Article

Theoretical Methane Emission Estimation from Volatile Fatty Acids in Bovine Rumen Fluid

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Abstract: Methane production from livestock farming is recognized as an important contributor to global GHGs. Volatile fatty acids (VFAs) found in bovine rumen may be utilized as a substrate for methanogens to form CH₄, and thus improvement of quantitative VFA measurements can help facilitate greater understanding and mitigation of CH₄ production. This study aims to contribute to the development of more accurate methods for the quantification and specification of VFAs in bovine rumen. The VFAs were analyzed using the conventional method and an alternative catalytic esterification reaction (CER) method. Substantial differences in the detected concentrations of the C3+ VFAs (chain length ≥ 3) were observed between both methods, especially for butyric acid. Evaluation of the sensitivity of both methods to detecting the VFA concentrations in standard solutions confirmed that the values resulting from the CER method were closer to the known concentrations of the standard solution than those from the conventional method. The results of this study provide the first quantitative proof to show the improved accuracy of the measurements of C3+ VFAs when using the CER method compared with the conventional method. Therefore, the CER method can be recommended to analyze the VFAs found in rumen, especially butyric acid and other C3+ VFAs.

Keywords: rumen; odor; volatile fatty acids; methane; catalytic esterification

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1. Introduction

Food and Agriculture Organization (FAO) statistics show that the worldwide supply of animal protein has risen from 34 to 43 kg per capita per year between 1993 and 2013 [1]. Large inequalities in protein consumption between countries mean that the annual consumption of animal proteins in many wealthier nations far exceeds this amount. For example, in 2013, the annual per capita animal protein supply in North America, the European Union, and Australia and New Zealand stood at 113 kg, 81 kg, and 114 kg, respectively [1]. To sate our massive demand for meat and dairy products, concentrated animal feeding operations (CAFOs) have inevitably served the long-term viability of the livestock industry over the last three decades [2–4]. Despite their economic benefits and production efficiencies, CAFOs have triggered unwanted environmental problems due to the large production of manure waste, far exceeding the capacity of land to assimilate the loadings of organic carbon and nutrients [5,6]. One of the recent urgent issues associated with CAFOs is the loss of gaseous species to the ambient air stream, which has contributed to climate change by means of emitting potent greenhouse gasses such as CH₄ and N₂O [7,8]. For instance, animal agriculture contributes 9% of anthropogenic CO₂ emissions, 37% of CH₄ emissions, and 65% of N₂O emissions, and the combined emissions expressed as a CO₂ equivalent amounts to about 18% of anthropogenic greenhouse gas (GHG) emissions [7]. Despite the well-defined guidelines to estimate GHG emissions from the Intergovernmental Panel on Climate Change (IPCC), the whole rationale for estimating GHG emissions from the livestock sector is only sensible with the support of robust and accurate data sets [9,10].

To this end, it is highly desirable to establish technical advancements to provide robust and accurate data sets for estimating GHG emissions from the livestock sector.

Among the various GHGs from the livestock industry, CH₄ is a major ubiquitous GHG during the normal digestive process in ruminant animals, and its global warming potential is 25 times that of CO₂ [11]. For instance, ruminal methanogens use the methanogenesis pathway to maintain low H₂ partial pressure and to facilitate fiber digestion in the rumen by converting H₂ to CH₄ [12]. There are two strategies to reduce CH₄ emissions from the livestock sector. One strategy is dietary manipulation [11], and another strategy is to improve the efficiency of ruminal function and to mitigate methane release [12]. However, quantification of CH₄ emissions from the livestock industry is challenging because a limited number of individual animals are monitored in any study, and correction factors are required to calculate actual CH₄ emission values [10]. Moreover, direct quantification of individual animal CH₄ emissions in open-circuit respiration chambers or using the sulfur hexafluoride (SF₆) tracer technique requires considerable investment in infrastructure and technical support and may impact animal feeding behavior [10,12]. VFA quantification methods through a catalytic esterification reaction (CER) requires a biochemical methane potential (BMP) test that allows alternative measurements compared with the conventional methane estimation methods such as respiration chamber techniques or direct stomach porthole treatment through the livestock.

It is urgent that a quantitative methodology for estimating CH₄ emissions from the livestock sector should be developed. Better accuracy in CER VFA measurements will provide compensation to methane emission inventory, especially from livestock. This case study aims to contribute to the development of more accurate methods for the quantification and specification of volatile fatty acids (VFAs) in bovine rumen. Improved quantitative measurements of VFAs could be correlated with the theoretical production of CH₄ due to the likelihood that VFAs can be utilized as a substrate for methanogens to form CH₄ [13,14]. Methanogens live in a variety of environments, including in the human and animal gut, and in ruminants that are responsible for emitting abundant amounts of methane during the digestion of food [15,16]. To ensure the accuracy of quantitative measurements of VFAs in bovine rumen and excreta, a reliable analytical technique should be developed. In addition, for quantification of VOCs using gas chromatography (GC), quantifying VFAs by their corresponding methyl esters via derivatization is favorable over their direct analysis as VFAs themselves, because the hydrogen bonds derived from their carboxyl group cause low resolution and tailing peaks [17]. However, conventional derivation methods contain several difficulties. First, a homogenous acid catalyst is needed, which means a washing process is required to remove salt [18–20]. Second, the yield of methyl ester is readily affected by impurities such as contaminants and moisture in the sample, thereby resulting in a low conversion efficiency [21–23]. Third, hazardous and potentially explosive solvents such as diazomethane are needed for the esterification reaction [24–26]. Moreover, the conventional analytical methods require extraction and isolation steps prior to analysis [27]. To overcome these technical challenges, quantification of VFAs was conducted via catalytic esterification [21,23–25,27] without the pretreatment of the bovine rumen and excreta samples. To evaluate the efficacy of this new approach, quantification of VFAs by the CER method was compared with that of the conventional method.

2. Materials and Methods

2.1. Chemical Reagents and Materials

Rumen fluids were obtained from the National Institute of Animal Science (NIAS) in Korea, and the samples were stored in a freezer at −37 °C. The VFA standard mixtures were prepared by using the pure VFAs and deionized water. All pure VFAs (acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, and isovaleric acid) and all pure VFA methyl ester standard solutions (acetic acid methyl ester, propionic acid methyl ester, butyric acid methyl ester, isobutyric acid methyl ester, valeric acid methyl ester, and isovaleric acid methyl ester) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Methanol and silica with pore size of 60 Å were purchased from Sigma-Aldrich (St. Louis, MO, USA) as well.

2.2. Conventional Method

Five milliliters of rumen fluid was mixed with 1 mL of a 25% metaphosphoric acid solution and 0.05 mL of saturated mercuric chloride solution in a 15 mL PTFE tube. The mixture was centrifuged at 4000 rpm for 20 min (20 °C). The sets of 1 mL of the supernatants were moved into 1.5-mL centrifuge tubes and then centrifuged at 12,000 rpm for 10 min (20 °C). After centrifugation, the supernatants were filtered using a 0.2-µm syringe filter and extracted to a 20-mL GC vial. The prepared samples were injected into a gas chromatography (GC-2010, Shimadzu, Tokyo, Japan) equipped with a flame ionization detector (FID) and DB-WAX column (30 m × 0.25 mm × 0.25 µm, Agilent, Santa Clara, CA, USA) by a auto injector (AOC-20i, Shimadzu, Tokyo, Japan) for the quantification of volatile fatty acid methyl esters (VFAMEs). This procedure was performed in triplicate for each sample. Multi-calibration was conducted using a VFAME standard mixture [28–30]. The volatile fatty acid methyl ester standard solutions were utilized for identification and quantification. Similarly, the VFA mixtures were also treated with identical procedures.

2.3. Catalytic Esterification Reaction Method

To extract VFAs from the cattle manure samples, 3 ± 0.02 g of the cattle manure samples were placed in a serum bottle, and methanol was added. The mass ratio of the sample to the methanol was 1:4. The serum bottle was carefully sealed and stored at room temperature (23 °C) for 1 d. For the CER, the bulkhead unit (Swagelok SS-400-61, Fremont, CA, USA) was used as a batch reactor. One side of the bulkhead union was sealed with the stopper (Swagelok SS-400-P, Fremont, CA, USA), and 200 mg of silica (surface area: $480 \text{ m}^2 \text{ g}^{-1}$; pore volume: $0.75 \text{ cm}^3 \text{ g}^{-1}$; pore size: 60 Å; particle size: 150–250 µm) was loaded into the bulkhead union. Both 400 µL of methanol (MeOH) and 50 µL of the samples (i.e., rumen fluid or the VFA mixtures prepared with pure components) were loaded into the bulkhead union, and then the other side of the reactor was filled with 200 mg of silica and sealed with a stopper. The reactor was placed in a muffle furnace with a temperature value around 520 °C. The replication of the sample preparation and analyses were conducted as per the conventional methods in Section 2.2.

3. Results and Discussion

3.1. VFA Concentrations in Rumen Fluid Using the Conventional Method

All prepared samples were analyzed by GC-FID for the quantification of VFAs in the rumen fluid. The determination of acetic acid, propionic acid, and butyric acid is important because they account for about 95% of the total VFAs in rumen fluid [24]. In this study, isobutyric acid, valeric acid, and isovaleric acid were included for the analysis as well (Figure 1), as these less-common VFAs may be subject to greater measurement errors. The average measured concentrations of acetic acid, propionic acid, isobutyric acid, butyric acid, valeric acid, and isovaleric acid using the conventional method were 2833, 865, 188, 156, 103, and 93 ng µL^{−1}, respectively. Among the six types of VFAs, acetic acid and propionic acid accounted for about 87% of the VFAs. The tendency of these results was confirmed by other previous studies. Filípek and Dvořák [25] reported that the acetic acid, propionic acid, butyric acid, and valeric acid concentrations were determined to be 4002, 2013, 1271, and 366 ng µL^{−1} for the rumen liquid using gas chromatography. The specific concentration value was different, but the fraction of acetic acid and propionic acid was 80%, which accounted for the majority of the VFAs. Hofírek and Haas [26] collected and analyzed ruminal fluid from dairy cows, with their results showing the percentage of acetic acid, propionic, isobutyric acid, butyric acid, isovaleric acid, and valeric acid concentrations to be 63, 21, 0.6, 13, 1.2, and 1.1, respectively, also indicating that the main components of rumen fluid were acetic acid and propionic acid. In addition, Sutton et al. [27] also reported the molar proportions of acetic acid, propionic acid, butyric acid, and valeric acid from the

rumen of dairy cows to be 67, 19, 12 and 18, respectively. Among these studies, there do not appear to be large differences in the composition ratios of rumen VFAs.

Figure 1. Volatile fatty acid concentration in rumen fluid using the conventional method.

3.2. VFA Concentrations in Rumen Fluid Using the Catalytic Esterification Reaction Method

The CER method without any pretreatment was used to analyze the concentrations of VFAs in rumen fluid (Figure 2). The concentrations of acetic acid, propionic acid, isobutyric acid, butyric acid, valeric acid, and isovaleric acid were 2680, 1031, 381, 782, 82, and 52 $\text{ng } \mu\text{L}^{-1}$, respectively. Among the six types of VFAs, acetic acid and propionic acid accounted for about 75% of them, which was a lower proportion than that found using the conventional method. Compared with the conventional method, the recovery rates of acetic acid, propionic acid, isobutyric acid, butyric acid, valeric acid, and isovaleric acid were 0.95, 1.19, 2.02, 5.03, 0.80, and 0.56, respectively (Figure 3). When using the catalytic esterification reaction method, the main VFAs, acetic acid and propionic acid, had similar detection rates to the conventional method. However, in the case of isobutyric acid and butyric acid, larger amounts were detected in the CER method. On the contrary, in the case of valeric acid and isovaleric acid, slightly lesser amounts were detected by the catalytic esterification reaction method than by analysis using the conventional method.

Figure 2. Volatile fatty acid concentration in rumen fluid using the catalytic esterification reaction method.

Figure 3. Ratio of the measured concentration to the known one for each component of the standard VFA solution to compare the catalytic esterification reaction method to the conventional method.

3.3. Confirmation Using Standard Mixtures Prepared with Purified VFAs

It is widely reported that the main constituents of acidogenic production from rumen fluid are acetic acid, propionic acid, butyric acid, and valeric acid [31]. Considering CH₄ production via methanogens, the importance of precise analysis of volatile fatty acids is becoming more important. In order to assess the precision of both methods, standard VFA mixture solutions containing known concentrations of each component were prepared at various concentrations, and confirmatory analyses were performed using the conventional method and CER method. For both methods, the measurement deviation for each component was evaluated using a ratio, defined as the measured concentration over the known concentrations of the VFAs in the standard solution. The ratios are presented in Figure 3. If the ratio is closer to one (dot line), the deviation is lower; that is, the measurement is more accurate.

Generally, the CER method resulted in lower measured deviations from known VFA concentrations. The results when measuring standard mixtures of acetic acid and propionic acid were relatively reliable for both methods, with measured-to-known concentration ratios of 1.08 and 1.03 for the conventional and CER methods, respectively. However, the deviation in results when using the conventional method was greater when measuring the less abundant VFAs; that is, the differences in measured concentrations between the two methods were especially apparent for butyric acid and other C3+ VFAs. For butyric acid, there was a factor-of-5 difference in sensitivity between the two methods, with the CER method detecting 91 percent of the known sample concentration compared with 18 percent for the conventional method. The discrepancy between the two methods and the substantially greater accuracy of the CER method was also apparent for valeric acid. However, the overall concentrations of the valeric and isovaleric acids in the rumen were the lowest observed among the measured VFAs for both methods (Figures 1 and 2). Consequently, the CER method displayed the largest improvement over the conventional method in measuring the absolute amount of butyric acid in the rumen. It has also been reported in previous research that the ratios of acetic acid and propionic acid appear to be overestimated [25–27]. However, their measured deviations from known concentrations in the CER method were consistent with the tendency of this overestimation (i.e., there was no substantial change in measurement accuracy for the acetic acid or propionic acid for the CER method vs. the conventional method). Using the evaluation of the frequency distribution, the comparison between the methods has been proven. The relative frequency of the ratios is presented in Figure 4.

The standard deviations of the distribution were 0.456 and 0.161 for the conventional method and the CER method, respectively. It was observed that the distribution for the conventional method was specifically high at both around 0.2 and 1.9. This shape is consistent with the results of Figure 3. The evaluation of the frequency distribution obviously suggests that the analysis described above is convincing.

To our knowledge, the results of this study provide the first quantitative proof to show the improved accuracy in the measurements of C3+ VFAs when using the CER method compared with the conventional method. Therefore, a new approach, the CER method, can be recommended when comprehensively analyzing not only the most important substances (acetic acid and propionic acid) but also others, including butyric acid. The more accurate analyses of these substances may provide a useful platform to predict the amount of methane generation from VFAs in rumen.

Figure 4. Ratio of the measured concentration to the known one for each component of the standard VFA solution, comparing the catalytic esterification reaction method to the conventional method.

4. Conclusions

In this study, the reliability of the CER method for measuring VFA concentrations in rumen was evaluated and compared with the conventional method. For C3+ VFAs, substantial differences in sensitivity and accuracy were observed between both methods, especially for butyric acid. When used to measure a standard mixture solution prepared with the known concentrations, the results show that both methods proved relatively reliable for acetic acid and propionic acid. However, the CER method appeared to be superior for measuring concentrations of the other less abundant VFAs, especially butyric acid. For this VFA, only the CER method allowed relatively accurate measurement, while the sensitivity of the conventional method for butyric acid was less than 20%. The results of this study provide the first empirical evidence that application of the CER method may result in more accurate measurements of C3+ VFAs compared with the conventional method. Therefore, a new approach using the CER method can be recommended to analyze not only the most important substances (acetic acid and propionic acid) but also others, including butyric acid.

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