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TECHNICAL NOTE

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Method development for the quantitative determination of short chain fatty acids in microbial samples by solid phase extraction and gas chromatography with flame ionization detection

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Abstract

Background: In order to quantitatively measure short chain fatty acids (SCFAs) in microbial samples, sample preparation and analytical conditions using gas chromatography with flame ionization detection (GC-FID) were established.

Methods: The extraction of SCFAs with ethyl ether-hexane (1:1, *v/v*) followed by the aminopropyl solid phase extraction (SPE) was carried out. An acid-modified poly(ethylene glycol) GC column was used for the chromatographic separation of four volatile fatty acids.

Results: Good linearity was obtained with $r > 0.999$ in the calibration range. The limits of quantification (LOQs) of the analytical method were in the ranges of 5.71 to 11.20 μg/mL. The overall recoveries excluding acetic acid ranged between 96.51 and 108.83% with relative standard deviations (RSD) below 10% for the entire procedure from the results of matrix spiking experiments at three concentration levels.

Conclusion: The analytical method was validated in accordance with the requirements of the international guideline. The method enables the use of the most commonly used technology without derivatization and the elimination of interferences in the sample matrix.

Keywords: Short chain fatty acids (SCFAs), Microbial samples, Gas chromatography with flame ionization detection (GC-FID), Solid phase extraction (SPE)

Introduction

As the importance of short chain fatty acids (SCFAs) increase, a lot of research is being done in various fields. SCFAs are the main products of bacterial metabolism in the human large intestine. Evidence for the occurrence of microbial breakdown of carbohydrate in the human colon, informing the healthy state, has been sought by measuring the concentrations of SCFAs. They, also referred to as volatile fatty acids (VFAs), have an important role in biogas production as well as the human body. Anaerobic digestion is essential for the production of biogas, and it takes place in the order of hydrolysis, fermentation, and methanogenesis. In the fermentation process, SCFAs are produced by the decomposition of amino acids, sugars, and some fatty acids and are a precursor of methanogenesis which is the final process of biogas production. For this reason, the measurement of SCFA in biogas production has been recognized as a quantitative indicator for monitoring biogas production efficiency (Sheveleva and Ramenskaya 2010, Madsen, Holm-Nielsen et al. 2011). Thus, the monitoring of SCFAs produced by microbial samples is necessary for various reasons and it can be measured from a

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culture medium which is required for microbial growth (Banel, Jakimska et al. 2012).

Various separation techniques have been used to determine SCFA in biological fluids, the most widely used being gas chromatography (GC) (McGrath, Weir et al. 1992). By combining selective GC detectors, a flame ionization detection (FID) is commonly used due to its inexpensive cost and operation, as well as its ability to detect a wide range of concentrations of organic compounds (Ahn, Jeon et al. 2018). Regarding the unique physicochemical properties of SCFAs, low vapor pressure and relatively high solubility in the aqueous phase cause the difficulties in the sample preparation (Park, Kim et al. 2017). Several types of derivatization typically done to change the analyte properties have been adopted to enhance the better separations with increased resolution and response (Klemm, Hintze et al. 1973, Moreau, Goupry et al. 2003). However, their procedures include the steps of chemical reaction or concentration, and it can lead to serious analyte loss due to the high volatility of SCFAs. Furthermore, risks of contamination and ghost peaks are sometimes limiting factors during SCFA analysis (Pouteau, Meirim et al. 2001). In this study, we report a combined method using solid phase extraction (SPE) followed by the chromatographic separation with acid-modified poly(ethylene glycol) column for the quantification of SCFAs in microbial samples. The method was validated with respect to linearity, limit of detection (LOD), limit of quantification (LOQ), and percentage recovery from the entire procedure using the control microbial samples.

Methods

Chemicals and reagents

Four SCFAs (acetic acid, propionic acid, butyric acid, and valeric acid) and pivalic acid as an internal standard were purchased from Sigma-Aldrich (St. Louis, MO, USA). Distilled water was filtered using a Milli-Q Reagent Water System (Millipore, Billerica, MA, USA). All organic solvents of GC analysis grade were purchased from Burdick & Jackson (Philipsburg, NJ, USA). Aminopropyl solid phase extraction cartridges (LC-NH2, 100 mg) were from Supelco (Bellefonte, PA, USA).

Preparation of samples

The quantitative determination of SCFAs produced by microorganism can be measured in culture media, and the reinforced clostridial medium was used as the experimental samples. Three grams of anhydrous sodium sulfate and 300 μL of sulfuric acid (2.5 mol/L) were added to 1 mL of sample containing 25 μg/mL of internal standard. Consecutively, the sample was extracted with 3 mL of ethyl ether-hexane mixture (1:1, *v*/*v*) for 10 min and centrifuged at 2500 rpm for 5 min. The upper phase was collected and was performed two times with the same procedure. The extract was loaded onto the aminopropyl SPE cartridge, which had been pre-conditioned with 10 mL of hexane. Ten milliliters of chloroform/2-propanol (2:1, *v*/*v*) was discarded to remove any interference, and the fraction for SCFAs was collected with elution of 6 mL of diethyl ether containing 2% formic acid.

Instrumentation

Agilent 6890 gas chromatographic system (Agilent Technologies, Santa Clara, California, USA) equipped with a flame ionization detector and an automated liquid sampler was used. Chromatographic separation was achieved using a Nukol™ capillary GC column (15 m × 0.53 mm × 0.5 μm film thickness) from Supelco (Bellefonte, PA, USA). The oven temperature was maintained at 80 °C for 2 min and then ramped to 190 °C at a rate of 5 °C per minute. Injection was performed at 190 °C; the injection volume is 1 μL with split ratio 10:1 and a linear velocity of 30 cm/s. The detector gases were air and hydrogen; their flow rates were regulated at 450 and 40 mL/min, respectively. Nitrogen was used as make-up gas at 40 mL/min.

Method validation

The validation of the analytical method was performed to determine the linearity of calibration, LOD, LOQ and evaluated the accuracy, precision from the results of spiking experiments. For the quantification, a standard mixture of four SCFAs in distilled water and the matrix-matched calibration standards in the concentration range of 0.1 to 100 μg/mL were prepared. For the spiking experiment, the reinforced clostridial medium provided from the biological disaster analysis group of Korea Basic Science Institute was used. It was the supernatant of broth culture of *A. rhamnosivorans sp.* under anaerobic conditions with pH 6.8 to 7.2 at 37 °C. The percent recovery of SCFAs was calculated by analyzing five replicate spiked samples and comparing with matrix-matched calibration standards for each concentration.

Results and discussion

Derivatization and analysis of SCFAs by GC-MS

Normally, the preparation of chemical derivatives has been performed using some reagents such as bromoacetophenone (L'Emeillat, Ménez et al. 1981), difluoroaniline (Breves and Krumscheid 1997), and phenyldiazomethane (Klemm, Hintze et al. 1973) for the analysis of SCFAs in biological samples. Among the various derivatization, silylation is the most common derivatization technique used in the analysis of SCFAs (Ng and Hupé 1993); however, the silylated derivatives have been reported that the results were not reproducible, and it

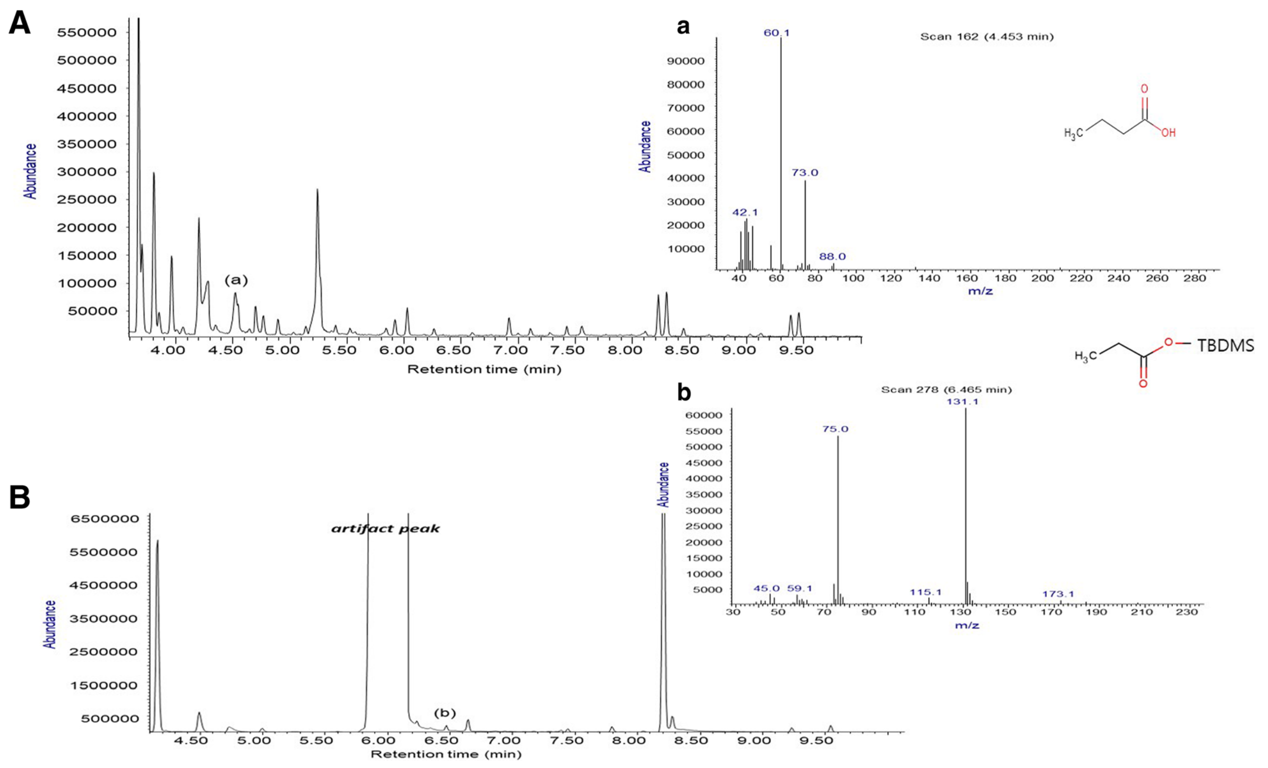

Fig. 1 Total ion chromatograms of the microbial sample spiked with SCFAs in the GC-MS scan mode (**A**) underivatized extract (**a**) Mass spectrum of partially separated butyric acid (50 μg/mL), (**B**) after TBDMS derivatization. (**b**) Mass spectrum of propionic acid-TBDMS eluted after the artifact

was thought that the hydrophilic nature of the reagent yielded an unstable product that was easily hydrolysable under conditions where water was present (Boppana, S 2013). Alternatively, tert-butyl dimethylsilyl (TBDMS) derivatives of SCFAs from the microbiological media sample were applied to separate on non-polar stationary phase, DB-5MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness, 5% diphenyl, 95% dimethylsiloxane phase, J&W Scientific, Folsom, CA, USA) with a gas chromatography/mass spectrometry (GC-MS). Figure 1 indicates the total ion chromatogram of the extract of microbial media

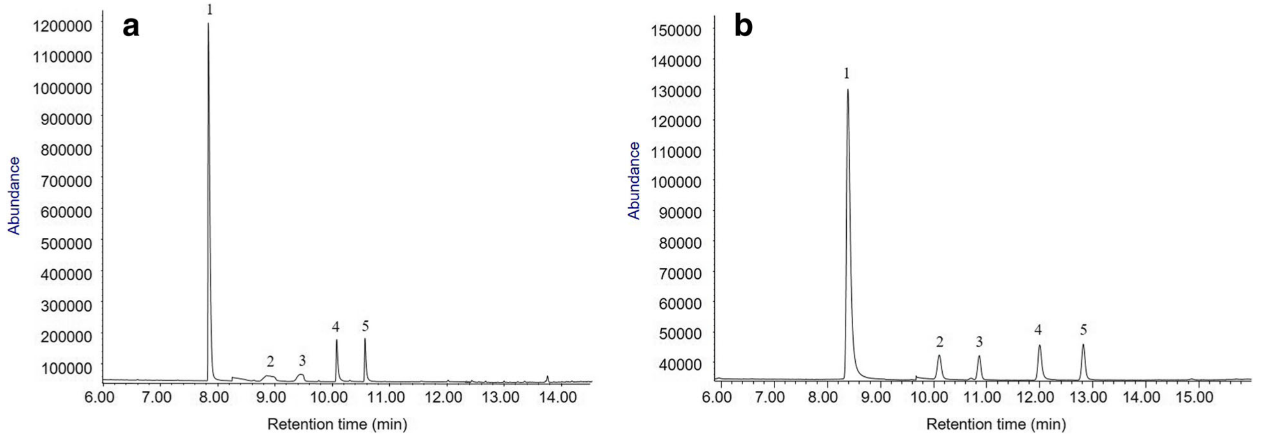

Fig. 2 Comparison of chromatograms of the eluent through SPE cartridge for the spiking control sample with SCFA standard (50 μg/mL) (**a**) DB-WAX, (**b**) Nukol™ column. Peak identities were as follows: (1) acetic acid, (2) propionic acid, (3) pivalic acid (IS), (4) butyric acid, (5) valeric acid

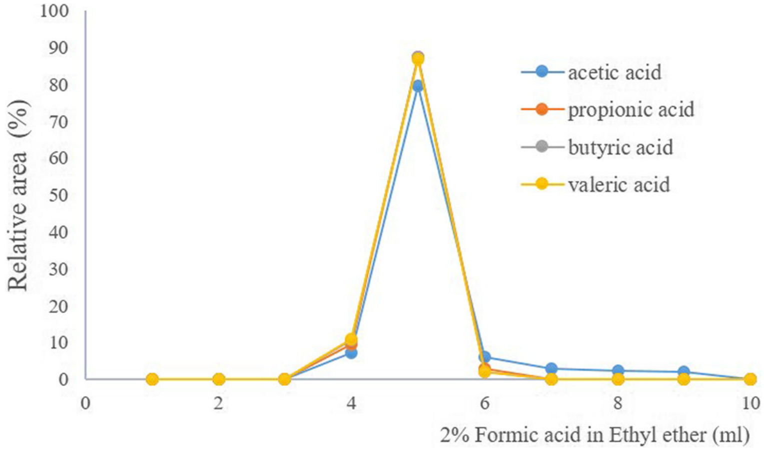

Fig. 3 Elution profile of SCFAs from aminopropyl SPE cartridge

spiked with 50 μg of SCFAs at the concentration of 50 μg/mL in the sample obtained by GC-MS. Acetic and propionic acids could not be found because of the early co-eluting compounds in the sample matrix even though the extracted ion chromatogram (EIC) was generated. Butyric acid was able to be found from the co-eluted other compounds in a state of partial separation by generating EIC process as shown in Fig. 1. After the TBDMS derivatization step, the chromatographic separation was improved; however, the artifacts from the derivatization reaction were inevitable. Especially, the separation of propionic acid was influenced by the artifacts and it could not completely remove even by GC-MS in selected ion-monitoring mode. For that reason, the use of polar stationary phase columns was considered. Instead, SPE cleanup combined with GC-FID which is the most popular GC detector was applied to separate and quantify underivatized SCFAs in the biological culture medium.

Chromatographic properties of underivatized SCFAs

For the separation of SCFAs, the polarity of the column stationary phase plays a critical role, and two types of GC column, DB-Wax and Nukol™, were compared. Figure 2 shows the comparison of chromatograms of the eluent through SPE cartridge for the spiking control sample with SCFA standard obtained by GC-FID in two kinds of column. Since the peak resolution of SCFAs in the Nukol ™ column was better than the peak resolution

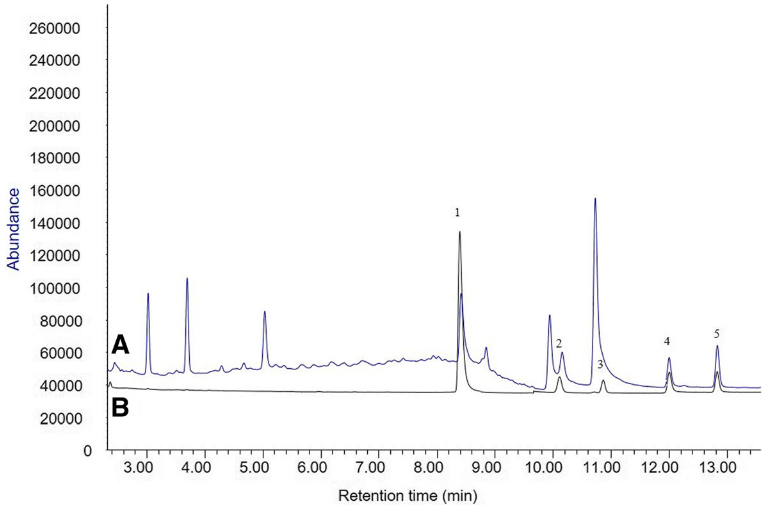

Fig. 4 Overlay chromatograms of the microbial medium sample spiked with SCFAs (50 μg/mL) prior to (A) and after (B) adopting SPE cleanup. Peak identities were as follows: (1) acetic acid, (2) propionic acid, (3) pivalic acid (IS), (4) butyric acid, (5) valeric acid

Table 1 Calibration, LOD, and LOQ of SCFAs obtained by GC-FID

Compound	Calibration curve			LOD (μg/mL)	LOQ (μg/mL)
	Slope	Intercept	Correlation coefficient		
Acetic acid	0.0088	0.3388	0.9994	3.00	9.09
Propionic acid	0.0241	0.1869	0.9992	3.70	11.20
Butyric acid	0.0333	0.1061	0.9998	1.88	5.71
Valeric acid	0.0331	0.1415	0.9995	2.79	8.46

in the DB-Wax column, therefore method validation was performed using a Nukol ™ column.

Optimization of SPE condition

The mixture of ethyl ether-hexane (1:1, *v:v*) as an extraction solvent of SCFAs to be transferred from an aqueous to an organic phase was chosen to increase the extraction efficiency (Mallatou, Pappa et al. 2003, Juan, Ferragut et al. 2008). In order to measure low concentrations of SCFAs from biological samples, the procedure of aminopropyl SPE which is known to be in the effective separation of lipids was applied (Valerie Walker 2002). Figure 3 shows the elution profile of SCFAs from the biological culture medium eluted with diethyl ether containing 2% formic acid after loading of the sample. From this result, 3 mL of elution solvent of diethyl ether containing 2% formic acid could be discarded, and afterward, 3 mL of eluent was enough for the efficient recovery to collect SCFAs. Figure 4 shows the GC chromatograms are overlaid for the biological culture medium sample before and after the SPE cleanup procedure obtained by GC-FID. After the procedure, the baseline noise on the chromatogram was reduced to increase the selectivity and sensitivity of SCFAs from the sample matrix.

Method validation

The calibration curves of SCFAs were generated in the range of 0.1~100 μg/mL using least-square linear regression analysis. Eight levels of standard mixtures of SCFAs (0.1, 0.5, 1, 5, 10, 25, 50, 100 μg and 25 μg of internal standard) in 1mL of distilled water were prepared and analytical procedures were carried out. The calibration equations were obtained by the peak areas for each analytes against the internal standard of pivalic acid. Detailed data is given in Additional file 1: Table S1 and Figure S1. The correlation coefficients to evaluate the degree of linear were all greater than 0.999. The LOD and LOQ were determined based on the standard deviation (SD) of y-intercept of the regression line (s) and the slope of the calibration curve (S) as $\text{LOD} = 3.3 \times (s/S)$ and $\text{LOQ} = 10 \times (s/S)$ in accordance with ICH (International Council for Harmonisation) guidelines (Dongala, Palakurthi et al. 2019). The linearity of calibration, LOD, and LOQ parameters are summarized in Table 1. For the recovery test, the pooled control sample of microbial culture medium was analyzed, and target analytes were not found excluding acetic acid. Acetic acid could not be evaluated because the presence of the analyte in the control sample was attributed to the cultivation media composition. Five replicate analyses of pooled medium samples spiked with SCFAs for three different levels of concentration (5, 25, and 50 μg/mL) were performed in accordance with the optimized procedure. The accuracy and precision were assessed by the average recovery and percentage RSD of five results at each concentration as shown in Table 2 (see Additional file 1: Table S2). The average recovery of SCFAs ranged from 96.51 to 108.83%, with RSDs ranged from 1.75 to 4.08%. The obtained results fell within the acceptance criteria except acetic acid.

Conclusion

In the present study, SPE cleanup coupled with GC-FID was applied for the quantification of SCFAs in microbial culture samples. The use of derivatization reagent led to artifacts and interfered with the separation of propionic acid. For the chromatographic separation of underivatized SCFAs, a Nukol™ column was effective to increase the selectivity for them among the polar stationary phase columns. This work shows the linearity, LOD, and LOQ based on the ICH validation guideline for the optimized method. The high percentage recoveries were obtained from the entire procedure using the control microbial samples, but excluding acetic acid because of the presence in the control sample. This method enables the use of the most common detector without derivatization and the elimination of interferences in the sample matrix.

Table 2 Accuracy and precision from the results of spiking experiments

Compound	5 μg/mL		25 μg/mL		50 μg/mL	
	Accuracy (%)	Precision (% RSD)	Accuracy (%)	Precision (% RSD)	Accuracy (%)	Precision (% RSD)
Propionic acid	103.52	1.95	102.75	4.08	108.50	3.36
Butyric acid	104.72	1.33	96.51	1.75	108.38	2.33
Valeric acid	98.47	3.18	108.83	3.14	99.86	3.76

Additional file

Additional file 1: Table S1. Descriptive data of Calibration, LOD and LOQ of SCFAs obtained by GC-FID. **Table S2.** Descriptive data of recovery experiment. **Figure S1.** Calibration curves of SCFAs. (DOCX 47 kb)

Abbreviations

EIC: Extracted ion chromatogram; GC-FID: Gas chromatography with flame ionization detection; GC-MS: Gas chromatography/mass spectrometry; ICH: International Council for Harmonisation; LOD: Limit of detection; LOQ: Limit of quantification; RSD: Relative standard deviation; SCFAs: Short chain fatty acids; SPE: Solid phase extraction; TBDMS: Tert-butyl dimethylsilyl; VFAs: Volatile fatty acids

Authors' contributions

YGA designed the study. The experiment was executed by HK and SYC. Supervision of experimental work and analysis of results were done by YGA and JK. HK and YGA contributed to the draft version of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Research data have been provided in the manuscript and supporting information file.

Competing interests

The authors declare that they have no competing interests.

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