Determination of volatile fatty acids in tofu wastewater by capillary gas chromatography with flame ionization detection: A Comparison of extraction methods

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Abstract. Volatile fatty acids (VFA) are intermediate products during the anaerobic digestion process of complex waste. Accurate quantification of VFA is a useful measurement of the process. This study evaluated two extraction methods for gas chromatography determination of the following VFA: acetic acid, propionic acid, butyric acid, and valeric acid to find a simple and efficient preparation approaching on Green Analytical Chemistry methods. Tofu wastewater was used as the sample. The extraction was performed using diethyl ether and carried out either by vortex mixing or shaken by hand. The measurement was performed using gas chromatography with HP-INNOWax column and flame ionization detector. The linearity, precision, accuracy, limit of detection and limit of quantification were evaluated. VFA determination was linear at the concentration range of 5-500 μg/mL for all VFA. The precision of 2.59-12.63 % was obtained for both methods. The recovery of the fortified sample (10 μg-VFA/mL) was 75.33-98.31 % for vortex extraction and 99.81 to 103.80 % for shaken by hand extraction. Limits of detection and quantification of vortex extraction were 0.38-2.78 μg/mL and 1.06-3.03 μg/mL, respectively. Limits of detection and quantification using hand shaken extraction were 0.09-1.13 μg/mL and 0.55-1.61 μg/mL, respectively. Based on the high recovery and low limits of detection and quantification, shaken by hand extraction method can be used for rapid and accurate quantification of VFA.

Keywords: Capillary gas chromatography, Extraction method, Tofu wastewater, Volatile fatty acid

1. Introduction

One of the monocarboxylic aliphatic acids containing 2 to 7 carbon atoms is volatile fatty acids (VFA) or also known as short-chain fatty acids (SCFA). In many biological processes, volatile fatty acids (VFA) act as intermediates and as metabolites, the presence of bacterial activity can also be demonstrated by the presence of these compounds in a sample [1]. The boiling point of VFA for ethanolic acid or acetic ranges from 117 °C to 235 °C for octanoic acid, with solubility in water is influenced by their chain length; acetic and propionic acids are well soluble, butyric acid is fairly soluble, while hexanoic and higher acids are poorly soluble in water [2]. Based on its solubility, at highest concentrations in the environment, acetic and propionic acids are quite mobile. Upon contact with heavy metals or radionuclides, they increase the mobility of these pollutants and very dangerous
for the environment [1]. Source of VFA emissions can be very disturbing for humans, because of the foul odour it produces mainly from butyric, propionic and acetic acids.

Separation and determination of individual VFAs in several types of samples can be done by several methods. Mass spectrometric detection or flame ionization and gas chromatography (GC), mainly capillary GC with polar or slightly polar stationary phases are commonly used [2,3]. Another study used high-performance liquid chromatography (HPLC) with reversed-phase column [4].

Gas chromatography can be used to determine the VFA from aqueous samples, after filtration or centrifuged the sample to remove solid materials inside, but this method can only recommended for samples with low colloidal and particulate matters and complex organic molecules, detection system and separation column are not sensitive to water [5–7].

VFA in several procedures are isolated from complex sample or original samples to be a new matrix which can be inert gas, organic solvent or stationary phase on fused silica fibre and desorbed thermally into GC injector. Solid phase micro extraction (SPME) was performed on water sample directly from the aqueous phase, and then followed by mass spectrometry/GC [8]. When it is necessary to determine only the organic acid, liquid-liquid extraction (LLE) is a selective technique and very sensitive that also contributes to increasing the useful life of chromatographic column [3]. Petroleum ether as solvent extraction was used to extract VFA from leachate, followed by GC determination [9]. Marine sediments after derivatization with 2-nitrophenylhydrazides can be determined by HPLC [4]. Manni and Caron [6] extracted VFA from an original aqueous sample with diethyl ether and then was analysed by GC. Methyl-tert-butyl ether (MTBE) was shown the same in a very similar way to [6] and used as a VFA extractant for their determination in aqueous samples [2]. However, because most of these method required sample preparation before determination by GC, its either time consuming and also the chromatographic column may be damage cause its uses a very acidic solution that are injecting directly into GC.

Tofu is popularly consumed for its health benefits and nutrition. It has been increasingly used in numerous culinary dishes. Tofu production process can produce waste which can cause problems in the environment. The liquid waste produced from tofu industries are often discharged directly into the river, which disrupts the life of the river biotic, degrades the quality of water from highly content of organic substances, pollution of the surface and groundwater, and causes unpleasant odours. VFA is generated during the anaerobic digestion process and, their parameters that must be controlled for methane production [10].

Based on these, it is necessary to develop an analytical method to detecting and measure VFA content accurately and precision based the Green Analytical Chemistry concepts. Two extraction methods for determination of VFA by GC in tofu wastewater were evaluated in this study. Both methods used diethyl ether as the extracting solvent, vortex mixing or shaken by hand was used for extraction process. GC with a flame ionization detector (GC-FID) is used for measurement purposes. The accuracy, precision, linearity, limit detection and also limit of quantification were evaluated.

2. Material and Methods

2.1. Tofu wastewater
Tofu wastewater was taken from a home-based tofu industry located next to Cikapundung River, Bandung, Indonesia. Tofu wastewater was freshly collected in the morning after tofu production. The wastewater had a pH of 4 and a temperature of 35 °C upon collection. The sample was collected in 500 mL PTFE bottles and immediately stored in a cold room at a temperature of ± 4 °C.

2.2. Chemicals and reagent
All VFA (valeric acid, propionic acid, acetic acid, butyric acid) standards and diethyl ether were of analytical grade and obtained from Merck.
2.3. Preparation of standard solution
Stock solutions of valeric acid, propionic acid, acetic acid, butyric acid, were prepared by dissolving each VFA in 5000 µg/mL diethyl ether. These solutions were subsequently mixed to prepare a 1000 µg/mL mixed standard solution in diethyl ether. For linearity determination, the concentration of mixed standard solution was diluted at range of 0.1 to 500 µg/mL in diethyl ether.

2.4. Sample extraction
Two extraction methods were tested, i.e. vortex and shaken by hand methods. Before the analysis, the sample was conditioned to reach room temperature. Vortex extraction was carried out in a pre-cleaned screw cap septum vial made from borosilicate glass and of 20 or 40 mL capacity. The vial was rinsed several times with tofu wastewater sample or standard. A volume of 8 mL sample was transferred to the vial, and then a thick septum lined with polytetrafluoroethylene (PTFE) was placed on top of the vial to ensure no air bubble trapped within, and the septum cap was firmly screwed down to seal the sample in the vial [11]. Before extraction, the sample was centrifuged at 4000 rpm for 3 min, and the supernatant was transferred to the vial, mixed with diethyl ether (1:1) by vortex at 2500 rpm for 10 min and left until two separate two layers were formed. From the supernatant, a volume of 1 µL was injected into the GC using manual injection [11]. Shaken by hand extraction was performed in a similar procedure as vortex extraction, but instead of mixing by vortex, the sample was mixed by handshaking for 10 s.

2.5. VFA determination
VFA composition of the samples was analysed using gas chromatography (GC) Agilent Technologies 7890B equipped with a split injector and flame ionization detection (FID) system. VFA were separated using HP-INNOWax column (15 m × 0.25 mm i.d, 0.25 µm thicknesses). Held the oven temperature at 40 °C for 2 min, then increased to 150 °C at 10 °C/min, held for 1 min. Temperatures for injector and detector were set at 250 °C and 300 °C, respectively. Sample (1 µl) was injected with a split ratio of 10:1 at column temperature of 40 °C. Helium was used as a carrier gas for the system at 3.0 mL/min, 16.793 psi. Hydrogen and air were used for carrier gases for FID, held at 30 and 400 mL/min, respectively.

Based on the retention time, all VFA peaks were identified. Determine the VFA concentration by plotting the width area against the calibration curve of each compound (valeric acid, propionic acid, acetic acid, butyric acid).

2.6. Recovery determination
Recovery determination was performed by the addition of mixed VFA standard solution to tofu wastewater sample at the concentration of 10 µg/mL. The fortified sample was then extracted and determined using the same method as the unfortified sample, and the results were compared. The determination was performed in seven replicates.

2.7. Determination of Limit of detection (LOD) and limit of quantification (LOQ)
The LOD and LOQ were determined by performing quantification on serial dilutions of a VFA mixture and evaluated based on the signal-to-noise ratio. The determination was performed in seven replicates. The concentration of LOQ was about 2 % of the individual VFA assay level and injected into the GC system to obtain a signal-to-noise ratio higher than 10. The limit of detection was estimated based on a limit of quantification injection to obtain a signal-to-noise ratio higher than 3.0.

3. Results and discussion
Figure 1 shows a clear separation between acetic acid, propionic acid, butyric acid and valeric acid with retention times of 0.868; 7.357; 8.394; 9.410 and 10.601 min, respectively. All peaks provided good resolution, as shown by distinct separation on base peaks. Diethyl ether as solvent was shown as a peak at 0.389 min. The analysis of VFA can be performed by using HP-INNOWax capillary column
with the Gas Chromatography equipped Flame Ionization Detector (GC-FID). The linearity of VFA determination using GC-FID was investigated and the results are presented in Table 1. For all VFA, the linear range was 5 to 500 µg/mL at correlation coefficients of ≥ 0.999.

![Figure 1. Standard chromatogram of VFA at 10 µg/mL](image)

**Table 1.** Calibration curve data for VFA

| Compound      | t_R (min) | Linear Range (µg/mL) | Correlation coefficient (R) | Equation          |
|---------------|-----------|----------------------|----------------------------|------------------|
| Acetic acid   | 7.342     | 5 - 500              | 1.000                      | y = 0.429x + 1.187 |
| Propionic acid| 8.395     | 5 - 500              | 0.999                      | y = 0.718x + 1.658 |
| Butyric acid  | 9.411     | 5 - 500              | 1.000                      | y = 0.944x + 0.438 |
| Valeric acid  | 10.602    | 5 - 500              | 1.000                      | y = 0.924x + 1.966 |

Tofu is produced by coagulation process of soybean milk. Traditional tofu production in Indonesia is often used fermented whey as coagulant [12], which contains various organic acid, mainly formic and acetic acids. Due to this process, acetic acid will be present in the wastewater, and the content of acetic acid is higher than other VFA compounds. The results of the VFA determination of tofu wastewater are presented in Figure 2 and Table 2. Coefficient of variance (CV) for repeatability of VFA was in the range of 7.32 to 12.63 % for vortex extraction and 2.59 to 9.54 % for shaken by hand extraction, (Table 2). For both methods, the CV values were less than CV Horwitz for all VFA. However, all average concentrations from vortex extraction, particularly acetic acid, were lower than shaken by hand extraction. This occurred because the very high pressure from the vortex caused the emergence of gas above the surface of the sample solution so that the septum bulged. Acetic acid is the lowest organic carbon atom in the measured VFA, which was more readily oxidized by air to CO₂ and H₂O, resulted in a lower acetic acid concentration in the sample. Acetic acid concentration from vortex extraction was significantly lower than shaken by hand extraction due to the higher volatility of acetic acid compared with other VFA. In fact, the sample should not be shaken excessively because the sample will be oxidised or hydrolysed so that it can reduce the VFA content.
Figure 2. Chromatogram of VFA in tofu wastewater

Table 2. The reproducibility data (precision) for VFA (n=7)

| Compound   | Vortex extraction | Shaken by hand extraction |
|------------|-------------------|---------------------------|
|            | Average concentration (µg/mL) | CV (%) | Horwitz CV (%) | Average concentration (µg/mL) | CV (%) | Horwitz CV (%) |
| Acetic acid| 99.86             | 7.74     | 7.97          | 207.53          | 2.59    | 7.97          |
| Propionic acid | 1.07             | 7.32     | 15.83         | 1.32            | 5.66    | 7.97          |
| Butyric acid | 0.91             | 12.63    | 16.23         | 1.28            | 3.59    | 16.75         |
| Valeric acid | 0.58             | 11.20    | 20.21         | 0.71            | 9.54    | 17.50         |

Fortification technique was used to determine the VFA recovery; the results of fortified tofu wastewater sample are presented in Figure 3 and Table 3. The results show that VFA recoveries were 75-98% for vortex extraction and 99-103% for shaken by hand extraction. VFA recoveries from shaken by hand extraction method (>$99\%$) were 80-110% for fortification unit 10 µg/mL [13] and were better compared with other studies that used cultured shakily method, which reported VFA recoveries of 87.5 and 95.86% [14,15]. On other hands, lower recoveries were achieved for vortex extraction that also suggests a loss of sample in this method because of the oxidation process. As expected in this case, acetic acid had the lowest recovery, and valeric acid had the highest recovery from vortex extraction.

Table 3. VFA recoveries from fortified tofu waste sample

| Compound    | Recovery (%) (Mean ± SD, n=7) |
|-------------|-------------------------------|
|             | Vortex extraction | Shaken by hand extraction |
| Acetic acid | 75.33 ± 8.19         | 101.74 ± 12.48          |
| Propionic acid | 86.70 ± 1.52     | 103.80 ± 5.79          |
| Butyric acid | 85.79 ± 8.84        | 99.81 ± 15.18          |
| Valeric acid | 98.31 ± 6.23        | 99.89 ± 1.90           |
Figure 3. Chromatogram of fortified tofu wastewater sample

Table 4 shows the limit of detection (LOD) and limit of quantification (LOQ) for both methods. LOD in common knowledge is obtained from three times of variation in the blank measurement of a standard curve. The LOD presented in this study was obtained by diluting the concentration of the sample as small as possible and injecting it into the GC until the concentration response fluctuated. LOD was established at 2.780 and 1.130 µg/mL for vortex and shaken by hand extraction methods, respectively. The LOQ presented in this study was obtained by diluting the concentration of the sample as small as possible and injecting it into GC but the concentration response must provide a good precision. The LOQ was established at 3.025 and 1.614 µg/mL for vortex and shaken by hand extraction methods, respectively. The vortex extraction had a higher level of detection and quantification limits because the lower recovery increased the signal-to-noise ratio.

Table 4. Limit of detection and quantification data for VFA

| Compound   | Vortex extraction | Shaken by hand extraction |
|------------|-------------------|---------------------------|
|            | LOD (µg/mL)       | LOQ (µg/mL)               | LOD (µg/mL) | LOQ (µg/mL) |
| Acetic acid| 2.780             | 3.025                     | 1.130       | 1.614        |
| Propionic acid | 0.718         | 1.144                     | 0.446       | 0.953        |
| Butyric acid  | 0.672            | 1.692                     | 0.089       | 0.739        |
| Valeric acid  | 0.382             | 1.058                     | 0.111       | 0.551        |

A previous method by Adorno et al. [3] also used diethyl ether at low volume (0.6 mL) and vortex mixing for VFA extraction. This method, however, used crotonic acid and sulfuric acid solutions to increase VFA solubility in the organic phase, as well as used NaCl to decrease the solubility in the water phase. The LOD and LOQ of this method were 3.97 and 36.45 µg/mL, respectively [3], which shows that the shaken by hand extraction performed better as shown by the lower LOD and LOQ (Table 4). Without acid and salt addition, our method consumed fewer chemicals and safer for the equipment.
4. Conclusion
Based on the results of measurements that have been obtained in this study show that the extraction method with shaken by hand had a limit of detection of 0.09 to 1.13 µg/mL and limit of quantification of 0.55 to 1.61 µg/mL, with the recovery of >99%. This shows that the shaken by hand extraction using diethyl ether as extracting solvent can be used for rapid and accurate quantification of VFA. In addition, the sample should not be shaken excessively because the sample will be oxidised or hydrolysed so that it can reduce the VFA content. The analysis does not require a long time and consumed fewer chemicals.

Acknowledgement
The authors would like to thank Siti Aisyah Zahrad and Meng Sophorn who assisted the preparation of equipment and chemicals. We are also grateful to Research Unit for Clean Technology LIPI, which provided the financial support for doing this research.

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