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Development of a static headspace gas chromatographic/mass spectrometric method to analyze the level of volatile contaminants biodegradation

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Abstract

Volatile compound biodegradation analysis usually requires the time-consuming step of extraction of the analytes from the matrix using organic solvents or costly radioactive-compounds. Thus, it is desirable to have a simple and fast technique to generate a good evaluation of bacterial biodegradation. The goal of this research was to develop a methodology on the basis of static headspace-gas chromatography/mass spectrometry (HS-GC/MS) to evaluate the level of volatile contaminant biodegradation. The effects of the following parameters were studied: temperature and time of equilibration. The biodegradation experiments were carried out with bacteria inoculation in mineral media in presence of volatile hydrocarbon compounds (toluene, *p*-xylene, nonane and naphthalene). Autoclaved inoculates were used as control and reference sample. The optimal headspace conditions were observed when the vials were heated at 80 °C for 20 min, the syringe at 81 °C and an injection volume of 0.4 mL was used. This methodology has the advantage of being relative free from matrix effects.

Keywords: Static headspace analysis; Biodegradation; Volatile organic compounds

1. Introduction

Volatile aromatic hydrocarbons such as toluene, *p*-xylene and naphthalene are hazardous contaminants to the environment. They are released from the volatilization of petroleum fuels, motor vehicle exhaust, oil spills and industrial waste disposal sites [1,2]. They are frequently analyzed in air, water, effluents, soils and sediments [3–5].

Several studies have demonstrated that microorganisms can degrade a great variety of contaminants in soils and sediments [6,7]. For biodegradation studies, there are many methods described in the literature [8,9]. Volatile hydrocarbons biodegradation may be tested using ^{14}C compounds [10]. This technique requires radiolabeled compounds, which makes it costly. Biodegradation efficiency can also be measured by analyzing the remainder contaminant from the culture medium [11]. In gas chromatography analysis the contaminants are extracted from the medium using laborious extraction techniques and ultra pure solvents. The headspace appears to be one of the best techniques for biodegradation analysis, especially for volatile contaminants because of the fast analysis, its practicability and for its minimal solvent influence.

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Static headspace/gas chromatography (HS-GC) has been used for the determination of volatile organic compounds (VOC) in soil and water [12–14]. However, there is no detailed description in the literature of the use of this method for the quantification of biodegradation.

The static HS method is simple and involves the thermodynamic equilibrium of volatile substances within the aqueous and the gas phase of the sample in a closed thermostated vial. The gas phase containing the volatile substances is injected into the GC column and analyzed [15] without the influence of the matrix. The concentration of the analyte (Cg) in the headspace depends on the partition coefficient (K) of the analyte, the initial analyte concentration (Co) and the volume ratio (β) of the gas phase to the liquid phase at equilibrium as described by Eq. (1) [16].

$$\mathrm{Cg} = \frac{\mathrm{Co}}{K + \beta} \quad (1)$$

Since K is temperature dependent, the temperature of equilibration influences the HS-GC.

The development of a static headspace-gas chromatographic/mass spectrometric (HS-GC/MS) method to determine the level of volatile contaminant biodegradation is described in this paper. The tests were performed using aerobic bacteria isolated from polluted sediments collected in the Santos Estuary (São Paulo State, Brazil), which receives large amounts of contaminants from urban and industrial sludge waste.

2. Experimental

2.1. Chemicals and standard preparation

The standard hydrocarbon solution was prepared in acetone containing the following volatile hydrocarbons: toluene, naphthalene, nonane and *p*-xylene at a concentration of 5000 mg L^{-1}.

Nonane (99%) and naphthalene (99%) were purchased from Aldrich (Milwaukee, USA). Acetone (residual grade), *p*-xylene (99%), toluene (residual grade) were obtained from Merck (Darmstadt, Germany) and octane (99%) from Across Organics (New Jersey, USA).

2.2. Headspace parameters

The vials (25 mL) used in the headspace analysis were hermetically closed by Teflon-coated rubber stoppers (Supelco) and sealed by crimped aluminum caps.

Vial temperature studies were performed at different temperatures (60, 80, 90 and 100 °C). This experiment was done with an injection volume of 400 μL, syringe temperature of 81 °C and 30 min equilibration time. During the equilibration time (the vial was heated for 20 and 30 min at 80 °C), the volatile analytes behavior was analyzed.

2.3. Calibration curve

The calibration curves (10, 20, 30, 40 and 50 mg L^{-1}) were prepared by diluting the standard solution in water. Octane was used as internal standard (30 mg L^{-1}). The static headspace injections were performed in 5 replicates under the best parameters obtained in the headspace optimization.

2.4. Headspace-gas chromatography/mass spectrometry

Analyses were performed using a Shimadzu Headspace Analysis System composed of GCMS-QP5050 and HSS-4A automated headspace sampler.

The separation was carried out using a DB-5 J&W column (30 m × 0.25 mm i.d.; film thickness 0.25 μm). The GC temperature program was 40 °C for 2 min; increasing 3 °C min^{-1} up to 65 °C (holding for 1 min), increasing again from 65 °C min^{-1} up to 120 °C, this temperature was kept for 3 min. The analysis was done in a split/splitless injector (split ratio 1:14) at 250 °C. The carrier gas was helium at a constant flow rate of 1.3 mL min^{-1}. The interface temperature to MS was 230 °C. Mass spectrometer was operated in scan mode (electron impact at 70 eV, 1000 V).

2.5. Bacterial enrichments

The 34 isolates used in this study were obtained by enrichment of sediment samples collected during summer time in Santos Estuary (Fig. 1), an important industrial, portuary and touristic pole, located at the southeastern coast of Brazil [17].

Enrichment cultures were prepared by adding 1 g of sample soil in mineral salt medium [18] with 0.05% of diesel fuel as carbon source. The mineral medium contained (g L^{-1} of deionized water) 1.0 g $(NH_4)_2SO_4$, 0.2 g KH_2PO_4, 1.6 g K_2HPO_4, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.1 g NaCl, 0.01 g $FeSO_4{\cdot}7H_2O$ and 0.02 g $CaCl_2{\cdot}2H_2O$. The cultures were incubated on a rotary shaker at 160 rpm and 28 °C

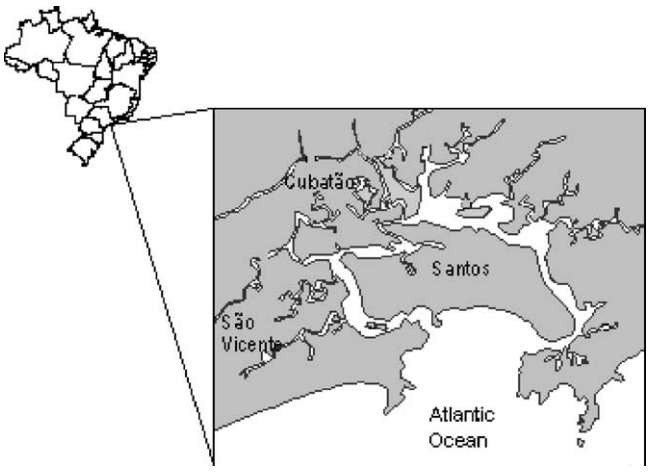

Fig. 1. Santos Estuary, São Paulo, Brazil.

until growth was observed, which occured approximately after 48 h. After three serial transfers in the enrichment medium, the cultures were streaked onto an agar mineral salt medium in a Petri dish with diesel fuel on the lid. All colonies that differed in appearance were picked and streaked two more times in order to isolate colonies. Isolates were also streaked onto tryptic soya agar (TSA) and selected by the rep-PCR technique [19] with Box A1R primer [20]. Strains with different band patterns were used in the biodegradation analysis. As control, flasks containing mineral medium and diesel fuel without sediments were incubated at the same temperature as the actual samples.

Biodegradation experiments were performed in 25 mL glass vials containing 4 mL of mineral medium and 1 mL of culture after growing in tryptic soya broth (TSB) medium, washed and resuspended in mineral medium to an optical density $(OD)_{600}$ of 1.0. Also 1 mL autoclaved inoculum was used as control. Mixed hydrocarbons (150 μg of toluene, *p*-xylene, nonane and naphthalene) were added from the standard solution to the vials (final concentrations of 30 mg L^{-1}). The bacteria were incubated for 4 days at 28 °C under rotary shaking. After incubation, the cultures were heated at 100 °C for 30 min to stop any further degradation and then, the internal standard (octane) was added (150 μg). Thirty-four bacteria and controls were studied; all experiments were done in triplicate. In total 204 vials were analyzed.

3. Results and discussion

3.1. Optimization of HS-GC/MS parameters

The important parameters on HS-GC/MS analysis were studied: heating temperature and the equilibration time. The other parameters like syringe temperature and volume are specific for this HS/GC equipment.

The sample heating changes the solubility of the analyte, driving the equilibrium to the gas phase. The temperature effect was estimated by heating the vials within the range of 60–100 °C.

Raising the vial temperature increases the vapor pressure of water and all studied volatile compounds and hence its concentration in the gas phase until 80 °C. Above 100 °C the vapor pressure of water contributes mostly to the internal pressure. The resulting internal pressure in the vial extends into the syringe and during the sample transfer, the pressurized headspace gas then expands to the atmosphere and part of the headspace gas gets lost [21] explaining the decreasing area value in Fig. 2. The effects of temperature also depend on the partition coefficient from each compound.

The time of equilibration is another important parameter that influences the method sensitivity and repeatability [22]. No significant difference in peak responses was observed between the two times of equilibration settings (20 and 30 min) during the experiments.

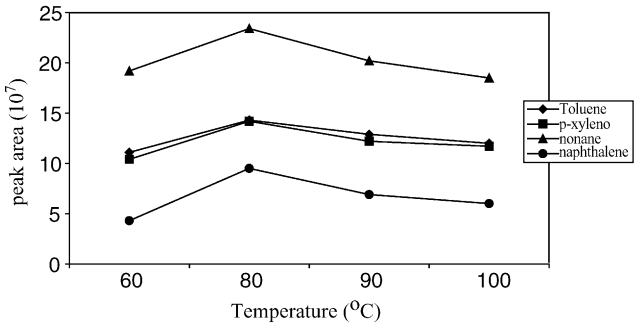

Fig. 2. The vial temperature optimization for the HS-GC/MS.

The syringe temperature of 1 °C above vial temperature was selected to avoid the analytes condensation.

3.2. Method evaluation

The calibration curve and the biodegradation of volatile contaminants studies were performed with the best parameters according to the optimization of this methodology which are: vial temperature 80 °C, equilibration time 20 min, syringe temperature 81 °C and injection volume 400 μL.

The resulting calibration curves were linear with correlation coefficients (r) > 0.995 for all analytes. The detection limit was 12 ppb and the quantification limit was 40 ppb.

3.3. Biodegradation of volatile contaminants

In order to evaluate the current HS-GC/MS methodology for volatile biodegradation analysis, the degradation of toluene, *p*-xylene, nonane and naphthalene was studied using thirty-four bacteria strains.

The bacteria were isolated from sediments that were collected during the summer time in the Santos estuary, the water temperature in this place varied between 25 and 30 °C, therefore 28 °C was chosen as test temperature. The mixed hydrocarbon concentration was selected according to Greer and co-workers [11]. Incubations were performed for 4 days at 28 °C under rotative shaking as described in the literature [23-25] that shows that aerobic microorganisms can degrade several hydrocarbons in less than 50 h. After that period the vials were heated at 100 °C for 30 min to stop the biodegradation process by sterilization.

By Henry's law, in a closed system, when the concentration of the volatile compound in the liquid phase is below 1%, the sample solution of volume Vl, with an initial concentration of C°l (solute mass M°l), is led to a gas-liquid equilibrium at a constant temperature. The mass of volatile organic compound in the gas phase (Mg) can be calculated by Eq. (2) [26]:

$$\mathrm{Mg} = \frac{K(\mathrm{Vt} - \mathrm{Vl})\mathrm{VlC}^{\circ}\mathrm{l}}{K(\mathrm{Vt} - \mathrm{Vl}) + \mathrm{Vl}} \quad (2)$$

where K is the partition coefficient and Vt is the vial volume. The solute mass in the liquid phase (Ml) can be calculated by

Eq. (3).

$$Ml = M^{\circ}l - Mg \quad (3)$$

Considering the partition coefficient for toluene as $K = 0.217$ [27], the mass of toluene in the liquid phase at equilibrium available for biodegradation was calculated as 12 μg using the Eqs. (2) and (3).

The values of K for xylene, nonane and naphthalene are equal to Ostwald solubility coefficient [28] and are 3.9, 0.005 and 56.2, respectively [29].

Vials which contained 1 mL of autoclaved inoculates were set up as control to monitor background loss of the hydrocarbons due to any retention and volatilization that could occur during the degradation experiment.

The HS-GC/MS analyses were performed at 80 °C for 20 min. This temperature did neither decompose the hydrocarbons [30] nor react with the mineral media, which contain just inert salts at these temperatures.

Among the isolates tested, there were several species from different genera, like *Serratia sp*, *Acinetobacter sp* and *Klebesiella sp* [20].

As no significant loss of volatile compounds was observed at the controls by the HS-GC/MS analysis (Fig. 3a), we considered that biodegradation occurred when the peak area of volatile hydrocarbon disappeared or partially decreased when compared to the control samples (Fig. 3b).

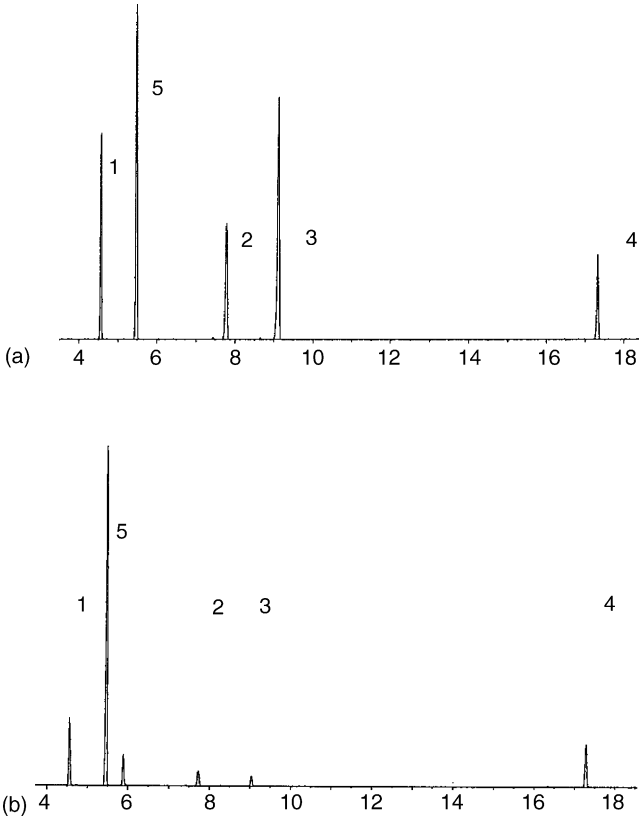

Fig. 3. (a) Control chromatogram: toluene (1), *p*-xylene (2), nonane (3), naphthalene (4) and standard octane (5) (30 mg L^{-1} from each compound). (b) Chromatogram after the biodegradation test: toluene (1) and naphthalene (4) were partially degraded, nonane (3) and *p*-xylene (2) were biodegraded by *Klebesiella sp*. Standard octane (5).

Table 1
Percentage of contaminants degradation by numbers of bacteria analyzed by HS-GC/MS

Degradation (%)	Toluene	*p*-Xylene	Nonane	Naphthalene
80–100	18	15	15	13
50–79	4	None	None	7
20–49	2	5	5	2
10–19	2	8	6	4
No degradation	7	5	7	7

The biodegradation percentage was calculated based on the difference between the concentration of samples and controls at the end of the experiment. For instance, according to the chromatogram (Fig. 3b), the level of biodegradation for toluene and naphthalene was calculated as 20–49% and for *p*-xylene and nonane as 80–100%. The level of biodegradation of toluene, *p*-xylene, nonane and naphthalene from 34 bacteria is shown on Table 1.

The analysis of 204 vials was performed in a few days. This was faster when compared to other techniques that require the volatile contaminants using extraction with organic solvents.

4. Conclusion

The results show that HS-GC/MS appears to be an efficient methodology for analyzing the level of volatile contaminants after biodegradation and for screening of hydrocarbon degrading bacteria. No potential interferences are introduced, minimizing the problems associated with the sample matrix. The vial temperature was the most important parameter that influenced the performance of the methodology. This method can be an important tool for obtaining a fast feedback about microbial degradation in a polluted area.

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References

[1] F. Solano-Serena, R. Marchal, T. Huet, J.-M. Lebeault, J.-P. Vandecasteele, Appl. Microbiol. Biotechnol. 54 (2000) 121.
[2] G. Witt, E. Trost, Chemosphere 38 (1999) 1603.
[3] C.W. Sweet, S.J. Vermette, Environ. Sci. Technol. 26 (1992) 165.
[4] T.A. Anderson, J.J. Beauchamp, B.T. Walton, J. Environ. Qual. 20 (1991) 420.
[5] G.A. Jungclaus, V. Lopeszavila, R.A. Hites, Environ. Sci. Technol. 12 (1978) 88.
[6] J.F. Leahy, R.R. Colwell, Microb. Rev. 54 (1990) 305.

[7] F. Dagher, E. Deziel, P. Lirette, J. Bisailon, R. Villemur, Can. J. Microbiol. 43 (1997) 368.
[8] U. Pagga, Chemosphere 35 (1997) 2953.
[9] J.D. Van Hamme, O.P. Ward, J. Ind. Microb. Biot. 25 (2000) 155.
[10] R.A. Miles, W.J. Doucette, Chemosphere 45 (2001) 1085.
[11] L.G. Whyte, L. Bourbonnière, C. Greer, Appl. Environ. Microbiol. 63 (1997) 3719.
[12] P. Kurán, L. Soják, J. Chromatogr. A 733 (1996) 119.
[13] A. Przyjazny, J.M. Kokosa, J. Chromatogr. A 977 (2002) 143.
[14] V.I. Safarova, S.V. Sapelnikova, E.V. Djazhenko, G.I. Teplova, G.F. Shajdulina, F.Kh. Kudasheva, J. Chromatogr. B 800 (2004) 325.
[15] T.C. Voice, Environ. Sci. Technol. 27 (1993) 709.
[16] B. Kolb, L.S. Ettre, Static Headspace-Gas Chromatography, Wiley-VCH, New York, 1997.
[17] S. Taniguchi, R.C. Montone, J. Bonetti Filho, C. Bonetti, Twenty-second Soc. Environ. Toxicol. Chem. 56 (2002) 131.
[18] K. Furukawa, F. Matsumura, K. Tonomura, Agric. Biol. Chem. 42 (1978) 543.
[19] J. Versolovic, T. Koeuth, J.R. Lupski, Nucleic Acids Res. 19 (1991) 6823.
[20] D.F. Rodrigues, S.K. Sakata, J.M. Tiedje, J.V. Comasseto, R. Vazoller, V.H. Pellizari, in preparation.
[21] B. Kolb, J. Chromatogr. A 842 (1999) 163.
[22] C.C. Camarasu, Chromatographia 56 (2002) 131.
[23] W.D. Weissenfels, M. Beyer, J. Klein, Appl. Environ. Microbiol. 32 (1990) 479.
[24] B.V. Chang, W.B. Wu, S.Y. Yuan, Chemosphere 35 (1997) 2807.
[25] D.L. McNally, J.R. Mihelcic, D.R. Lueking, Chemosphere 38 (1999) 1313.
[26] T. Hino, S. Nakanishi, T. Hobo, J. Chromatogr. A 746 (1996) 83.
[27] B.V. Ioffe, A.G. Vitenberg (Eds.), Headspace Analysis and Related Methods in Gas Chromatography, Wiley, New York, 1984.
[28] Y. Seto, J. Chromatogr. A 674 (1994) 25.
[29] M.H. Abraham, J. Andonian-Haftvan, G.S. Whiting, A. Leo, R.S. Taft, J. Chem. Soc. Perkin Trans. 2 (1994) 1777.
[30] R.T. Morrison, R.N. Boyd, Organic Chemistry, fourth ed., Allyn and Bacon Inc., 1983.