>
<td>8</td>
<td>Isovaleric acid</td>
<td>9.03</td>
<td>503–74–2</td>
<td>CH₃(CH₂)₃COOH</td>
<td>60, 42, 39</td>
</tr>
<tr>
<td>9</td>
<td>Valeric acid</td>
<td>9.80</td>
<td>109–52–4</td>
<td>CH₃(CH₂)₃COOH</td>
<td>60, 73, 42</td>
</tr>
<tr>
<td>10</td>
<td>Hexanoic acid</td>
<td>10.99</td>
<td>142–62–1</td>
<td>CH₃(CH₂)₄COOH</td>
<td>60, 73, 42</td>
</tr>
</tbody>
</table>

Additional compounds found in other samples

<table>
<thead>
<tr>
<th>Peak</th>
<th>Compound</th>
<th>GC Column Retention Time (min)</th>
<th>CAS No.</th>
<th>Formula</th>
<th>Qualifier Ions</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>Octanal</td>
<td>4.43</td>
<td>124–13–0</td>
<td>CH₃(CH₂)₃COH</td>
<td>41, 39, 56</td>
</tr>
<tr>
<td>12</td>
<td>Nonanal</td>
<td>5.38</td>
<td>124–19–6</td>
<td>CH₃(CH₂)₃COH</td>
<td>41, 57, 39</td>
</tr>
</tbody>
</table>

[a] CAS = Chemical Abstract Service.
One-minute sampling with SPME was sufficient to extract enough mass to be detected on a GC–MS for several low molecular weight VFAs. Further optimization and reduction of the GC run time could also reduce the overall sample/analysis turnaround time. Solid phase microextraction sampling of in vitro ruminal cultures to characterize the end products of digested feeds presents great advantages over conventional methods. Solid phase microextraction is relatively fast, easy to use, requires very little sample preparation, and the sample can be collected from the same flask, assuming that the mass of analytes in the headspace is far greater than the amount extracted. This new sampling method based on SPME would require fewer culture vials sampled repeatedly over time with SPME and then directly analyzed with a GC–FID or GC–MS. By combining sampling and sample preconcentration into one step, SPME could reduce the amount of personnel, resources, and time needed to complete testing of a closed in vitro system. The approach presented in this research could be extended to development of an alternative method for in vitro characterization of feed additives. This method could be used to characterize microbial metabolic activities and their kinetics, and the effects of other variables via relatively simple, indirect characterization of headspace gases and their production trends.

The collected mass spectroscopy data was limited to molecular weights from 35 to 200 amu; therefore, compounds with lower molecular weight such as methane and hydrogen sulfide were not identified. Carbon dioxide and formic acid were not identified because low partitioning coefficients with the fiber hindered the pre–concentration of detectable masses of the aforementioned compounds. The low partitioning coefficient for low molecular weight analytes is one of the limitations of SPME. Other compounds present in the headspace were also extracted with SPME and detected with the MS, as evidenced by the unknown peaks in figure 3. However, positive identification of these compounds was not within the scope of this initial experiment.

Development of a quantitative method for headspace rumen gases should account for important variables affecting their production. These variables include pH of the ruminal fluid and feed, fluid temperature, rate of stirring, sorption to particles in fluid, partitioning of gases between the liquid, solid and gas phases, and gas production rate. Other variables associated with SPME include the partitioning coefficients of individual gases to a particular SPME coating, extraction time, temperature and humidity, and gas velocity in the headspace (particularly for short extraction times). The potential use of a headspace SPME/in vitro approach could include: (1) development of novel ways of testing feed digestibility and its kinetics, (2) determination of the rumen component of eructated gases in breath, (3) determination of the production of odorous gases in the rumen to assess feed additives for the control of odor, (4) determination of specific metabolic end products that may be important for food safety, and (5) monitoring of a wide range of bioreactor processes.

**CONCLUSIONS**

The following conclusions resulted from this study:

- Volatile fatty acids including acetic, propionic, butyric, isobutyric, valeric, isovaleric, and hexanoic and other gases including toluene, pentadecane, and dimethyl disulfide were identified in the headspace of a rumen closed in vitro fermentation system using SPME. The presence of these compounds is consistent with compounds previously detected in the liquid phase (Church, 1988).
- Solid phase microextraction technology facilitated rapid sampling and immediate analysis with a GC–MS to identify specific end products of microbial digestion in the headspace. The new approach required much less sample preparation, allowed for near immediate analysis, and provided very sensitive gas sampling without much disturbance to the headspace environment. The overall sampling/analysis process can be reduced to minutes and is limited by the GC–MS analysis time. The SPME–based approach could serve as a novel technique for the development of an alternative method for characterization of ruminal fermentation end products and the effects of important variables on kinetics of their production. Similarly, the SPME–based approach could be adapted for monitoring of a wide variety of biological processes including bioreactors.
- The approach used in this research is novel for the following reasons: (1) characterization of headspace VFAs instead of conventional liquid sampling, (2) the use of fewer samples that are repeatedly sampled and analyzed over time, (3) detection of other gases at very low quantities that were not previously thought to be relevant in evaluating cultures, and (4) the use of SPME for rapid sampling of headspace gases from live rumen cultures.

**REFERENCES**


