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GC/MS method for determining carbon isotope enrichment and concentration of underivatized short-chain fatty acids by direct aqueous solution injection of biogas digester samples

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ABSTRACT

In anaerobic digestion of organic matter, several metabolic pathways are involved during the simultaneous production and consumption of short-chain fatty acids (SCFA) in general and acetate in particular. Understanding the role of each pathway requires both the determination of the concentration and isotope enrichment of intermediates in conjunction with isotope labeled substrates. The objective of this study was to establish a rapid and simple GC/MS method for determining the isotope enrichment of acetate and concentration of underivatized short-chain fatty acids (SCFA) in biogas digester samples by direct liquid injection of acidified aqueous samples. Sample preparation involves only acidification, centrifugation and filtration of the aqueous solution followed by direct injection of the aqueous supernatant solution onto a polar column. With the sample preparation and GC/MS conditions employed, well-resolved and sharp peaks of underivatized SCFA were obtained in a reasonably short time. Good recovery (96.6–102.3%) as well as low detection (4–7 μmol/L) and quantification limits (14–22 μmol/L) were obtained for all the 6 SCFA studied. Good linearity was achieved for both concentration and isotope enrichment measurement with regression coefficients higher than 0.9978 and 0.9996, respectively. The method has a good intra- and inter-day precision with a relative standard deviation (RSD) below 6% for determining the tracer-to-tracee ratio (TTR) of both [2-^{13}C]acetate and [U-^{13}C]acetate. It has also a good intra– and inter-day precision with a RSD below 6% and 5% for determining the concentration of standard solution and biogas digester samples, respectively. Acidification of biogas digester samples with oxalic acid provided the low pH required for the protonation of SCFA and thus, allows the extraction of SCFA from the complex sample matrix. Moreover, oxalic acid was the source of formic acid which was produced in the injector set at high temperature. The produced formic acid prevented the adsorption of SCFA in the column, thereby eliminating peak tailing and ghost peaks. The applicability of the validated GC/MS method for determining the concentration of acetate and its ^{13}C isotope enrichment in anaerobic digester samples was tested and the results demonstrated the suitability of this method for identifying the metabolic pathways involved in degradation and production of acetate.

1. Introduction

The conversion of organic compounds from waste materials to methane is an important strategy for sustainable energy supply. The production of methane involves a consortium of several microorganisms, which undertakes major biological processes such as hydrolysis/acidogenesis, acetogenesis and methanogenesis steps. Fermentative bacteria hydrolyze polymers to simpler monomers and oligomers, which afterward are fermented to alcohols, short-chain fatty acids (SCFA), carbon dioxide and hydrogen. Then acetogenic bacteria convert the SCFA other than acetate to acetate, carbon dioxide and hydrogen. Finally, methanogens produce methane from the direct cleavage of acetate through acetoclastic methanogenesis pathway as well as the reduction of carbon dioxide with hydrogen via hydrogenotrophic methanogenesis pathway [1,2].

SCFA including acetate are key intermediates in an anaerobic digestion of organic matters to methane [2–6]. In particular to acetate, fermentative, acetogenic and homo-acetogenic bacteria are the main contributors to its production. Fermentative and acetogenic bacteria are involved in the production of H_2, CO_2, acetate and other products [3]. On the other hand, homo-acetogenic bacteria utilize H_2 and CO_2 for the production of acetate via homo-acetogenesis pathway according to Eq. (1). The feasibility of the homo-acetogenesis pathway is highly sensitive to the amount of hydrogen. In the

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presence of H_2-consuming microorganisms (e.g. hydrogenotrophic methanogens), the amount of hydrogen is kept very low. In such an environment, homo-acetogenic bacteria are outcompeted by the hydrogenotrophic methanogens due to the latter having a lower hydrogen threshold than the former [7]. However, homo-acetogenic bacteria has been shown to be stimulated in a high hydrogen concentration environment such as a biohydrogen producing digester [8] as well as actual land fill [9]. Understanding the role of different metabolic pathways involved in the production and consumption of acetate is paramount important for the efficient operation and optimization of biogas process. This is because of an imbalance in the production and consumption of acetate could lead to biogas process inhibition [4–6,10]. In this regard, an analytical technique that helps us to quantify the concentration of acetate and its isotope species from a biogas digester sample is highly needed.

$$2CO_2 + 4H_2 \rightarrow CH_3COOH + 2H_2O \quad (1)$$

Radio isotopes are commonly used as tracers to study the fate of acetate in anaerobic digester [7,11]. Radio isotope tracer is not a method of choice due to the strict safety requirement in handling radio isotopes [3]. An alternative method is the use of stable isotope pairing and the measurement of isotopically enriched products with hyphenated mass spectrometry techniques (GC/MS and HPLC/MS). GC/MS and HPLC/MS have been successfully employed for determining isotope enrichment of SCFA from plasma and urine samples in the field of clinical chemistry [12]. The separation of individual SCFA from a complex biological sample is provided by either HPLC or GC, whereas the tracer/tracee ratio (TTR) which is proportional to the ion-current ratio of the labeled/unlabeled species is determined by the mass spectrometer.

GC/MS is a widely employed technique for determining the isotope enrichment of SCFA [12–14]. Previous GC/MS method for SCFA measurement involves different sample preparation procedures, such as, extraction with organic solvents [13], purge and trap technique [15], steam distillation [13], ultrafiltration [13] and solid-phase microextraction [16] as the most common cleanup pre-treatments followed by derivatization step [12,13]. Derivatization of SCFA with different derivatizing reagents is usually performed for increasing the volatility of the analytes. Although these sample preparation procedures were demonstrated to be a good cleanup strategy, they are relatively time-consuming and labor-intensive. They may also reduce analyte recovery and affect the accuracy and repeatability of the method due to the requirement of multiple sample preparation procedures [13,17,18]. Moreover, derivatization of the analytes potentially increases natural isotope background due to the introduction of additional isotopomers from the derivatizing reagents which challenges accurate determination of low isotope enrichment assay [12].

An alternative method to derivatization of SCFA is a direct injection of aqueous sample into a GC/MS system after a minor pre-treatment of the liquid sample. GC method for determining the concentration of fermentation products including SCFA from the direct injection of aqueous solution into GC was shown to be rapid, accurate and reliable [4,13,18–20]. However, direct aqueous solution injection could lead to peak tailing and ghosting due to contamination of the GC column. Biogas digester liquid samples are complex in nature containing several organic and inorganic compounds and solid particles. Therefore, an appropriate choice of glass liner, cleaning solvent and sample preparation strategy should be employed to avoid column contamination which ultimately provides good baseline separation of individual SCFA and peak shape.

To date there has not been any report on the application of GC/MS method for determining the isotope enrichment of underivatized SCFA from the direct injection of aqueous biogas digester sample into GC/MS system. To our knowledge this is the first time GC/MS was applied for determining the isotope enrichment of acetate in a biogas digester samples without involving any derivatization step, though GC/MS method was employed in plasma samples in one study [14]. In this study we report a very simple, accurate, reproducible and rapid GC/MS method for determining both the isotope enrichment of acetate and concentration of underivatized SCFA in biogas digester sample by direct aqueous sample injection into the GC system. A water resistant polar column was used for separating underivatized SCFA from the complex biogas digester matrix. In this study, oxalic acid was used for acidification of biogas digester samples and other purposes such as to improve peak sharpness, reduce peak tailing and to clean unwanted residues coming from the complex sample matrix. As an example of application of this method, this paper also presents that a stable isotope tracer experiment in combination with tracer-to-tracee ratio (TTR) determination by the GC/MS method can be used to verify the activity of homo-acetogenic bacteria in a biogas digester process.

2. Experimental

2.1. Reagents

A short-chain fatty acids (SCFA) mixture containing acetic acid (C_2), propionic acid (C_3), isobutyric acid (*i*-C_4), *n*-butyric acid (C_4), isovaleric acid (*i*-C_5) and *n*-valeric acid (C_5) was obtained from Sigma-Aldrich (Denmark). The concentration of all the analytes was 10 mmol/L in deionized water. Sodium acetate, acetic acid, propionic acid, butyric acid, *tert*-pentanoic acid (*t*-C_5), oxalic acid as well as sodium salts of [2-^{13}C]acetate (99 at%) and [U-^{13}C]acetate (99 at%) were purchased from Sigma-Aldrich (Denmark). Fluoromethane as specific inhibitor to acetoclastic methanogens was obtained from Sigma-Aldrich (Denmark).

2.2. Instrumentation and analytical condition

The GC/MS analysis was performed using a CP-3800 gas chromatograph (Varian Inc.) coupled to a Saturn 2000 ion trap mass spectrometer (Varian Inc.). The gas chromatograph was equipped with an electron impact ion source, split–splitless injector and an autosampler. A high polarity capillary column with a cross-linked and bonded polyethylene glycol (PEG) phase (HP-INNOWax, 30 m × 0.25 mm i.d coated with 0.25 μm film thickness, Agilent Technologies Inc., Denmark) was used for separation of the SCFA. GC/MS conditions for determining both the isotope enrichment and concentration were as follows: positive electron impact mode; injector temperature, 285 °C; helium constant flow, 1 ml/min; initial column temperature was 100 °C and hold for 1 min, then increased at the rate of 10 °C/min to 120 °C and hold for 5 min, and finally increased at the rate of 30 °C/min to 220 °C and kept at 220 °C for 3 min; solvent delay time, 3 min; total run time, 14.3 min. The volume injected was 1 μL in 1:10 split mode. All the samples were analyzed in full scan mode with a mass range of *m/z* 40–300. Finally, the following extracted ions were used for determining the concentration and isotope ratio of SCFA: *m/z* 60 for unlabeled C_2, C_4, *i*-C_5 and C_5; *m/z* 61 for [1-^{13}C]acetate/[2-^{13}C]acetate; *m/z* 62 for [U-^{13}C]acetate; *m/z* 57 for *t*-C_5; *m/z* 73 for C_3 and *i*-C_4. The total concentration of acetate was determined from sum of ion currents of *m/z* 60, 61 and 62. Data acquisition and analysis was done with Saturn GC/MS workstation software (Varian Inc.)

2.3. Operation of biogas digester

Inoculum was obtained from batch anaerobic digesters fed with ^{13}C fully labeled maize leaf (IsoLife BV, Wageningen, the