J. Dairy Sci. 99:9313–9318
http://dx.doi.org/10.3168/jds.2016-11447

Technical note: Analytical refinements of the methane indicator archaeol in bovine feces, rumen fluid, and feedstuffs

S. Görs,* B. Kuhla,* N. Krattenmacher,† G. Thaller,† and C. C. Metges*[1]
*Institute of Nutritional Physiology, Leibniz Institute for Farm Animal Biology (FBN), 18196 Dummerstorf, Germany
†Institute of Animal Breeding and Husbandry, Christian-Albrechts University of Kiel, 24118 Kiel, Germany

ABSTRACT

Archaeol (1,2-di-*O*-phytanyl-*sn*-glycerol) is a cell membrane lipid component of methanogens that has the potential to be used as a biomarker for methane production in ruminants. However, its analysis via gas chromatography–mass spectrometry (GC-MS) is challenging because of its molecular size and structure. In this study, 2 different sample preparation methods were tested, Soxhlet and sonication-aided extraction, and the methods were compared for extraction efficiency using the internal standard (IS; 1,2-di-*o*-hexadecyl-*rac*-glycerol). The extraction efficiency of the Soxhlet method for fecal archaeol was twice that of sonication. With the use of a high-temperature GC column, the retention times of IS and archaeol were 17.6 and 19.4 min, respectively, with a total run time of only 25 min. The molecule ions m/z 611.4 (IS) and m/z 725.8 (archaeol), or alternatively the fragment ion of the glycerol moiety m/z 130.0, were used for identification and quantification via GC-MS in positive chemical ionization mode. The intra-assay coefficients of variation for fecal archaeol measurements were 1.3% (m/z 725.8) and 2.1% (m/z 130.0) (n = 3), respectively. Fecal archaeol quantifications did not differ between the use of the molecule or glycerol moiety ions (paired *t*-test, n = 156). Archaeol concentrations tended to be 3.3% greater in samples stored at −20°C before drying compared with samples that were immediately dried after collection (paired *t*-test, n = 5). The detection limit of archaeol was 0.5 μg/g of fecal dry matter (DM); no archaeol could be detected in feed samples. In different fractions of rumen fluid, archaeol levels ranged from 1.9 to 24.0 μg/g of DM. In 10 cows fed the same grass and corn silage/hay-based ration, diurnal variations of fecal archaeol levels (5 time points over 2 d) were cow dependent and ranged from 26.2 to 77.2 μg/g of DM (mean 48.4 μg/g of DM). Thus, within-animal variation in cows on the same diet was between 4 and 27%. We suggest that this finding is related to the amount and time of the latest feed intake event before the fecal sampling. Feeding pattern can determine the passage rate of digesta through the alimentary tract and thus the duration of contact time of archaea with their substrate.
Key words: archaeol, methane biomarker, feces, rumen fluid, dairy cow

Technical Note

Methane is a greenhouse gas contributing to global warming, and on a global scale, an estimated 17% is attributable to enteric production, mostly from ruminants (Knapp et al., 2014). The gold standard to quantify methane emission in ruminants is the measurement of individual animals in respiration chambers, which is laborious and does not allow the screening of a large number of animals, especially under farming conditions. Currently, proxies for individual methane emission that are easier to measure are under investigation to provide better estimates for greenhouse gas inventories or phenotypic traits. One of these proxies is archaeol (1,2-di-*O*-phytanyl-*sn*-glycerol), a cell membrane lipid constituent of methanogenic microorganisms that is excreted with bovine feces (Gill et al., 2011). The aims of this study were to test the reproducibility and precision of different archaeol analytical methods and to identify analytical refinement factors. A further objective was to investigate whether time of the day of fecal sampling and manner of feces conservation affect archaeol values.

German Holstein dairy cows (average BW of 570 kg, first and second lactation, 100–140 DIM) fed ad libitum a TMR that was composed of (per kg of DM) 28.4% grass silage, 14.4% corn silage, 4 and 8% grass hay and wheat straw, respectively (17% RP, 99 g of starch, 349 g of NDF, 205 g of ADF per kg of feed DM; 6.7 MJ of NE_L; Aguinaga Casañas et al., 2015) were used to sample feces. Two further German Holstein cows equipped with a rumen fistula and fed a similar ration were in their second lactation and at 180 DIM when

Received May 11, 2016.
Accepted June 23, 2016.
[1]Corresponding author: metges@fbn-dummerstorf.de

rumen fluid was sampled. All cows were given fresh feed at 0700 and 1500 h and were milked twice a day at 0630 and 1630 h.

Five rectal grab samples of approximately 200 g of feces each from 10 cows were collected on 2 consecutive days. Fecal samples were thoroughly mixed and divided into 2 subsamples, one was stored at −20°C until further preparation, and the other was immediately dried at 60°C for 72 h, ground (1 mm), and kept in a cool, dry place. In addition, rumen fluid from 2 cows was sampled at 1000 h using a tailored device consisting of a syringe attached to a sieve probe that was introduced into the rumen cannula, always at the same depth, to obtain 1 L of fluid from the saccus ventralis. Rumen fluid was fractionated by filtration and centrifugation into (1) large particles (>2 mm), (2) sifted fraction (1–2 mm), (3) protozoa fraction (10 min, 100 *g*), and (4) bacteria fraction (45 min, 4°C, 22,800 *g*). Feed samples [corn silage, soy extract meal, concentrate (MF2000; Getreide AG, Güstrow, Germany), grass hay, dried melassed pellets (rape and corn pulp)] were collected.

Archaeol contents in bovine feces, rumen fluid, and feedstuffs were analyzed by modifications of methods previously published by Bull et al. (2003), Gill et al. (2010, 2011), and McCartney et al. (2013) as follows. Dried and ground (to 1 mm) fecal (1 g), rumen fluid fractions (1–5 g), and feed (5 g) samples were spiked with 1,2-di-*O*-hexadecyl-*rac*-glycerol (25 μg, Santa Cruz Biotechnology Inc., Santa Cruz, CA) as internal standard (**IS**). The Soxhlet extraction was performed by 200 mL of dichloromethane (**DCM**)/acetone (9:1, vol/vol) under reflux for 24 h using a Soxhlet apparatus according to Gill et al. (2010, 2011) but using about half the amount of IS as reported by McCartney et al. (2013; 43.4 μg of IS with 300 mg of sample). The total lipid extract was dried under a gentle N_2 stream and saponified with 5 mL of 5 *M* KOH in 90% methanol for 1 h at 120°C. After addition of 10 to 15 mL ultrapure water, pH was adjusted to 3 to 4 by addition of 6 *M* HCl. The saponified total lipid extract was extracted twice with 10 mL of chloroform, and the solvent of the combined organic phases was evaporated under N_2. The residue was dissolved in 5 mL of DCM/methanol (2:1, vol/vol) and dried by addition of anhydrous Na_2SO_4 (Bull et al., 2003). After removing the solvent, the sample was dissolved in 1 mL of DCM/isopropanol (2:1, vol/vol), applied to a pre-eluted (6 mL of *n*-hexane) solid phase extraction (**SPE**) column (Strata NH_2 0.5 g/6 mL, Phenomenex, Aschaffenburg, Germany), and eluted with 5 mL of the same solvent mixture. Finally, the solvent was evaporated under N_2, and samples were stored at 4°C.

Lipid extraction by sonication and the removal of polar head groups was performed according to McCartney et al. (2013) but with a lower amount of IS. The combined extracts were dried with Na_2SO_4 before evaporation. The extract was dissolved in 1 mL of DCM and purified by elution using a Strata NH_2 SPE column with another 4.5 mL of DCM.

The cleaned Soxhlet and sonication extracts were derivatized by adding 50 μL of pyridine and 50 μL of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane to yield trimethylsilyl (**TMS**) derivatives (Gill et al., 2010, 2011). The mixture was heated at 70°C for 1 h, and solvents were evaporated under a gentle N_2 stream. Derivatives were dissolved in 500 μL of ethyl acetate and proved to be stable at −20°C for at least 6 mo. They were separated by GC-MS (QP 2010 ULTRA coupled with GC 2010 plus; Shimadzu Deutschland GmbH, Duisburg, Germany) on a 30 m × 0.25 mm × 0.25 μm high-temperature column (ZB-5HT, Phenomenex). This method is in contrast to the earlier reports in which no high-temperature columns were used (Gill et al., 2010, 2011; McCartney et al., 2013). The GC was operated with helium as the carrier gas in the flow control mode (linear velocity 45 cm/s). A 1-μL sample was injected (injector AOC-5000 plus, Shimadzu) at a split ratio of 5:1. Injector and interface were held at 280°C, ion source was set at 200°C, detector gain at 1.4 kV, and emission current at 100 μA. The GC conditions for separation of the TMS derivatives were 70°C held for 1 min; 70–350°C, increased at 20°C/min; and 350°C held for 10 min (total run time 25 min). Mass spectra of IS and archaeol were recorded by selected-ion monitoring after positive chemical ionization (methane), but they were also checked for key ions by a scan in electron impact ionization mode. Archaeol and IS were identified and quantified by their specific molecule ions m/z 611.4 and 725.8, respectively, and their retention times of 17.6 and 19.4 min (Figure 1A). Alternatively, quantification was done using ions m/z 130.0, representing the glycerol moiety of archaeol and IS alike.

For quantification, calibration curves were constructed by derivatization of 0, 10, 20, 25, 30, 40, 50, 60, 70, and 80 μg of archaeol (1,2-di-*O*-phytanyl-*sn*-glycerol; Avanti Polar Lipids Inc., Alabaster, AL) together with a fixed amount of 25 μg IS (1,2-di-*O*-hexadecyl-*rac*-glycerol, corresponds to 50 ng/μL nonderivatized substance injected). The molecule peak area ratios as well as the ratios for the glycerol moiety of archaeol divided by the peak area of IS were plotted against archaeol amounts (Figure 2). The calibration curve was fitted polynomial (TableCurve 2D v5.01.01; Systat Software Inc., Chicago, IL) because the relationship was nonlinear in the lower part of the curve. The resulting equation was used to convert peak area ratios of the samples to archaeol concentrations (μg/g of DM) and