Article

OPTIMIZATION AND VALIDATION OF LIQUID-LIQUID EXTRACTION (LLE) METHOD AND THE APPLICATION OF GAS CHROMATOGRAPHY WITH FID DETECTOR FOR THE DETERMINATION AND QUANTIFICATION OF VOLATILE FATTY ACIDS IN EFFLUENTS FROM ANAEROBIC REACTORS

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Controlling the chemical parameters of the anaerobic digestion (AD) process is essential for the bioconversion of organic matter to methane; among these parameters include the presence and concentration of volatile fatty acids (VFAs). It is thus necessary to use efficient analytical methods that are capable of identifying and quantifying VFAs in reactor effluents in order to obtain an immediate response to their conditions. In this study, the liquid-liquid extraction (LLE) method was optimized and validated – through an adaptation of the official method, using acetone and $KHSO_4$, and gas chromatography with flame ionization detector (GC-FID) was used for the determination of acetic, propionic, isobutyric, butyric, isovaleric and valeric acids present in the effluents. The limit of detection (LOD) and limit of quantification (LOQ) obtained were 1.2-2.5 mg L^{-1} and 1.9-3.7 mg L^{-1}, respectively, for all analytes. These low LODs and LOQs are essentially important because the presence of isobutyric and isovaleric acids above 5.0 mg can be considered indicative of imbalances in AD. The proposed method, which presented satisfactory results with good repeatability (4.2-20.7%) and recovery (90.9-104.0%) rates, was applied for the analysis of effluent samples from three continuous stirred-tank reactors (CSTR).

Keywords: biogas; fatty volatile acids; effluents from biodigester; monitoring of anaerobic digestion; gas phase chromatography.

INTRODUCTION

One of the most suitable and environmentally-friendly ways to obtain energy involves the reuse of organic waste for the production of biofuels; among the biofuels generated through this mechanism is biogas, which is produced from anaerobic digestion (AD). The AD process promotes proper disposal of waste along with the production of methane, and this technique helps control environmental pollution, while at the same time contributing toward the generation of renewable energy. Biogas is considered a viable substitute for fossil fuels, and this renewable source of energy contributes toward the diversification of the energy matrix of the country where it is used.[1,2]

Degradation of organic matter in wastes occurs through the action of a number of microorganisms up to the formation of biogas. The wide range of biological reactions that take place in a reactor can be grouped into four different sequential steps/processes: (*i*) hydrolysis; (*ii*) acidogenesis; (*iii*) acetogenesis; and (*iv*) methanogenesis.[3,4] Under the hydrolysis process, complex organic polymers (carbohydrates, proteins and lipids) are converted into simpler molecules (sugars, amino acids and fatty acids). In the acidogenesis process, these molecules are transformed into short-chain carboxylic acids, which are widely known in the literature as volatile fatty acids (VFAs). Subsequently, in the acetogenesis process, the acetogenic microorganisms consume the VFAs and produce acetate. Finally, in the last step – the methanogenesis process leads to the production of methane and carbon dioxide.[5-7]

VFAs are carboxylic acids that possess about 2 to 7 carbons and which can be saturated or branched.[8-10] The most highly representative of these acids include the following: acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, and valeric acid. As the carbon chain increases, these compounds become less soluble in aqueous medium; this clearly explains why it is difficult to find carboxylic acids with structures above 6 carbons in aqueous media.[11,12] Although these compounds are essentially important in the AD process, their excessive quantity can be associated with operational instability in the system.[13,14] The accumulation of VFAs in the system can lead to a drastic drop in pH, and this impedes the effective buffering/plugging of the reactor, which consequently affects microbial growth and diminishes the efficiency of the biogas.[2,4,15] In view of that, VFAs are considered the main important intermediate compounds when it comes to biogas production. According to literature reports, the total amount of VFAs regarded as ideal for the anaerobic digestion process ranges between 1000 and 4000 mg L^{-1}.[5]

The presence of VFAs can be determined through titration methods. Although these methods are well-known for being simple, straightforward and inexpensive to execute, the response they provide is found to be limited to total organic acids (TOAs) and total inorganic carbon (TIC).[1,16] Several studies have pointed out the need to identify and quantify VFAs individually so as to ensure an effective monitoring of the AD process, since the compounds are required to be produced and consumed during the process.

As it has been well established in the literature, acetic acid concentrations above 800 mg L^{-1}, elevated propinic acid/acetic acid ratio (> 1.4), and individual concentrations of isobutyric and/or isovaleric acids in the range of 5.0-15.0 mg L^{-1} point to an impending failure in the anaerobic digestion process.[17-19] Furthermore, the accumulation of acetic acid is considered the main inhibitor of the AD process when it comes to the treatment of food waste, once they cause an imbalance between the routes/mechanisms that lead to the production of methane, and by doing so, reduce the amount of methane in the final product – biogas.[20] In this sense, chromatographic methods are used to monitor the AD process and to determine

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the individual concentration of VFAs and their presence at low concentrations with high precision and accuracy.[4,11,21,22]

Among the chromatographic methods widely employed in the literature, the use of high-performance liquid chromatography (HPLC) coupled with UV/Vis detector and refractive index detector (RID) has been found to be advantageous when it comes to sample preparation, as the technique is less rigorous and does not require the application of high temperatures in analytes determination.[11,23,24] For comparison purposes, the methods involving the use of gas chromatography with flame ionization detector (GC-FID) or gas chromatography-mass spectrometry (GC-MS) allow one to determine the analytes in a more precise manner and are highly more recommended when working with complex matrices, since these methods exhibit low limits of detection (LOD) and wide linear dynamic range (LDR).[10] In addition, the monitoring of VFAs by GC-FID is the technique recommended officially for the determination of VFAs.[9,10,12,25] It should be noted however that both the GC-FID and GC-MS require a rigorous sample preparation procedure in order to ensure the durability of the equipment and yield good analytical results.[4,21,22,26]

Clean-up procedure, such as filtration and centrifugation, are usually employed in order to remove solid particles present in the samples so as to pave the way for executing the direct aqueous injection procedure through GC-FID;[22] this is the officially recommended approach for determining VFAs in sludge samples treated in sewage treatment plants. Considering the high amount of impurities that can be present in the chromatographic system, repeated blank tests are recommended to be performed in order to ensure that the equipment is thoroughly clean.[12] The direct injection mechanism allows the deposition of inorganic compounds present in the samples into the chromatographic column, and this helps reduce the sensitivity of the detector, while increasing the need for the maintenance of the equipment.[8-10] In this context, it is clear that there is a need for sample pre-treatment, mainly by extraction, in order to enhance the analytical performance and reduce the costs of the technique.[4,10,21]

Headspace (HS), solid-phase microextraction (SPME) and liquid-liquid extraction (LLE) are extraction techniques that have been widely employed in the literature.[4,21,26] Several studies have employed the LLE-based methods for the preparation of samples from sewage wastewater, swine farm waste, and landfill leachate using organic solvents (dimethylcarbonate and methyl-*tert*-butylic ether) in combination with inorganic salts ($KHSO_4$ and NaCl) in order to promote a salting-out effect and stimulate the extraction of VFAs from the aqueous phase to the organic phase, and to subsequently perform the determination analysis by GC-MS.[4,26] Hexane extraction has also been used for sample preparation with a view to monitoring VFAs by GC-MS in acidogenic and methanogenic reactors; the findings of the study showed that, in both reactors, the VFAs did not exhibit a stable behavior throughout the AD process, and this reinforces the need for monitoring.[27] Thus, analytical methods with low limits of detection and quantification are an important tool for decision making when it comes to monitoring VFAs.[27]

It is worth noting however that, to date, there are no reports in the literature regarding the use of LLE-based methodologies for the determination of VFAs through the application of GC-FID.

Taking into account the importance of VFAs for the efficiency of the AD process and the monitoring of the process, as well as the need to conduct analyses that are capable of identifying and quantifying these compounds with high precision and rapidity, the present study sought to investigate the optimization and validation of an LLE-based method, and the use of GC-FID for the simultaneous determination of acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, and valeric acid in samples of effluents obtained during the AD process in continuous stirred-tank reactors (CSTRs).

EXPERIMENTAL

Chemical reagents and materials

The experiments were conducted using the following analytical standards obtained from Sigma-Aldrich (Saint Louis, MO, USA): acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid and octanoic acid, with purity ≥ 99.0-99.5%. For the acidification of the samples, we employed 85% phosphoric acid (v/v) (Sigma-Aldrich, P.A.). The following salts were used in the experiments: potassium bisulfate (Merck, ≥ 99.0%) and sodium chloride (Dinâmica, P.A., 99.0%). The solvents used in the experiments included the following: acetone (Merck, HPLC grade ≥ 99.8%), dimetylcarbonate (Sigma-Aldrich, HPLC grade ≥ 99.0%) and ultrapure water with controlled resistivity of 18.2 MΩ cm (ELGA, Purelab Option Q). The ultrapure water was also used to wash the glassware and to prepare the solutions. For the chromatographic analyses, we employed the following gases: nitrogen 5.0 (Air liquid, purity of 99.999%), hydrogen 5.0 (Air liquid, purity of 99.999%) and synthetic air (Air liquid, purity of 99.999%).

Effluents from reactors

Samples of effluent from CSTR-type reactors were used in the development of the analytical method proposed in this study. The substrate for the feeding of the reactors was constituted by a mixture of ground organic restaurant waste, powdered cocoa, and corn bran. The reactors were operated in batches until the volumetric production of biogas was stabilized. Subsequently, the reactors were fed again with a mixture of substrate and inoculum in another cycle of acclimatization/temperature, which lasted 10 days.

After this period, the reactors were operated in semicontinuous mode using a volumetric organic load (VOL) of 0.5 g of volatile solids (VS) L^{-1} day^{-1}, which was later increased to 0.83 g VS L^{-1} day^{-1} from the 15th day of the experiment. The samples of effluent were collected three times a week during the operation period of the reactors, according to the collection and storage procedures described in VDI 4630.[28]

The study was conducted in three bench-top reactors, which were named R1, R2, and R3. The R1 reactor had a stainless steel operating tank containing 9.0 L of useful volume, while the reactors R2 and R3 were constructed using acrylic material with useful volume of 5 L each.

Parameters for the optimization of liquid-liquid extraction (LLE) of VFAs

The LLE technique was used for the extraction of the VFAs from aqueous media; the technique employed was adapted from the methodologies described by Banel *et al.*[21] and Ghidotti *et al.*[4] For the optimization of the procedure involving the extraction of VFAs, we employed a complete factorial planning with three variables: extracting solvent (3 levels), pH value (2 levels), and ionic strength (2 levels); these variables were tested directly in the matrix (Table 1).

For the optimization of the LLE parameters, the room temperature was kept at 20 °C, the effluent samples with aqueous aspect were homogenized for 2 min, with the aid of a vortex (Phoenix, AP 56), and separated into two parts. With the aid of a paper indicator, the pH value of one of the aliquots was adjusted to 3.0 using phosphoric acid solution at 85.0% (v/v), while the pH value of the other was kept at 7.0 (from the original sample).

An amount of 0.50 g of the samples was weighed in a 15 mL conical centrifugal tube. Subsequently, the following materials were added into the mixture: 0.10 mL of saturated solution of

Table 1. Levels of the variables used in the complete experimental planning targeted at the optimization of LLE of the VFAs in CSTR reactor effluent

Variable	Levels of the variables		
	−1	0	1
Solvent	Dimethylcarbonate	Acetone	Ultrapure water
pH value	3.0	-	7.0
Ionic strength	$KHSO_4$	-	NaCl

LLE: Liquid-liquid extraction. VFA: Volatile fatty acids. CSRT: Continuous stirred-tank reactors.

$KHSO_4$ (0.49 g mL^{-1}) or NaCl (0.36 g mL^{-1}), 0.10 mL of 50 mg L^{-1} octanoic acid (internal standard, IS), and 1.0 mL of solvent fortified with 175.0 mg L^{-1} of the analytes. Ultrapure water, acetone or dimethylcarbonate were employed as solvents, depending on the experiment. The mixture was homogenized in a vortex at an average velocity of 6000 rpm for 2 min and centrifuged at 4000 rpm (Hettich, Rotina 380), for 30 min. After this procedure, the samples were left idle for 3 h. An amount of approximately 500 µL of the supernatant was collected, filtered with 0.22 µm PTFE hydrophobic filter (Millex HPF®, Merck) in 2.0 mL vials, and stored at –18 ºC prior to the conduct of the chromatographic analysis.

The LLE-based sample preparation was performed using the variables applied in the experimental procedure (Table 1); this resulted in a sequence of 24 experiments, which were executed in order to investigate the interactions of the variables in the extraction of the analytes (Table 2).

Table 2. Sequence of experiments conducted for the optimization of the process involving the LLE of VFAs in effluents from CSTR-type reactors

Experiment	Solvent	pH	Ionic strength
1	1	−1	1
2	1	−1	−1
3	0	1	−1
4	0	−1	1
5	0	1	−1
6	−1	1	−1
7	−1	1	1
8	−1	−1	−1
9	0	−1	1
10	0	−1	−1
11	1	1	−1
12	0	1	1
13	−1	1	−1
14	−1	−1	−1
15	0	1	1
16	1	1	−1
17	1	1	1
18	−1	1	1
19	−1	−1	1
20	1	1	1
21	−1	−1	1
22	0	−1	−1
23	1	−1	1
24	1	−1	−1

LLE: Liquid-liquid extraction. VFA: Volatile fatty acids. CSRT: Continuous stirred-tank reactors.

Validation of methodology

Based on the results obtained from the optimization analysis, one is able to obtain the parameters considered to be optimal for the validation of the method. With this in mind, the following parameters were thoroughly evaluated: linearity, limit of detection (LOD), limit of quantification (LOQ), repeatability, recovery, and homoscedasticity, based on the spreadsheet validation method.[29]

To satisfy the validation criteria, solutions of intermediate standards, in acetone, of individual VFAs were prepared; this was done in order to construct the analytical curves for acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, and valeric acid at 5.0 g L^{-1}. Based on these individual analyte solutions, a mixed standard acetone solution was prepared for each point of the curves. For the other solutions, dilution calculations were performed based on the concentration of the intermediate standard. By doing so, we were able to prepare solutions for the construction of two analytical curves through the fortification of the matrix (effluent before operating point in semi-continuous mode) in order to satisfy the working range. One of the curves with concentrations of 0.4, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, and 7.0 mg L^{-1} (8 points) was used to determine the LOD and LOQ of the method, and the other curve with concentrations of 10.0, 20.0, 30.0, 50.0, 65.0, 85.0, and 100.0 mg L^{-1} (7 points) was used for the concentration range of the samples.

Octonic acid, which was used as internal standard (IS), was prepared separately, based on its density, in acetone at the concentration of 5.0 g L^{-1}, and this solution was subjected to 50 mg L^{-1} dilution in the same solvent and applied directly in the samples during the preparation. The volume and concentration of the IS were kept fixed for all the samples, according to the optimization procedure.

The matrix was spiked by adding the previously prepared VFAs solution at the predetermined concentration of the curves. The replicates of the points were repeated 3 or 7 times, depending on the parameter evaluated, according to the procedure previously described.

Linearity was evaluated using the low and high curves; in addition, the homoscedasticity of the residuals was also investigated. The LOD and LOQ were evaluated using the data from the 8 points of the lower concentration curve (0.4-7.0 mg L^{-1}) and were calculated mathematically; the estimate was obtained based on the regression confidence interval.[30] For the analysis of repeatability (precision), three levels of concentration were evaluated: 10, 50 and 100 mg L^{-1} of the working curve; the tests in these points were performed in seven replicates, and the results were analyzed using relative standard deviation (RSD%).

For the recovery analysis, low, medium, and high concentration points (10, 50 and 100 mg L^{-1}, respectively) were evaluated for each of the analytes, according to Equation 1 below:

$$\text{Recovery } \% = \frac{(C1 - C2)}{C3} \times 100 \quad (1)$$

where C1 stands for the concentration of analyte determined (calculated through the curve) in the fortified matrix, C2 is the concentration determined in the unfortified matrix (blank), and C3 is the known concentration of the analyte added in the matrix (addition of the standard).

Regarding the results obtained, the densities of all the samples evaluated in this study were found to be between 0.99 and 1.05 g mL^{-1}; based on that, we converted the sample mass to solution volume, and the results were expressed in milligrams of analyte *per* liter of solution (mg L^{-1}).

GC-FID analysis

The analysis involving the determination and quantification of VFAs was conducted by gas chromatography (Agilent Technologies model 7890 B), equipped with flame ionization detector (GC-FID), in accordance with the official standard methods,[12] with minor adjustments. The analytes were separated in a Supelco silica-bound phase capillary column – model SPB®-1000 (30 m × 0.53 mm × 0.5 μm). The injection volume employed was 1 μL; the volume was applied in split mode, in the ratio 1:20. The injector temperature was set at 150 °C. The elution ramp employed was as follows: starting from 95 °C (2 min) to 140 °C at 10 °C min^{-1}, then moving further up to 200 °C at 40 °C min^{-1}. Hydrogen was used as carrier gas at flow rate of 18 mL min^{-1}, and nitrogen was used as a makeup gas at flow rate of 12 mL min^{-1}. Regarding the detector, we employed hydrogen and synthetic air for the flame composition at flow rates of 30 mL min^{-1} and 300 mL min^{-1} for hydrogen and synthetic air, respectively. The temperature of the detector was kept at 240 °C. The identification of the VFAs was conducted based on a comparison of the retention times of the analytical standards with the analytes of the samples. For analytical response, we applied a ratio between the chromatographic area of the peak of each analyte and the peak area of the internal standard.

Statistical analysis of the data

In the experimental design, the data were subjected to One-Way ANOVA test, using the ActionStat Pro software.[31]

RESULTS AND DISCUSSION

The liquid-liquid extraction (LLE) technique was used for the extraction of the analytes from the complex matrix of the samples to an organic phase which can be readily filtered. The procedures involving the cleaning up of the samples, for the removal of the solids, were executed initially before the LLE process (centrifugation) and after the process (filtering); this was done in order to reduce any interfering effects during the chromatographic analyses, as recommended by the official method for the determination of VFAs.[12] To effectively determine the optimal extraction conditions, the VFAs were evaluated separately using the ANOVA test (Table 3).

Table 3. Results obtained from the complete factorial planning analysis conducted using the VFAs and the respective *p*-values for the variables

VFA	Solvent	pH	Ionic strength
Acetic	9.5×10^{-1}	9.7×10^{-1}	6.8×10^{-2}
Propionic	9.1×10^{-13}*	3.9×10^{-1}	5.3×10^{-1}
Isobutyric	1.6×10^{-14}*	8.9×10^{-1}	4.1×10^{-1}
Butyric	8.6×10^{-16}*	8.8×10^{-1}	4.9×10^{-1}
Isovaleric	7.0×10^{-13}*	4.4×10^{-1}	1.5×10^{-1}
Valeric	7.2×10^{-12}*	3.9×10^{-1}	2.4×10^{-1}

*p-values ≤ 0.05 are statistically significant. VFA: Volatile fatty acids.

Regarding the variables, only the variable solvent exhibited statistically significant values in the LLE process. Among the solvents, acetone displayed the highest efficiency in terms of the extraction of propionic, isobutyric, butyric, isovaleric and valeric acids, in comparison with ultrapure water and DMC; as such, acetone was chosen as the most suitable solvent for application in the extraction procedure. The application of different pH values (3.0 and 7.0) did not exert any significant influence over the extraction of the VFAs ($p > 0.05$) (Table 3). It should be noted however that ionization of the analytes tends to favor their transfer to the organic phase. Moreover, according to the literature, acidification of samples helps ensure the predominance of the non-ionized form of carboxylic acids, which is enhanced during vaporization through injection, and this contributes toward improving the quality of the chromatographic peaks.[10,26] In this sense, although the factorial planning analysis did not point to the influence of pH in the extraction of VFAs, pH 3.0 was chosen for the execution of our proposed method as a way to ensure that the addition of acids in large volumes does not change the physical-chemical characteristics of the samples (dilution).

The inorganic salts exhibited no significant differences ($p > 0.05$) in the extraction of VFAs; however, for the continuity of our studies, we thought of using $KHSO_4$ because of the slope of the straight line segment that corresponded to this salt and which was found to be more efficient in the extraction of acetic acid – one of the main acids produced in the biodigesters. The graph of effects related to the extraction of each analyte was constructed; this can be found in Figures 1S-8S in the supplementary material. The addition of inorganic salt in the sample preparation decreases the solubility of the VFAs in aqueous solution, and this enhances the extraction to organic phase.[26,28] In a study conducted by Ghidotti *et al.*,[4] the authors employed 1.0 mL of DMC as extracting solvent and $KHSO_4$ for salting-out. Banel *et al.*[21] employed LLE for the extraction of VFAs (C2 to C8 carbons) in matrices of wastewater from pig farms, municipal sewage wastewater, and leached landfill; in the preparation procedure, these authors employed the following: 1.0 g of NaCl and 2.0 mL of methyl *tert*-butyl ether (MTBE).

To further our analysis in the present study and to validate the proposed method, it is worth pointing out the parameters that were optimized: acetone employed as extracting solvent; acidification of the samples at pH 3.0; and $KHSO_4$ used for salting-out.

Validation of the proposed method

By optimizing the parameters of the LLE technique, we were able to estimate the following: linearity, limit of detection (LOD), limit of quantification (LOQ), and repeatability, and to plot the graph of residuals for the construction of the curves.[29]

The proposed method exhibited selectivity and the chromatographic peaks were visually noticeable; also, the times of retention were noticeably different between the analytes – this effectively allowed the individual identification and quantification of the VFAs (Figure 1 and Table 4). In the chromatogram shown in Figure 1, one will observe that the internal standard (IS) was added at the same concentration in both samples in order to quantify the analytes in the ratio VFA/IS.

The plots of residuals for all the analytes in both curves showed that the errors were uniformly distributed and without discrepant values – this clearly points to homoscedasticity. The plots of residuals are shown in Figures 8S-13S in the supplementary material. The proposed method exhibited linearity within the working concentration range, with R^2 between 0.998 and 0.999 (Table 4), while the curve related to the lowest concentration (0.4 to 0.7 mg L^{-1}) recorded R^2 between 0.957 and 0.992. As mentioned previously, two curves were constructed to cover two working ranges: one for higher concentration (10 to 100 mg L^{-1}) and the other one for lower concentration (0.4 to 0.7 mg L^{-1}). However, during the experiments, we noted that the concentrations of the analytes were higher than 10 mg L^{-1}; for some few samples, the concentrations were below this value (10 mg L^{-1}), so we applied the LOQ and LOD of the lowest curve, but even so, we were only able to detect them but not quantify them.

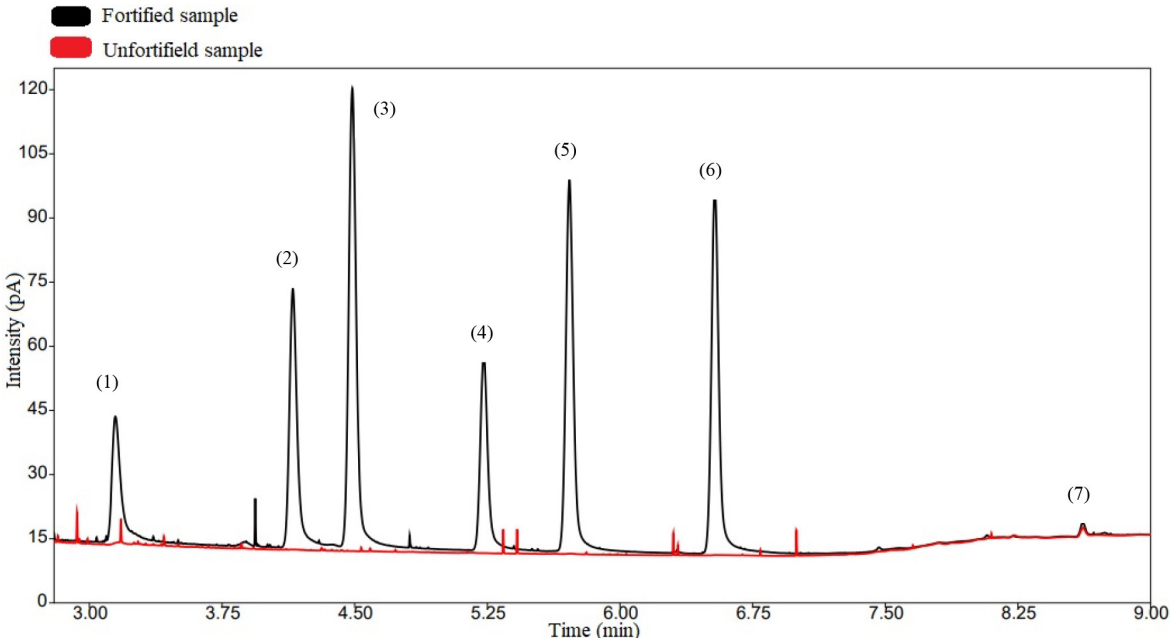

Figure 1. *Chromatograms for the samples of unfortified effluent (matrix) and effluent fortified with the VFAs investigated in this study. Acids: (1) acetic, (2) propionic, (3) isobutyric, (4) butyric, (5) isovaleric, (6) valeric, and (7) octanoic (internal standard)*

The values obtained for LOD varied between 1.2 and 2.5 mg L^{-1}; propionic acid and valeric acid were the analytes that exhibited the lowest LOD – with values ranging from 1.2 to 1.6 mg L^{-1}, respectively. The values obtained for LOQ ranged from 1.9 mg L^{-1} for propionic acid to 3.7 mg L^{-1} for butyric acid (Table 4).

Several methodologies reported in the literature related to the extraction and determination of the same VFAs in samples of wastewater, animal waste, and leached landfill by GC-MS obtained LOD values ranging from 0.017 to 0.064 mg L^{-1} and LOQ values ranging from 0.051 to 0.5 mg L^{-1}; these values are clearly inferior to the values obtained in our present study.[21,26] Other studies conducted using digestate samples reported to have obtained LOD of 2.5, 3.8, 0.30, 0.68, 0.79 and 0.42 mg L^{-1} for acetic, propionic, isobutyric, butyric, isovaleric and valeric acids, respectively;[4] the values obtained for acetic acid and propionic acid are superior to the values obtained in the present study (1.9 and 1.2 mg L^{-1}, respectively).

Table 4. Validation parameters for VFAs in digestate samples for the proposed LLE/GC-FID method

Analyte	TR (min)	Linear range (mg L^{-1})	Equation of the line	R^2	LOD (mg L^{-1})	LOQ (mg L^{-1})	Recovery		Repeatability	
							Level (mg L^{-1})	(%) n = 3	Level (mg L^{-1})	RSD (%) n = 7
Acid acetic	3.2	10.0-100.0	y = 0.0567x – 0.1465	0.999	1.9	2.9	10.0	98.2	10.0	11.2[a]
							50.0	99.5	50.0	7.2
							100.0	99.3	100.0	5.2
Propionic acid	4.2	10.0-100.0	y = 0.0866x – 0.2019	0.999	1.2	1.9	10.0	104.0	10.0	12.1[a]
							50.0	92.0	50.0	5.4
							100.0	100.4	100.0	4.7
Isobutyric acid	4.5	10.0-100.0	y = 0.1712x – 0.5387	0.999	2.1	3.1	10.0	109.2	10.0	4.2
							50.0	92.9	50.0	5.9
							100.0	101.0	100.0	4.8
Butyric acid	5.2	10.0-100.0	y = 0.0592x – 0.4040	0.997	2.5	3.7	10.0	106.0	10.0	5.4
							50.0	100.3	50.0	6.6
							100.0	100.6	100.0	5.1
Isovaleric acid	5.7	10.0-100.0	y = 0.1505x – 0.7617	0.998	2.0	2.9	10.0	106.6	10.0	20.7[a]
							50.0	99.1	50.0	5.4
							100.0	100.9	100.0	5.3
Valeric acid	6.5	10.0-100.0	y = 0.1509x + 1.0707	0.998	1.6	2.4	10.0	109.4	10.0	13.3[a]
							50.0	97.8	50.0	5.2
							100.0	100.8	100.0	7.0[a]

[a]Values above the thresholds stipulated by AOAC.[30] TR: Time retention. R^2: Coefficient of determination. LOD: Limit of detection. LOQ: Limit of quantification. RSD: Relative standard deviation. LLE: Liquid-liquid extraction. VFA: Volatile fatty acids.

Furthermore, the LOD values obtained for the remaining acids, namely, isobutyric, butyric, isovaleric and valeric acids, were inferior to the values obtained in our present study (2.1, 2.5, 2.0 and 1.6 mg L^{-1}, respectively).

Regarding LOD, Ghidotti *et al.*[4] obtained LOD of 8.3 and 13.0 mg L^{-1} for acetic acid and propionic acid, respectively; these values are above the values obtained in the present study (2.9 and 1.9 mg L^{-1}). It is worth noting that while lower LOD and LOQ values have been reported in the literature for some of the analytes investigated here, this was possible because MS detectors were employed in these studies; MS detectors are clearly more sensitive than the FID detector employed in the present study.

In a recent study, Raposo *et al.*[9] evaluated the results of validation analyses conducted by nine research laboratories which employed different GC-FID equipment for the determination of VFAs in aqueous samples. The LOD values obtained ranged from 2.6 to 4.3 mg L^{-1} for acetic acid, 1.2 to 3.4 mg L^{-1} for propionic acid, 1.2 to 11.5 mg L^{-1} for isobutyric acid, 1.1 to 3.5 mg L^{-1} for butyric acid, 0.8 to 4.3 mg L^{-1} for isovaleric acid, and 1.1 to 2.5 mg L^{-1} for valeric acid. In this same study, the LOQ values recorded were as follows: 7.8 to 12.4; 3.7 to 10.3; 3.6 to 8.3; 3.3 to 10.7; 2.3 to 13.1; and 3.4 to 7.5 mg L^{-1} for the same aforementioned VFAs, respectively. These values are found to be quite similar to the results obtained in our present study related to the application of LLE/GC-FID.

The repeatability of the proposed method was evaluated using RSD% (n = 7), in three levels of concentration of the analytes. The results obtained for the lowest level of concentration (10.0 mg L^{-1}) for acetic acid (11.2%), propionic acid (12.1%), isovaleric acid (20.7%) and valeric acid (13.3%) pointed to RSD values superior to the values recommended. For the highest concentration level (100.0 mg L^{-1}), only valeric acid (7.0%) presented RSD value superior to the value stipulated by the Association of Official Analytical Chemists (AOAC)[30] – according to AOAC, the RSD value is required to be lower than 7.3% for the analytes in the concentration range of 1.0 to 99.0 mg L^{-1}, and inferior to 5.3% for analytes at the concentration of 100.0 mg L^{-1} (Table 4).

In another study conducted by Ghidotti *et al.*,[4] the digestate samples were subjected to organic solvent extraction and the VFAs were determined by GC-MS; the authors reported to have obtained RSD% ranging from 6.0 to 9.0% for samples with concentration of 0.1 mg L^{-1}. In another related study conducted by Raposo *et al.*[9] where they determined VFAs by GC-FID using aqueous samples, the authors obtained the following RSD% for acetic, propionic, isobutyric, butyric, isovaleric, and valeric acids: 0.4-1.3, 0.2-2.1, 0.3-0.9, 0.2-1.2, 0.3-1.1 and 0.2-2.5 mg L^{-1}, respectively; these analytes were evaluated in three levels of concentration: 80.0, 200.0, and 600.0 mg L^{-1} (with n = 3) – these levels of concentration are quite different from those evaluated in our present study: 10.0, 50.0, and 100.0 mg L^{-1}, with n = 7 (Table 4). One will notice that the application of higher concentration of analytes resulted in lower RSD values.

Regarding recovery analysis, all the analytes evaluated exhibited recovery percentages ranging from 90.9 to 104.0%. Thus, all the recovery values are in line with the values recommended by AOAC – which determines that values between 80.0 and 110.0% should be acceptable for the concentration range investigated in this study (Table 4).

The recovery values obtained in this study are also compatible with those described by the American Public Health Association (APHA)[12] for the determination of VFAs in ETE sludge samples, based on the application of GC-FID with direct injection. The official method presents recovery values of 95.2, 93.6, 90.3, 89.8, 88.9, and 87.5% for acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, and valeric acid, respectively. For their interlab analysis of VFAs in aqueous samples by GC-FID, the authors obtained the following range of recovery values: 90.7-100.5, 79.9-105.9, 93.8-105.4, 95.6-104.7, 95.9-105.5 and 93.7-105.5% for acetic, propionic, isobutyric, butyric, isovaleric, and valeric acids, respectively; these analytes were evaluated in the following concentrations: 80.0, 200.0, and 600.0 mg L^{-1}, with n = 3. It is worth pointing out that the method proposed in our present study evaluated the analytes in the concentration range of 10.0-100.0 mg L^{-1}, these concentration levels are lower than those employed in other studies reported in the literature (Table 4).

Another point that is worth mentioning here is that, in the present study, we employed mass of samples of 0.5 g, which is inferior to the amount recommended in the official method (10.0 to 30.0 mL).[12] Furthermore, the use of acetone as solvent is advantageous in LLE because the density of this acid is inferior (0.791 g mL^{-1}, 25 °C) to the density of water (0.997 g mL^{-1}, 25 °C) and is characterized by the separation between the organic phase and the aqueous phase (quite more easily), which allows the extracting solvent that contains the analytes to be the superior phase. In this sense, the extract is collected quite more easily with the aid of pipettes.

Determination of VFAs in samples of effluents from CSTR reactors

Table 5 shows the values obtained from the analyses conducted aimed at the quantification and monitoring of VFAs in samples of CSTR digesters R1, R2, and R3, based on the application of the method proposed in this study. In R1 (reactor 1), only acetic acid was determined; this corresponded to the total VFA in the reaction medium. The assays conducted in reactors R1 and R2 pointed to the presence of acetic acid and propionic acid, the sum of which represents the total VFAs. The main differences observed between the reactors lie in the material used in the construction of the operating tank – stainless steel for R1 tank, and acrylic for R2 and R3 tanks; the useful volume – 9.0 L for R1 and 5.0 L for both R2 and R3; and the automation system – the system in R1 is more sophisticated than that of R2 and R3.

Looking at Table 5, one will observe that in the R1 samples, acetic acid was detected and quantified in the concentration ranges of 23.5 ± 0.2 and 45.1 ± 1.3 mg L^{-1}. In the R2 biodigester, acetic acid was identified in the range of 29.7 ± 0.9-45.3 ± 2.2 mg L^{-1}, and propionic acid from < LOD-34.6 ± 1.0 mg L^{-1}. Similar patterns were observed in R3, where we noted the presence of acetic acid in concentrations ranging between < LOD and 188.1 ± 13.4 mg L^{-1} and propionic acid in concentrations ranging from < LOD to 176.3 ± 12.7 mg L^{-1}. It should be noted that fatty acids considered to be in minority in the mesophilic range (butyric, isobutyric, valeric, and isovaleric acid) were neither identified nor quantified in the digesters (below the LOD of the proposed method).

Another point that deserves mentioning here is that acetic acid is considered the main VFA produced in the biodigesters, and the presence of this acid is regarded as suitable at the concentration of up to 800.0 mg L^{-1}.[19] Studies reported in the literature[20] investigated the accumulation of acetic acid during anaerobic digestion of food waste, and this acid was considered the VFA with the highest potential for inhibition when in excessive quantity – this resulted in a decrease in methane production.

More recently, studies reported in the literature[19] investigated the presence of propionic acid in two reactors; the results obtained showed the presence of concentrations of propionic acid in the range of 8.9 and 9.2 mg L^{-1}. Despite the fact that the values obtained in our present study were superior to the values obtained in the aforementioned study – R2 (14.9 to 24.6 mg L^{-1}),

Table 5. Concentration of VFAs obtained from the CSTR-type biodigesters

Digestion time (days)	Concentration of VFAs (mg L^{-1})				
	R1	R2		R3	
	Acetic	Acetic	Propionic	Acetic	Propionic
0	23.5 ± 0.2	44.6 ± 1.0	< LOD[a]	62.2 ± 4.4	< LOD[a]
3	< LOD[a]	35.9 ± 0.3	32.9 ± 1.5	188.1 ± 13.4	176.3 ± 12.7
6	45.1 ± 1.3	30.6 ± 1.3	17.8 ± 1.7	35.0 ± 1.1	21.9 ± 0.9
8	33.5 ± 2.0	29.7 ± 0.9	14.9 ± 0.7	36.7 ± 2.2	16.7 ± 0.9
10	34.0 ± 0.5	36.1 ± 2.5	15.9 ± 0.0	31.8 ± 1.4	15.3 ± 0.5
13	29.4 ± 0.4	39.3 ± 0.1	< LOD[a]	35.5 ± 3.0	< LOD[a]
15	30.0 ± 2.0	33.6 ± 2.0	< LOD[a]	< LOD[a]	< LOD[a]
17	28.3 ± 3.0	45.3 ± 2.2	31.1 ± 1.0	44.4 ± 1.8	30.5 ± 1.8
20	35.6 ± 2.7	37.6 ± 2.3	< LOD[a]	44.9 ± 0.5	28.5 ± 2.0
22	33.7 ± 2.8	41.8 ± 2.9	34.6 ± 1.0	48.2 ± 3.0	26.4 ± 0.6
24	29.8 ± 0.7	31.2 ± 4.0	< LOD[a]	44.0 ± 2.1	21.5 ± 1.4

[a]Below the limit of detection of the proposed method. VFA: Volatile fatty acids. CSRT: Continuous stirred-tank reactors.

R3 (15.3 to 173.0 mg L^{-1}), and no concentration of the acid was detected in R1 – the propionic/acetic acid ratio was below 1.4, as recommended in the literature.

In another related study, 17 samples of AD reactors were evaluated, and the results obtained showed significant variations in the total amount of VFAs relative to the type of reactors. The reactors in the aforementioned study were divided into three types: primary reactors – which comprised the hydrolysis and acidogenesis phases; secondary reactors – which were responsible for the acetogenesis and methanogenesis phases; and single reactors. The total amount of VFAs obtained in the primary reactors varied between 137.0 and 3776.0 mg kg^{-1}. In the secondary reactors, the total amount of VFAs obtained ranged from 53.0 to 346.0 mg kg^{-1}. Regarding the single reactors, the digestate derived from the use of slaughterhouse residues as substrate exhibited a total amount of VFAs of 74.0 mg kg^{-1}, while the other single reactors that employed samples of cattle waste exhibited a total amount of VFAs of 63.0 mg kg^{-1}. These values are in line with the maximum concentrations of VFAs obtained in our present study: 45.0 and 76.4 mg L^{-1} in R1 and R2, respectively. The R3 reactor exhibited a maximum concentration of VFAs of 364.4 mg L^{-1} (quite similar to the amount obtained in the secondary reactors) in the third day of AD – this value is considered higher than the values obtained for other reactors and considerably different from the values obtained for the same reactor in the other days evaluated (R2). Despite the differences observed in the units employed in the methods, the results obtained can be compared once they are mostly related to aqueous samples.

Another interesting work[25] that is worth mentioning is the study that evaluated real samples from different sludges of wastewater based on the application of the official method (APHA)[12] by GC-FID; the study obtained the concentration range of 0.8-1.89, 0.03-2.0, 0.03-1.98, and 0.11-0.25 g L^{-1} for acetic acid, propionic acid, butyric acid, and valeric acid, respectively. This study showed a large concentration range for the analytes in different samples derived from AD. Most of the samples evaluated in the aforementioned study recorded analytes concentrations above those found in our present study.

Although low concentrations of VFAs were recorded in all the reactors, we noted a slight increase in VFAs concentrations in R2 and R3 when the reactors started operating at higher VOL (0.83 g VS L^{-1} day^{-1}), after the 15th day of operation. We also observed the re-appearance of propionic acid in the reactors R2 and R3, following the increase in VOL; this points to the importance of determining the VFAs individually, as this primarily helps identify any possible imbalances in the reaction medium caused by the change in the operating conditions of the reactors.[4,28]

The total concentration of VFAs obtained in the three reactors was less than the values reported in the literature (between 1000 and 4000 mg L^{-1})[5] due to the low VOL employed in the initial phase of the implementation of the bench-top reactors. It is worth noting that low VOL (between 0.5 and 0.83 g VS L^{-1} day^{-1}) is recommended by VDI 4630[28] for the initial operating procedure of the reactor.

CONCLUSION

In the present study, the LLE method was optimized and validated and GC-FID analysis was applied for the simultaneous determination of acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid and valeric acid in samples of effluents obtained from anaerobic digestion in CSTR-type bench-top reactors.

Under the method proposed in this study, the samples were prepared by the LLE technique, where acetone was used as solvent and $KHSO_4$ as ionic force modifier; the application of this technique promoted an effective, selective extraction of the analytes of interest. The LLE-based preparation technique is a simple, fast alternative method for the extraction of analytes. The extraction procedure, which is followed by the filtering of the extract, prevents impurities from being inserted directly into the chromatograph, and this enhances the durability of the chromatographic column and helps ensure the quality of the assays conducted.

The application of the LLE-based method yielded satisfactory values with LOD ranging from 1.2 to 2.5 mg L^{-1}, LOQ ranging from 1.9 to 3.7 mg L^{-1}, recovery percentages between 90.9 and 104.0%, and repeatability ranging from 4.2 to 20.7%. The proposed method was found to be simple and straightforward, apart from consuming lower amount of solvents and being fast to perform. Thus, based on the results obtained here, one can conclude that the proposed method can be suitably applied for the conduct of routine assays involving the analysis of samples of effluents from different reactors targeted at the simultaneous determination of VFAs using GC-FID.

The assays conducted in real samples allowed the successful identification and quantification of acetic acid and propionic acid in the reactors; these VFAs are considered the most predominant acids in the AD process. The least predominant acids were not identified.

Isobutyric and isovaleric acids, which when present in concentrations higher than 5.0 mg L^{-1} may indicate an imbalance in the reaction medium, were also not identified. The LOD obtained for the VFAs ranged from 2.1 to 2.0 mg L^{-1}, while the LOQ ranged between 3.1 and 2.9 mg L^{-1}. Thus, based on the findings of this study, one can conclude that the proposed method is highly suitable for use in monitoring individual concentrations of VFAs which are considered essentially critical in AD.

SUPPLEMENTARY MATERIAL

Some images related to the results obtained in this work are available at http://quimicanova.sbq.org.br, in file PDF form, with free access.

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REFERENCES

1. Sun, H.; Wu, S.; Dong, R.; *Chem. Eng. Technol.* **2016**, *39*, 599. [Crossref]
2. Alavi-Borazjani, S. A.; Capela, I.; Tarelho, L. A. C.; *Biomass Bioenergy* **2020**, *143*, 105833. [Crossref]
3. Deublein, D.; Steinhauser, A.; *Biogas from Waste and Renewable Resources: An Introduction*, 2nd ed.; Wiley-VCH: Deggendorf, 2011.
4. Ghidotti, M.; Fabbri, D.; Torri, C.; Piccinini, S.; *Anal. Chim. Acta* **2018**, *1034*, 92. [Crossref]
5. Korres, N. E.; Nizami, A. S. In *Bioenergy Production by Anaerobic Digestion: Using Agricultural Biomass and Organic Wastes*; Korres, N. E.; O'Kiely, P.; Benzie, J. A. H.; West, J. S., eds.; Routledge: Abingdon, 2013.
6. Kunz, A.; Steinmetz, R. L. R.; Amaral, A. C.; *Fundamentos da Digestão Anaeróbia, Purificação do Biogás, Uso e Tratamento do Digestato*, 2ª ed.; Embrapa Suínos e Aves: Concórdia, 2019.
7. Tabatabaei, M.; Ghanavati, H.; *Biogas: Fundamentals, Process, and Operation*, 1st ed.; Springer: Switzerland, 2018.
8. Raposo, F.; Borja, R.; Cacho, J. A.; Mumme, J.; Orupõld, K.; Esteves, S.; Noguerol-Arias, J.; Picard, S.; Nielfa, A.; Scherer, P.; Wierinck, I.; Aymerich, E.; Cavinato, C.; Rodriguez, D. C.; García-Mancha, N.; Lens, P. N. T.; Fernández-Cegrí, V.; *TrAC, Trends Anal. Chem.* **2013**, *51*, 127. [Crossref]
9. Raposo, F. Borja, R.; Cacho, J. A.; Mumme, J.; Mohedano, A. F.; Battimelli, A.; Bolzonellaf, D.; Schuit, A. D.; Noguerol-Arias, J.; Frigoni, J. C.; Penuela, G. A.; Muehlenberg, J.; Sambusiti, C.; *J. Chromatogr. A* **2015**, *1413*, 94. [Crossref]
10. Fernandez, R.; Dinsdale, R. M.; Guwy, A. J.; Premier, G. C.; *Crit. Rev. Environ. Sci. Technol.* **2016**, *46*, 209. [Crossref]
11. Wagner, A. O.; Markt, R.; Puempel, T.; Illmer, P.; Insam, H.; Ebner, C.; *Eng. Life Sci.* **2017**, *17*, 132. [Crossref]
12. APHA Standard Methods for the Examination of Water and Wastewater; *Method 5560D Volatile Organic Acids*, 22nd ed.; American Public Health Association: Washington DC, 2017.
13. Dong, L.; Chen, L.; Liu, X.; Mei, Z.; Ren, H.; Cao, Q.; Yan, Z.; *Bioresour. Technol*. **2017**, *245*, 90. [Crossref]
14. Lee, W. S.; Chua, A. S. M.; Yeoh, H. K.; Ngoh, G. C. A.; *Chem. Eng. J.* **2014**, *235*, 83. [Crossref]
15. Al-Sulaimi, I. N.; Nayak, J. K.; Alhimali, H.; Sana, A.; Al-Mamun, A.; *Fermentation* **2022**, *184*, 1. [Crossref]
16. Mézes, L.; Biró, G.; Sulyok, E.; Petis, M.; Borbély, J.; Tamás, J.; *International Symposia Risk Factors for Environment and Food Safety & Natural Resources and Sustainable Development*, Oradea, Romania, 2011, p. 802. [Link] accessed in November 2023
17. Ma, J. M.; Carballa, P.; Van, C.; Verstraete, W.; *Water Res.* **2009**, *43*, 3239. [Crossref]
18. Capson-Tojo, G. D.; Ruiz, M.; Crest, M.; Steyer, J.; Bernet, N.; Delgenès, J. P.; Escudié, R.; *Bioresour. Technol.* **2017**, *245*, 724. [Crossref]
19. Hill, T.; Holmberg, R. D.; *Biol. Wastes* **1998**, *23*, 195. [Crossref]
20. Xu, Z.; Zhao, M.; Miao, H.; Huang, Z.; Gao, S.; Ruan, W.; *Bioresour. Technol.* **2014**, *163*, 186. [Crossref]
21. Banel, A.; Wasielewska, M.; Zygmunt, B.; *Anal. Bioanal. Chem.* **2010**, *399*, 3299. [Crossref]
22. Aramrueang, N.; Lomwongsopon, P.; Boonsong, S.; Kingklao, P.; *Fermentation* **2022**, *202*, 2. [Crossref]
23. Vannecke, T. P. W.; Lampens, D. R. A.; Ekama, G. A.; Volcke, E. I. P.; *Environ. Technol.* **2015**, *36*, 861. [Crossref]
24. Falk, H. M.; Reichling, P.; Andersen, C.; Benz, R.; *Bioprocess Biosyst. Eng.* **2015**, *38*, 237. [Crossref]
25. Robert-Peillard, F.; Boudenne, J.; Coulomb, B.; *Talanta* **2013**, *115*, 737. [Crossref]
26. Boe, K.; Batstone, D. J.; Angelidaki, I.; *Biotechnol. Bioeng.* **2007**, *96*, 712. [Crossref]
27. Náthia-Neves, G.; Neves, T. A.; Berni, M.; Dragone, G.; Mussatto, S. I.; Carneiro, T. F.; *Biofuel Res. J.* **2018**, *18*, 813. [Link] accessed in November 2023
28. VDI Standards; *VDI 4630*: *Fermentation of Organic Materials. Characterisation of the Substrates, Sampling, Collection of Material Data, Fermentation Tests*, VDI HandbuchEnergietechnik, Germany, 2016. [Link] accessed in November 2023
29. Ribeiro, F. A. L.; Ferreira, M. M. C.; Morano, S. C.; Silva, R. L.; Scheneider, R. P.; *Quim. Nova* **2008**, *31*, 164. [Crossref]
30. Association of Official Analytical Chemists (AOAC); *Official Methods of Analysis: Appendix F: Guidelines for Standard Method Performance Requirements*; AOAC International: Maryland, 2016.
31. *ActionStat Pro*; EstatCamp - Software e Soluções Estatísticas, Brasil, 2016.