Artigo

LIMONENE QUANTIFICATION BY GAS CHROMATOGRAPHY WITH MASS SPECTROMETRY (GC-MS) AND ITS EFFECTS ON HYDROGEN AND VOLATILE FATTY ACIDS PRODUCTION IN ANAEROBIC REACTORS

Franciele Pereira Camargo[a, ID], Arnaldo Sarti[b,c], Alberto Camilo Alécio[b], Carolina Aparecida Sabatini[a], Maria Angela Tallarico Adorno[a], Iolanda Cristina Silveira Duarte[d] and Maria Bernadete Amâncio Varesche[a,*]

[a]Departamento de Hidráulica e Saneamento, Escola de Engenharia de São Carlos, Universidade de São Paulo, 13566-590 São Carlos – SP, Brasil

[b]Departamento de Engenharia, Física e Matemática, Instituto de Quimica, Universidade Estadual Paulista, 14800-900 – Araraquara – SP, Brasil

[c]Instituto de Pesquisa em Bioenergia, Universidade Estadual Paulista, 13500-230 Araraquara – SP, Brasil

[d]Departamento de Biologia, Universidade Federal de São Carlos, 18052-780, Sorocaba – SP, Brasil

The inhibition effect of limonene on the organisms involved in anaerobic reactors may affect obtaining H_2 in this process. Rapid and precise limonene quantification is desirable to characterize wastewaters. The objective of this research was to develop a Gas Chromatography Coupled to Mass Spectrometry (GC-MS) method to determine limonene concentration in liquid samples from batch reactors applied to H_2 production at 30 °C. Method validation was carried out through calibration curves (3 to 20 mg L^{-1} of limonene), in addition to evaluating its precision (CV = 0.1%), linearity (R^2= 0.979), limits of detection (1.7 mg L^{-1}) and quantification (4.2 mg L^{-1}). Its inhibitory effect was evaluated with increasing concentrations of limonene (without addition to 2000 mg L^{-1}). Limonene affected the starting time of H_2 production (λ), since λ was 8.7 h in control assays and 15 h with 2000 mg L^{-1} of limonene. The same was observed for obtaining H_2, since 19.5 mmol H_2 L^{-1} was obtained in control assays and 8.7 mmol H_2 L^{-1} was obtained in assays with 2000 mg L^{-1} of limonene. This method was effective for limonene determination in liquid samples from batch reactors and could be used for characterization and control of anaerobic reactors.

Keywords: wastewater; sewage sludge; anaerobic digestion; hydrogen; volatile fatty acids; limonene.

INTRODUCTION

Essential oils (EOs) are natural and volatile aromatic compounds, formed as secondary metabolite by some plant species, which account for 0.3-0.5% in *in natura* citrus peel waste (CPW).[1] Its synthesis occurs in all plant organs (stem, flowers, leaves, seeds, fruits, roots, wood), and it is stored in specialized secretory cells or trichomes. Because of its medicinal, antiseptic and microbicidal properties, in addition to its fragrance, essential oils are used as additives in pharmaceutical and food industry products, such as anesthetics, analgesics, sedatives, anti-inflammatories, among others.[2,3]

Since essential oils are metabolized mainly as plant defense mechanism, these compounds may present toxicity to other organisms because of their lipophilic affinity.[4] The lipophilic nature causes their passage through both the cell wall and the cytoplasmic membrane, destabilizing polysaccharides and fatty acids, in addition to the loss of ions in bacterial cells, leading them to lysis.[5] Limonene, a monocyclic monoterpene known as D-limonene or (R)-(+)-4-isoproprenyl-1-methylcyclohexane (Figure 1), is the main constituent of essential oils in most citrus species, such as lemon, orange, mandarin, lime and grapefruit.[6,7]
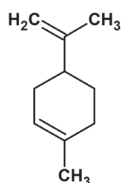
Figure 1. Chemical structure of limonene. Drawn in ChemSketch (ACD, 2019)

*e-mail: varesche@sc.usp.br

The limonene content in citrus processing waste is considered as one of the major obstacles to its reuse for biogas production. Its minimum concentration for inhibition is estimated to be approximately 200 mg kg^{-1} [1,7,8] and since the concentrations in CPW generally exceed this limit, the inhibitory effect of this substance is always expected.[9]

Under aerobic conditions, limonene degradation occurs in approximately 14 days, however there is still insufficient data regarding this process under anaerobic conditions. In anaerobic environment, limonene has greater toxicity; however, the mechanisms are still unclear. Limonene toxicity can be attributed both to the greater sensitivity of anaerobic organisms and to low partial pressure of oxygen in the system, resulting in less oxidation.[9]

Calabrò *et al.*[1] described the formation of p-cymene after limonene biodegradation in anaerobic environments, in which limonene is more toxic than p-cymene,[10] justifying the longer biomass adaptation time, proportional to higher limonene concentrations and to microbial adaptation after this period. The authors observed longer λ in assays with higher limonene concentration; however, methane production reached final production values (370 CH_4 g^{-1} TVS) similar to control assays.

There are few studies regarding limonene content in anaerobic reactors for the inhibition of microbial activity. However, Bakkali *et al.*[5] described the effect of several essential oils and their cytotoxicity on model microorganisms, concluding that citrus essential oils are toxic in concentrations between 0.2-25.6 mg L^{-1} for *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Bacillus subtilis*. Lotito *et al.*[2] observed methanogenic inhibitory effect at initial concentrations above 150 mg L^{-1} of limonene. Thus, the quantification of wastes or wastewater limonene content is essential for process control.

Several methods could be used to determine limonene content, such as Molecular Absorption Spectroscopy (UV-Vis),[11] Gas Chromatography with Flame Ionization Detection (GC-FID), Selected Ion Monitoring Mass Spectrometry (SIM),[12] Gas Chromatography with glass capillary Columns,[13] Ultraviolet Ion Mobility Spectrometry (IMS),[14] High Performance Liquid Chromatography (RP-HPLC)[15] and different Gas Chromatography conditions Coupled to Mass Spectrometry (GC-MS).[13,16,17] According to Adorno *et al.*,[18] methods that involve direct headspace analysis are more advantageous when compared to those with direct injection of liquid sample into the chromatographic column, preserving its life time.

GC-MS was used for limonene determination in citrus samples,[19] however, data regarding the application of this method in samples from anaerobic reactors, which are commonly fed with wastewater, are still scarce. The objective of this study was to develop and validate a new GC-MS method for limonene quantification, as well as to apply it in samples from anaerobic reactors. Therefore, batch assays were carried out to verify the possible inhibition of hydrogen production in reactors without limonene addition to 1500 mg L^{-1} of limonene.

EXPERIMENTAL

Reagents

Limonene standard solution was made with (R)-(+)-limonene ($C_{10}H_{16}$) with 90% purity (Sigma Aldrich-62122). All other reagents (ethanol and n-hexane) were of analytical purity (>98%).

Equipment and conditions

Limonene content was determined by Gas Chromatography, MassHunter software, coupled to Mass Spectrometer (GC-MS Agilent), model 7890B (gas chromatograph) and MS 5977A (mass spectrometer), injecting 250 µL of headspace samples, and using the parameters shown in Table 1. The chromatographic condition was defined by peak resolution analysis.

Sample preparation

Liquid anaerobic reactor samples were collected at the end of the operating cycle and centrifuged at 9000 rpm for 5 minutes, and preserved at -20 °C, without adding any substance. Biogas samples from the headspace were collected periodically (every 2 hours) with a butyl cap using a manual gas-tight syringe (Figure 2).The samples were diluted in MilliQ® water as needed, for later analysis in the calibration curve developed and described in the subsequent items.

Calibration curve

Calibration curve of standard limonene solutions were performed in triplicate. Dilutions were performed in ultra-purified water and ethanol p.a. was used as solvent to obtain concentrations of 3.0; 6.0; 9.0; 12.0; 15.0; 18.0 and 20.0 mg L^{-1}.

Method validation

Linearity, instrumental precision, detection and quantification limits were established and conducted according to recommendations in Ribani *et al.*,[20] Duarte *et al.*[21] and Adorno *et al.*,[18] following ANVISA (*Agência Nacional de Vigilância Sanitária*) specifications.[22]

Linearity
Detector response linearity for calibration curve was evaluated by

Table 1. Operation specifications for GC/MS

Gas Chromatograph	
Analytical Column	Agilent HP 5 MS (30 m x 0.25 mm x 0.25 µm)
Injector-Port	Split: 10:1
GC Inlet Temp	230 ºC
Carrier-Gas	He
Injection Volume	250 µL
Automatic injector	
Model	PAL RSI 85
Incubation Temperature	100 ºC
Time of incubation	10 min
Oven Program	
Temperature	40 ºC
Hold Time	3 min
Rate	10 ºC
Temperature	100 ºC
Rate	60 ºC
Temperature	220 ºC
Mass Spectrometer	
Auxiliary heat	240 ºC
Full scan Method Mass range	m/z 50 to 550
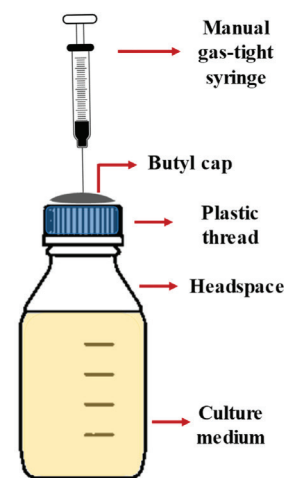
Figure 2. Static headspace sampling. The biogas content in the headspace was collected periodically (every 2 hours) through the butyl cap using a manual gas-tight syringe

linear regression, from samples with concentrations of 3 to 20 mg L^{-1} of limonene. Three replicates of each standard level were used to build the analytical curve.

Instrumental precision
Instrumental precision (as coefficient of variation, CV%) was estimated by the retention time of nine repeated injections of the standard solution with 12 mg L^{-1} of limonene.

Limit of detection
Detection limit (LD) was calculated according to Equation 1.

$$LD = 3 \times SD/m \quad (1)$$

where: SD = standard deviation; m = angular coefficient.

Limit of quantification

The lowest possible concentration of measurement was established by quantification limit (LQ), calculated according to Equation 2.

$$LQ = 10 \times SD/m \qquad (2)$$

where: SD = standard deviation; m = angular coefficient.

Adsorption assay

To estimate the adsorption potential of limonene to organic matter, its recovery was measured by the addition of known limonene concentration (2000 mg L^{-1}) to the sewage sludge used as inoculum in anaerobic reactors. The results were calculated by Equation 3.

Recovery (%) = (estimated concentration/theoretical concentration) $\times$ 100   (3)

Limonene adsorption was estimated according to Calabrò *et al.*,[1] in a control sample (with ultrapure water instead of sewage sludge) and in a sample with 2000 mg L^{-1} of limonene. To extract this compound, 1 mL of n-hexane (of analytical purity >98%) was added to each 1 mL of biomass, and vortexed for 5 minutes, and the procedure was repeated three times. The organic fraction was collected, and the recovery percentage was estimated by the ratio between the estimated concentration in the organic phase and the theoretical concentration.

Applicability

Kinetics

The assays were conducted in batch reactors with 5 g L^{-1} of glucose in Duhran flasks with 50% headspace and 50% reaction volume, in PCS medium,[23] under static conditions, at pH adjusted to 7.0 with 1 mol L^{-1} NaOH or HCl, with addition of 0 to 2000 mg L^{-1} of limonene (without limonene addition; 50; 100; 250; 500; 1000; 1500 and 2000 mg L^{-1} of limonene). The inoculum used was granulated sludge from thermophilic Upflow Anaerobic Sludge Blanket (UASB) applied to sugarcane vinasse treatment (Pradópolis, Brazil) (2 g TVS L^{-1}). The inoculum was chosen based on previous research.[24]

After inoculation, a mixture of N_2/CO_2 (70/30%) was fluxed, and the reactors were closed with butyl cap and plastic thread. Limonene concentration was estimated in liquid samples at the beginning and at the end of operation time.

Analytical methods

Total Solids (TS), Total Volatile Solids (TVS) and pH determination was performed according to methods 2540B and 2540E, respectively.[25] Total carbohydrates were quantified according to Dubois *et al.*,[26] and phenolic compounds were quantified according to Buchanan and Nicell.[27] Volatile fatty acids and alcohols determination was conducted on a Shimadzu® gas chromatograph (GC-2010) according to Adorno *et al.*[18]

Biogas composition analysis was performed on a Shimadzu® gas chromatograph (GC-2010), equipped with thermal conductivity detector (DCT). The column used was Carboxen 1010 PLOT, 30 m x 0.53 mm. Injector, furnace and detector temperatures were 220 °C, 130 °C – 135 °C (5.5 min), at 46 °C /min and 230 °C, respectively. Argon was used as carrier gas, with column flow of 5.66 mL min^{-1} and Make-up volume of 12 mL min^{-1}.

Data analysis and adjustment

The time to start the H_2 production, maximum H_2 production rate and maximum H_2 production potential were determinated with the modified Gompertz model.[28] The kinetic parameters were calculated using Equation 4 in OriginPro 9.0 software.

$$P_{ac} = P.\exp\left\{-\exp\left(\frac{Rme}{P}[\lambda - t] + 1\right)\right\} \qquad (4)$$

where: Pac= cumulative H_2 production, Rm= maximum H_2 production rate (mmol h^{-1}), e= Euler number (2.71828182), λ = time to start the H_2 production (h), P= maximum H_2 production potential (mmol L^{-1}).

Shapiro-Wilk normality test was applied to verify data parametricity, that is, if they presented normal distribution ($p \geq 0.05$) or non-normal distribution ($p \leq 0.05$). Comparisons were carried out using Kruskal-Wallis or Tukey's test, evaluating statistical relevance at 95% confidence interval. Experimental data were adjusted to mean values from replicates.

RESULTS AND DISCUSSION

Calibration curve and validation parameters

Standard deviations for calibration curve were between 0.5 and 1.8 mg L^{-1}. The method was considered linear for the concentration range studied (3 to 20 mg L^{-1} of limonene). The calibration plot showed good linearity, since the coefficient of correlation (R^2) was 0.979 (Figure 3).



y = 1E+06x + 570933
R^2 = 0.979
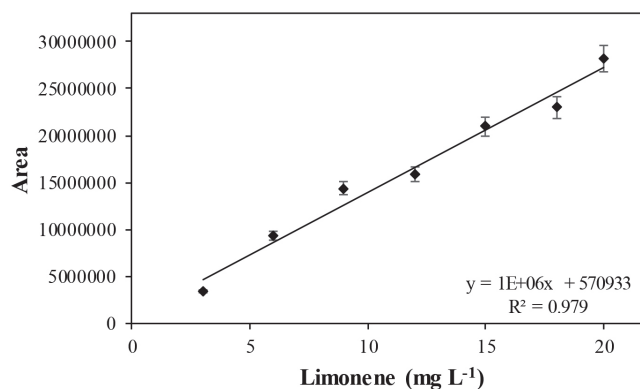
Figure 3. *Calibration curve of limonene for 3.0; 6.0; 9.0; 12.0; 15.0; 18.0 and 20.0 mg L^{-1} of limonene*

The peak area corresponding to limonene was determined by chromatogram and mass spectra analysis of a known limonene concentration standard solution. Figure 4.A shows the chromatogram for the standard with 15 mg L^{-1} of limonene, which corresponds to 15.3 mg L^{-1} of limonene according to the calibration curve. Figure 4.B shows the chromatogram for assays with 17 mg L^{-1} of limonene. In addition, the National Institute of Standards and Technology (NIST) library search supported the identification of limonene.

Instrumental precision, estimated as 0.1 (CV %), LD and LQ (1.7 and 4.2 mg L^{-1}, respectively) demonstrates the reliability of the method. The tabulated value for Ftest (ANOVA) was 2.6, while the calculated value was 233.51. The Fcalculated>Ftabulated values are further evidence of method linearity, at a significance level of 95%. The validation parameters evaluated are in accordance with Resolution-Re N. 899[22] and IUPAC Technical Report.[29]

Compared to Davidowski and DiMarco,[19] the method presented here is advantageous over both GC injector temperature (230 °C) and Oven Program temperature (40 °C), while in the study mentioned, the temperature range was 250 and 80 °C, respectively. Moreover, the method presented in this study allows the analysis of liquid samples
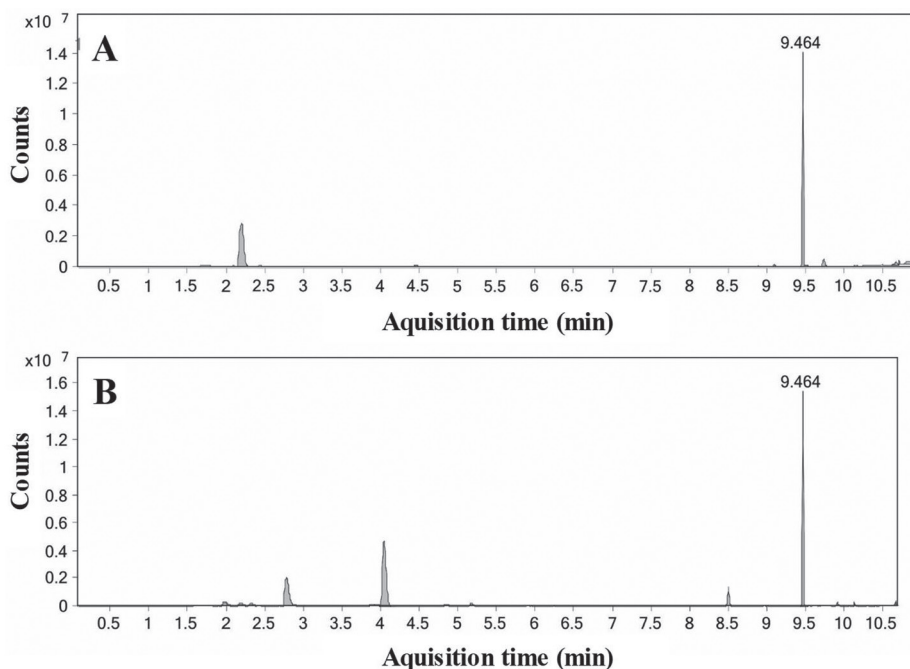
Figure 4. *Typical chromatogram of a standard limonene solution (15 mg L⁻¹) (A) and chromatogram for assays with 17 mg L⁻¹ of limonene (B)*

from anaerobic reactors, inoculated with impure materials such as sewage sludge, while in Davidowski and DiMarco[19] the authors used the GC-MS technique for quantification of limonene in orange extract.

Adsorption assay

Limonene recovery in the control assays (without sewage sludge) was $106.1\pm13\%$ of the theoretical value (2000 mg L⁻¹), while in assays with sewage sludge, $92.3\pm0.4\%$ was recovered. However, this difference was not statistically significant (p=0.179) for a 95% confidence interval.

Applicability

The proposed GC-MS method was applied to determine the limonene concentration at the beginning (pI) and at the end (pF)

of the fermentation process of batch anaerobic reactors (Figure 5). There was no significant difference (p>0.05) between pI and pF limonene content. It can be inferred that there was no significant limonene degradation throughout the fermentation process, as well as no significant adsorption of limonene in the sewage sludge used as inoculum, as shown in the adsorption assay. In addition, it can be concluded that the method developed was effective for limonene content analysis determination in anaerobic reactors.

Previous studies indicate that the sorption/desorption equilibrium between limonene and organic matter strongly affects the process.[1] Calabrò *et al.*[1] observed that approximately 76% of the limonene added to the assays was adsorbed by the solid phase of citrus peel waste. However, a recovery of 97% of the compound added was observed in this study, with no significant difference between the control (2123 mg L⁻¹) and assays with sewage sludge (1746 mg L⁻¹). This difference may have occurred due to the substrate composition
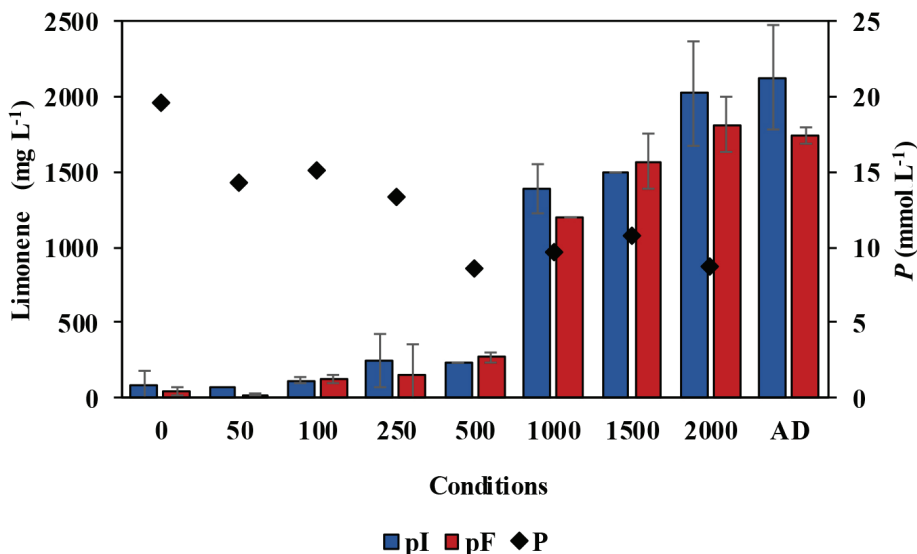
Figure 5. *Limonene content at the beginning (pI) and at the end (pF) of the fermentation process of batch anaerobic reactors operated with different concentrations of limonene (without limonene addition to 2000 mg L⁻¹) for about 160 hours, adsorption (AD) and H₂ production potential (P)*

used by the aforementioned authors, since citrus peel waste is rich in long chain waxes, which has a strong affinity with hydrophobic D-limonene.

The effect of different limonene concentrations was determined (without limonene addition to 2000 mg L^{-1}) on H_2 production potential (P mmol L^{-1}), maximum H_2 production rate (Rm mmol h^{-1}) and start time of H_2 production (λ h) (Figure 6). Maximum H_2 production was 19.5 mmol L^{-1} in control assays, while the minimum was 8.6 mmol L^{-1} in assays containing 2000 mg L^{-1} of limonene. For 95% confidence interval, it ca be stated that the increase in limonene to 50 mg L^{-1} and 50 to 100 mg L^{-1} was not significant. There was no inhibitory effect caused by this compound, while the increase in intervals between 100 and 2000 mg L^{-1} showed a significant inhibitory effect ($p \geq 0.05$) (Table 2).
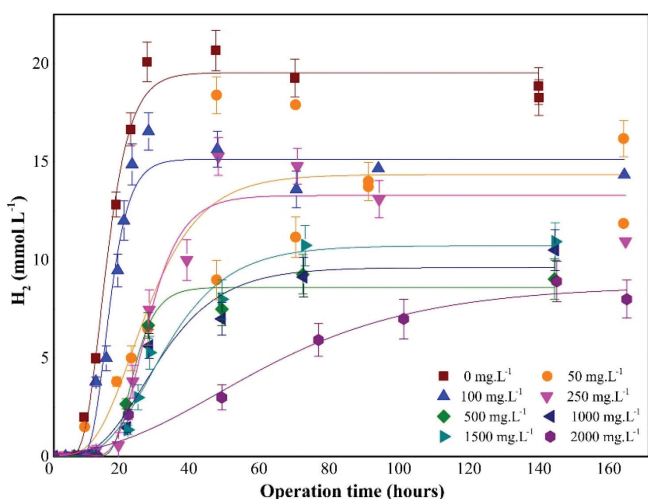
Figure 6. *Kinetics of accumulated H_2 production in different limonene concentrations (without limonene addition to 2000 mg L^{-1})*

Table 2. Kinetic parameters of H_2 production under different concentrations of limonene (without limonene addition to 2000 mg L^{-1}). Means followed by same letter do not differ from each other for a 95% confidence interval using Kruskal-Wallis test

Limonene (mg L^{-1})	Parameters			
	P (mmol L^{-1})	Rm (mmol h^{-1})	λ (h)	R^2
without	19.5±0.4[a]	1.7±0.2[a]	8.7±0.7[a]	0.99
50	14.3±1.1[a]	0.5±0.2[a]	11.6±5.9[a]	0.97
100	15.1±0.5[a b]	1.5±0.2[b]	10.8±0.9[b]	0.97
250	13.3±0.7[c]	0.7±0.2[c]	17.8±2.1[b]	0.96
500	8.6±0.3[d]	0.7±0.2[d]	17.0±1.3[b]	0.98
1000	9.6±0.7[e]	0.3±0.1[e]	13.1±3.5[b]	0.95
1500	10.7±0.5[c]	0.3±0.1[f]	14.7±2.1[b]	0.98
2000	8.7±0.5[f]	0.1±0.1[g]	15.0±2.3[b]	0.97

Likewise, Rm was not significant when 50 mg L^{-1} of limonene was added (Rm= 0.5 mmol h^{-1}), while the addition in the intervals between 100 and 2000 mg L^{-1} of limonene delayed the reaction in all assays. The maximum Rm observed was 1.6 mmol h^{-1} in control assays and the minimum Rm was 0.1 mmol h^{-1} with 2000 mg L^{-1} of limonene.

For λ, although there is a proportional increase in limonene concentration, the minimum λ was observed for control assay (17.1 h) and the maximum λ for 2000 mg L^{-1} of limonene assay (37.9 h). However, only the increase from 50 to 100 mg L^{-1} of limonene

showed significant difference for λ, where λ = 11.6 h to 50 mg L^{-1} and λ = 10.8 h to 100 mg L^{-1}. This could be explained based on the larger standard deviations of this parameter[1].

According to Calabrò *et al.*,[1] only concentrations above 200 mg L^{-1} of limonene were inhibitory for methane production with CPW as substrate. In addition, these authors observed an increase in λ proportional to limonene concentration increase, as observed in this study.

The aforementioned authors observed significant reduction in λ after the biomass adaptation period; however, methane production reached final production values (370 CH_4 g^{-1} TVS) similar to control assays. A greater adaptability of methanogenic organisms after certain period may have occured because of its crystalline surface layer, which confers greater resistance to lipophilic compounds, such as limonene.[9] These results may have occurred because of the longer reaction time required for methane production (about 4 days) when compared to H_2 in this study (about 17.1 h in control assays), and possible limonene volatilization and/or degradation, minimizing its inhibitory effect over time.

Soares *et al.*[30] characterized the same sludge used as inoculum in the present study, observing a relative abundance of 78.2% for Firmicutes, a bacterial phylum related to H_2 production and volatile fatty acids (VFA) as the main metabolites. However, its Gram-positive characteristics may be disadvantageous due to the presence of limonene and other essential oils during fermentation since, according to Ruiz and Flotats,[9] Gram-positive bacteria can tolerate less concentrations of lipophilic compounds when compared to Gram-negative.

According to Burt,[31] several operational parameters can affect and increase essential oils toxicity, especially acidic pH. Therefore, since the optimal pH for acidogenesis and H_2 production is considerably lower than the optimum pH of methanogenic step, it is expected that antimicrobial action in limonene will be potentiated in the first case. In this study, initial pH was adjusted to 7.0, while pH after fermentation it reached approximately 5.8, which may have potentiated the limonene inhibitory effect.

Srilatha *et al.*[32] studied the effect of limonene concentration on citrus residues at mesophilic temperature (30 °C) and 1500 L digesters, concluding that the maximum daily dosage of limonene would be 24 mg L^{-1}, corresponding to 2.5 kg TVS m^3 d^{-1} residue per digester. The maximum dosage described by these authors was considerably lower when compared to the present study; however, it should be noted that the authors used CPW, containing approximately 8.9 g kg^{-1} of limonene, whereas in this study the reactors were evaluated by the addition of limonene p.a in batch reactors. Therefore, it is important to consider the inhibitory effect of other essential oils on CPW, not only limonene.[9]

In relation to physical-chemical analysis, TS production was not significant ($p > 0.05$), as well as the TVS content variation. The variation of other parameters (phenolic compounds, pH variation and carbohydrate contente) was considered significant ($p \leq 0.05$), and phenolic compounds were produced in all assays, approximately 2 mg L^{-1}, independent of limonene initial concentration. Carbohydrate removal was statistically significant in all assays, with approximately 80% of the initial carbohydrate concentration (about 5 g L^{-1}), also independent of initial limonene concentration (Table 3).

The pH variation in different assays was significant ($p \leq 0.05$). In addition, an inversely proportional relation between initial limonene concentration and pH reduction was observed, since in control assays the pH was 5.9 and in assays with 2000 mg L^{-1} of limonene the pH was about 6.3 at the end of the experiments. This result can be related to VFA and H_2 production, where in assays with lower limonene concentration (50 mg L^{-1}), higher H_2 production was observed

Table 3. Results of physical-chemical analyses at different concentrations of limonene (without limonene addition to 2000 mg L^{-1}) at the beginning (pI) and at the end (pF) of the fermentation process of batch anaerobic reactors.

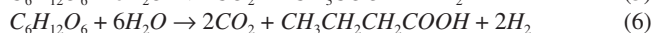| Limonene (mg L^{-1})	Carbohydrates (g L^{-1})		Phenols (mg L^{-1})		pH		TVS (mg L^{-1})	
	pI	pF	pI	pF	pI	pF	pI	pF
0	4.9±0.4	0.3±0.0	8.0±0.0	9.1±0.1	7.2±0.1	5.9±0.2	33±7	42±5
50	5.2±0.1	0.2±0.0	8.0±0.0	8.9±0.1	7.4±0.1	5.8±0.3	35±2	34±5
100	5.2±0.1	0.2±0.0	8.1±0.1	9.6±0.2	7.4±0.2	6.3±0.1	49±13	28±12
250	3.2±0.3	0.1±0.0	8.3±0.1	9.8±0.0	7.1±0.1	6.1±0.1	11±8	23±15
500	4.9±0.6	0.2±0.0	8.9±0.1	9.4±0.0	7.0±0.1	6.0±0.2	54±6	35±7
1000	3.5±0.2	0.1±0.0	7.8±0.0	9.3±0.1	7.3±0.3	6.4±0.2	56±6	47±11
1500	3.8±0.1	0.2±0.0	7.8±0.0	9.1±0.0	7.3±0.1	6.3±0.1	42±6	37±7
2000	3.5±0.1	0.1±0.0	7.8±0.0	9.5±0.3	7.2±0.1	6.3±0.1	95±2	49±22

(14.45 mmol L^{-1}) when compared to higher limonene concentrations (2000 mg L^{-1} of limonene; 8.7 mmol H_2L^{-1}) (Table 3).

By monitoring the accumulation of VFA and alcohols at the end of operation time, it was observed that there was a difference between the metabolites produced in different limonene concentrations (Figure 7). No production of HBu was observed in control assays and in assays with 50 mg L^{-1} of limonene. However, the difference in H_2 production between these assays was not significant. Maximum HBu production was 886 mg L^{-1} in assays with 100 mg L^{-1} of limonene.

HAc was the major metabolite in all conditions, with maximum production in the control assay (1771 mg L^{-1}) and lower production in the assay with 2000 mg L^{-1} of limonene (1024 mg L^{-1}). HAc production was proportional to H_2 production, since in control assays 19.5 mmol H_2L^{-1} was obtained and in assays with 2000 mg L^{-1} of limonene 8.68 mmol H_2L^{-1} was obtained.
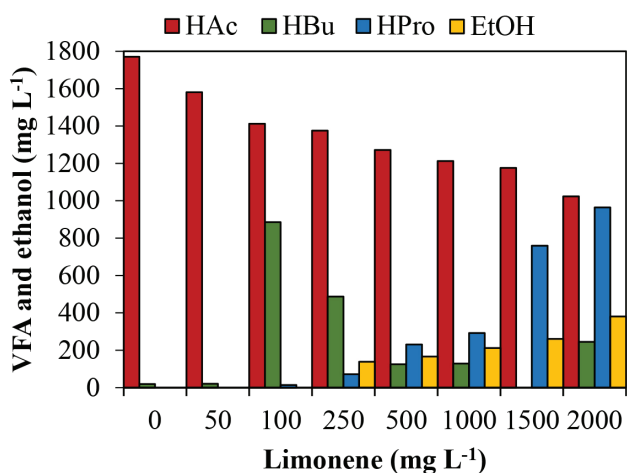
Figure 7. VFA and alcohols accumulated under different conditions. Acetic acid (HAc), butyric acid (HBu), propionic acid (HPro) and ethanol (EtOH)

It is known that acetogenic pathway is more advantageous for H_2 production in relation to HBu pathway, since in the first case, H_2 production is about 4 mols per mol of glucose (Equation 5), while in the second case, H_2 production is about 2 mols of H_2 per mole of glucose (Equation 6).[33,34] In this study, 0.70 mol of H_2 per mol of glucose was obtained in the control assays, while in assays with the maximum dosage of limonene evaluated (2000 mg L^{-1}), the production was only 0.3 mol H_2 per mol of glucose.

$$C_6H_{12}O_6 + 6H_2O \rightarrow 2CO_2 + 2CH_3COOH + 4H_2 \qquad (5)$$
$$C_6H_{12}O_6 + 6H_2O \rightarrow 2CO_2 + CH_3CH_2CH_2COOH + 2H_2 \qquad (6)$$

Likewise, a proportional increase in HPr production was noticed, with an increase of limonene concentration: 14 mgHPr L^{-1} in assays with 100 mg L^{-1} of limonene and 964 mgHPr L^{-1} in assays with 2000 mg L^{-1} of limonene. In the HPr metabolic pathway, there is a consumption of 2 mols of H_2 for each mol of glucose oxidized (Equation 7).[35,36]

$$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O \qquad (7)$$

Ruiz and Flotats[7] indicate a proportional relation between limonene concentration and HPr production. These authors obtained 284 mgHPr L^{-1} after CPW autofermentation, besides 805 mg HAc L^{-1} and 909 mgHBu L^{-1}. Similar results were obtained by Su *et al.*,[37] where in 30 days of fermentation with batch reactors operated with swine manure as inoculum, the main VFA produced from CPW were HAc (2610.7 mg L^{-1}), HPr (56.2 mg L^{-1}) and HBu (27.5 mg L^{-1}). In this study, although HAc was the main metabolite pathway in all assays, it was possible to observe a proportional relation between limonene concentration and HPr production, with concomitant reduction in H_2 production.

The same pattern was observed for EtOH production, since 139 mgEtOH L^{-1} was obtained in assays with 250 mg L^{-1} of limonene and 380 mg L^{-1} of EtOH in assays with 2000 mg L^{-1} of limonene. The solvetogenic pathway is not desirable in processes whose main objective is to obtain H_2, since there is production of EtOH from HAc, instead of H_2 (Equation 8).[33,34]

$$CH_3COOH + 2H_2 \rightarrow CH_3CH_2OH + H_2O \qquad (8)$$

Regarding H_2 and VFA production results, it can be inferred that the presence of limonene caused substantial changes in the metabolic pathway prevailing during fermentation in anaerobic reactors.

CONCLUSIONS

The developed method allowed the determination of limonene in anaerobic reactors, without prior preparation. Because of the simplicity and efficiency of this analysis, this method can be indicated for wastewater characterization and for anaerobic reactors control.

An inhibition effect was observed in H_2 and HAc production after limonene addition. This inhibitory effect was significantly proportional to the increase of limonene concentrations, possibly because of changes of metabolic pathway, stimulating EtOH and HPr production. The presence of limonene significantly affected the start time of the H_2 production, since in control assays $\lambda = 8.7$ h was observed and in assays with 2000 mg. L^{-1} of limonene, λ was 15.0 h.

REFERENCES

1. Calabrò, P. S.; Pontoni, L.; Porqueddu, I.; Greco, R.; Pirozzi, F.; Malpei F; *Waste Manage.* **2016**, *48*, 440.
2. Lotito, A.; M.; De Sanctis, M.; Pastore, C.; Di Iaconi, C.; *J. Environ. Manage.* **2018**, *215*, 366.
3. Negro, V.; Ruggeri, B.; Fino, D.; Tonini, D.; *Resour. Conserv. Recycl.* **2017**, *127*, 148.
4. Araújo, A.C. J.; Freitas, P. R.; Barbosa, C. R. S.; Muniz, D. F.; Rocha, J. E.; Silva, A. C. A.; Oliveira-Tintino, C. D. M.; Ribeiro-Filho, J.; Silva, L. E.; Confortin, C.; Amaral, W.; Deschamps, C.; Barbosa-Filho, J. M.; Lima, N. T. R.; Tintino, S. R.; Coutinho, H. D. M.; *Food Chem Toxicol.* **2020**, *136*, 111023.
5. Bakkali, F.; Averbeck, S.; Averbeck, D.; Idaomar, M.; *Food. Chem. Toxicol.* **2008**, *46*, 446.
6. Lopresto, C. G.; Petrillo, F.; Casazza, A. A.; Aliakbarian, B.; Perego, P.; Calabrò, V.; *Sep. Purif. Technol.* **2014**, *137*, 13.
7. Ruiz, B.; Flotats, X.; *Biochem. Eng. J.* **2016**, *109*, 9.
8. Pourbafrani, M.; Forgács, G.; Horváth, I. S.; Niklasson, C.; Taherzadeh, M. J.; *Bioresour. Technol.* **2010**, *101*, 4246.
9. Ruiz, B.; Flotats, X.; *Waste Manage.* **2014**, *34*, 2063.
10. Dorman, H. J. D.; Deans, S. G.; *J. Appl. Microbiol.* **2000**, *88*, 308.
11. Ahro, M.; Hakala, M.; Sihvonen, J.; Kauppinen, J.; Kallio, H.; *J. Agric. Food Chem.* **2001**, *49*, 3140.
12. Blanch, G. P.; Nicholson, G. J.; *J. Chromatogr. Sci.* **1998**, *36*, 19.
13. Song, H. S.; Sawamura, M.; Ito, T.; Kawashimo, K.; Ukeda, H.; *Flavour. Fragr. J.* **2000**, *15*, 245.
14. Vautz, W.; Sielemann, S.; Baumbach, J. I.; *Anal. Chim. Acta.* **2004**, *513*, 393.
15. Villa, C.; Gambaro, R.; Mariani, E.; Dorato, S.; *J. Pharm. Biomed. Anal.* **2007**, *44*, 755.
16. Marine, S. S.; Clemons, J.; *J. Chromatogr. Sci.* **2003**, *41*, 31.
17. Miller, J. A., Hakim, I. A.; Thomson, C.; Thompson, P.; Chow, H. S.; *J. Chromatogr. B* **2008**, *870*, 68.
18. Adorno, M. A. T.; Hirasawa, J. S.; Varesche, M. B. A.; *Am. J. Anal. Chem.* **2014**, *5*, 406.
19. Davidowski, S.; DiMarco, B.; *PerkinElmer Application Note* **2009**, 1.
20. Ribani, M.; Bottoli, C. B. G.; Collins, C. H.; Jardim, I. C. S. F.; *Quim. Nova* **2004**, *27*, 771.
21. Duarte, I. C. S.; Oliveira, L. L.; Buzzini, A. P.; Adorno, M. A. T.; Varesche, M. B. A.; *J. Braz. Chem. Soc.* **2006**, *17*, 1360.
22. Anvisa, Brasil, *Diário Oficial da União*, 2003, p. 56.
23. Haruta, S.; Cui, Z.; Huang, Z.; Li, M.; Ishii, M.; Igarashi, Y.; *Appl. Microbiol. Biotechnol.* **2002**, *59*, 529.
24. Camargo, F. P.; Sakamoto, I. K.; Duarte, I. C. S.; Varesche, M. B. A.; *Int. J. Hydrogen. Energy.* **2019**, *44*, 22888.
25. APHA/AWWA/WEF; *Standard Methods for the Examination of Water and Wastewater*, 22th ed., American Public Health Association: Washington, DC, 2012.
26. Dubois, M.; Gilles, K.; Hamilton, J.; Rebers, P.; Smith, F.; *Anal. Chem.* **1956**, *28*, 350.
27. Buchanan, I. D.; Nicell, J. A.; *Biotechnol. Bioeng.* **1997**, *54*, 251.
28. Zwietering, M. H.; De Wit, J. C.; Cuppers, H. G. A. M.; Van't Riet, K.; *Appl. Environ. Microbiol.* **1994**, *60*, 204.
29. Thompson, M.; Ellison, S. L. R.; Wood, R.; *IUPAC Techinal Reports* **2002**, *74*, 835.
30. Soares, L. A.; Braga, J. K.; Motteran, F.; Sakamoto, I. K.; Monteiro, P. A. S.; Seleghim Jr., P.; Varesche, M. B. A.; *Waste Biomass Valorization* **2019**, *7*, 1899.
31. Burt, S.; *Int. J. Food Microbiol.* **2004**, *94*, 223.
32. Srilatha, H. R.; Nand, K.; Babu, K. S.; Madhukara, K.; *Process. Biochem.* **1995**, *30*, 327.
33. Mäkinen, A. E.; Nissilä, M. E.; Puhakka, J. A.; *Int. J. Hydrogen Energy* **2012**, *37*, 12234.
34. Łukajtis, R.; Hołowacz, I.; Kucharska, K.; Glinka, M.; Rybarczyk, P.; Przyjazny, A.; Kamiński, M.; *Renewable Sustainable Energy Rev.* **2018**, *91*, 665.
35. Ghimire, A.; Frunzo, L.; Pirozzi, F.; Trably, E.; Escudie, R.; Lens, P. N. L.; Esposito, G.; *Appl. Energy* **2015**, *144*, 73.
36. Li, C.; Fang, H. H. P.; *Crit. Rev. Environ. Sci. Technol.* **2007**, *37*, 1.
37. Su, H.; Tan, F.; Xu, Y.; *Fuel* **2016**, *181*, 843.