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Solid phase microextraction, sand flies, oviposition pheromones, plaster of Paris and siloxanes—What is in common?

Thais Marchi Goulart[a], Christiann Davis Tosta[b], Vicente Estevam Machado[c], Flávia Benini da Rocha Silva[c], Camila Feitosa de Castro[c], Dennys Ghenry Samillan Ortiz[a], Wanderson Henrique Cruz Oliveira[c], Mara Cristina Pinto[c,*]

[a] Universidade Estadual de Campinas (UNICAMP), Departamento de Zoologia Animal, Campinas, SP, Brazil
[b] Instituto Federal de São Paulo, Campus Matão, Matão, SP, Brazil
[c] Universidade Estadual Paulista (UNESP), Faculdade de Ciências Farmacêuticas, Araraquara, São Paulo, Brazil

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ABSTRACT

Sand flies are natural hosts of various microorganisms. Due to their epidemiological importance, sand fly colonies are kept in laboratories to be studied in terms of their biology and vector/host/parasite interactions. In order to investigate the presence of oviposition pheromones in *Nyssomyia neivai*, experiments using Solid Phase Microextraction (SPME) were performed. However, siloxanes which is an external class of contamination, present in breeding containers made by plaster used to maintain sand flies in colonies, may be hindered the experiments.

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1. Introduction

Solid Phase Microextraction (SPME) is a solvent-free method for the extraction of volatile and semi volatile compounds first used for detecting traces of organic compounds in liquids analyses (Pawliszyn, 1995; Horák et al., 2009). Nowadays it has been used to find sexual pheromones in many insects orders, as Lepidoptera (Frérot et al., 1997), Coleoptera (Fujiwara-Tsujii et al., 2012), Diptera (Farine et al., 2012) and Hemiptera (May-Concha et al., 2015). However, SPME for detecting oviposition pheromones in insects has been found only in Hemiptera (Verheggen et al., 2008).

Oviposition pheromones studies in Phlebotomine sand flies (Diptera: Psychodidae) have been restrict to *Lutzomyia longipalpis*, vector of visceral leishmaniasis. Experiments with *Lu. longipalpis*, using hexane extract, showed a positive response to eggs (Dougherty et al., 1993; Elnaiem and Ward, 1991) and latter on, dodecanoic acid was identified as the oviposition pheromone (Dougherty and Hamilton, 1997). Nevertheless, there are over 900 sand flies species identified without any other oviposition pheromone detected.

Nyssomyia neivai, is a species strongly incriminated to be a vector of *Leishmania (Viannia) braziliensis*, etiological agent of American cutaneous leishmaniasis (Córdoba-Lanús et al., 2006; Casanova et al., 2009; Marcondes et al., 2009) and some previous behaviour experiments in our laboratory (data not shown) indicated a greater oviposition rate where previous eggs were placed. Some experiments were performed to detect a possible oviposition pheromone present in this sand fly species. Solid Phase Microextraction (SPME) and gas chromatography were used but high peaks of a contaminant, siloxane, made the analysis unfeasible. After some attempts we suspect that the plaster of Paris, the base of the sand flies breeding pots, which are used in routine to breed sand flies in laboratory conditions (Modi and Tesh, 1983), might have been the contamination source. Our aim is report and discuss the non-viability of using this method in such specific context.

* Corresponding author at: Faculdade de Ciências Farmacêuticas, UNESP – Universidade Estadual Paulista, Araraquara, Departamento de Ciências Biológicas, Laboratório de Parasitologia, Rodovia Araraquara-Jaú km 01, 14801-902 Araraquara, SP, Brazil.
E-mail address: marap@fcfar.unesp.br (M.C. Pinto).

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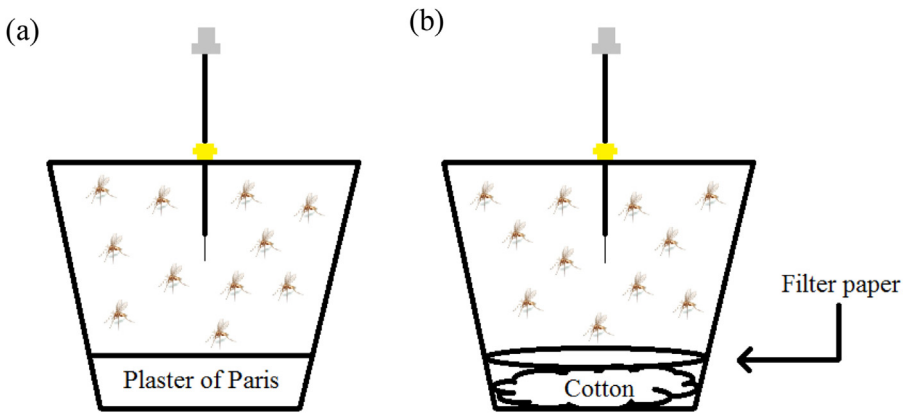

Fig. 1. Extractions using SPME methodology. Fiber in a pot with sand flies and plaster of Paris in the bottom as substrate (a), fiber in a pot with sand flies and cotton plus filter paper in the bottom as substrate (b).

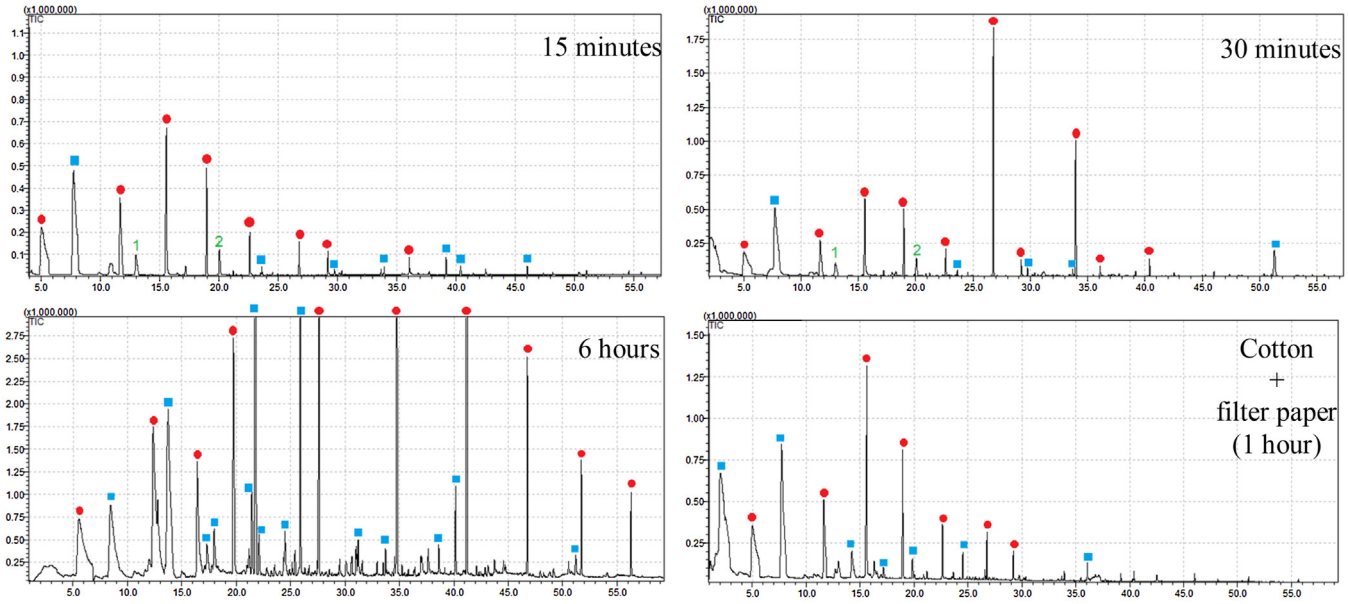

Fig. 2. Chromatograms of different times of extraction with plastic of Paris and extraction of the pot with cotton and filter paper in the bottom as a substrate, with all showing contamination of siloxanes (●). Peaks with low similarity or no identification are represented by (■), and peaks 1 and 2 were identified as 2-ethyl-1-hexanol and naphthalene, respectively.

2. Material and methods

Sand flies were obtained from a colony maintained at Biological Sciences Department of School of Pharmaceutical Sciences, Araraquara, São Paulo state, Brazil (Goulart et al., 2015).

All experiments were performed using SPME to extract possible oviposition volatiles.

2.1. First trial

The bottom of polystyrene tubes (15 mL) was covered with quick-drying plaster of Paris (Fortaleza®), a round piece of its cover was removed and a mesh was glued in the empty space. Sandflies were inserted into the vials and the top was closed. A piece of cotton soaked with 30% sugar solution was placed over the mesh to allow the insects to feed. A piece of Parafilm® paper was used to seal the top and prevent the volatiles to disperse. Three treatments were performed: a) three couples of sand flies – females without blood feeding; b) three couples – females after blood feeding; c) and empty vials (as blank control). After four days, time for oviposition, silica fibers (50/30 μm Divinylbenzene/Carboxen/Polydimethylsiloxane – DVB/CAR/PDMS – Sigma Aldrich®) were inserted to extract volatiles. The exposition time to extraction was 15, 30 and 45 min for the three treatments, in order to detect the better time. The fibers were inserted in Gas Chromatography–Mass Spectrometry (GC–MS, QP2010 Plus) for analysis on an ZB-5 column (30 m × 0.25 mm, 0.25 μm; temperature program: 50 °C for 3 min; 50–210 °C at 3 °C/min; 210 °C for 3 min) and the tests were carried out in triplicates (Fig. 1a).

Second trial

Considering the low number of eggs of the first experiment (49 eggs) the same procedure was repeated but at this time six couples were used and also the extraction time was increased to six hours (Fig. 1a).

Third trial

One thousand and two hundred eggs were placed inside a 2 mL vial. As a control, thin pieces of plaster of Paris, where the females laid the eggs, were transferred to another vial. Three fibers were

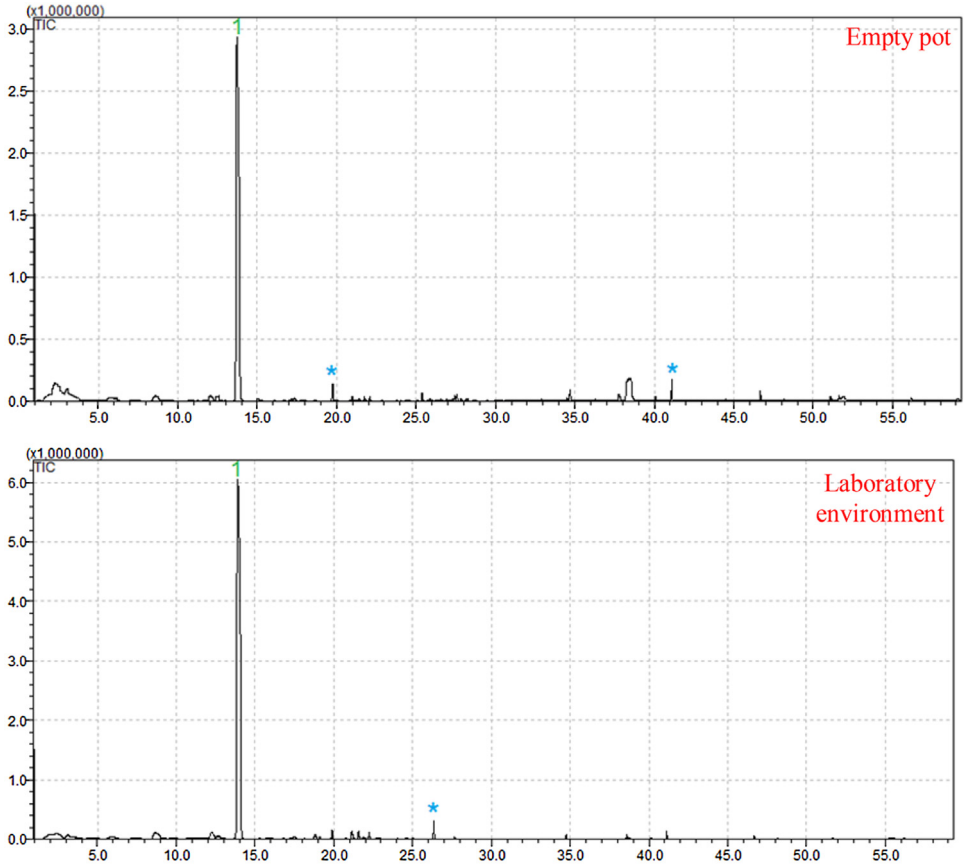

Fig. 3. Chromatograms of the extractions in an empty pot with no substrate and in the laboratory environment, showing no contamination of siloxanes. Peaks with low similarity or no identification are represented by *, and peak 1 was identified as limonene.

inserted in each vial for one hour and the analysis was carried out according to the first trial.

Fourth trial

Twenty bloodfed females were added inside a 150 mL polypropylene cup without plaster of Paris. Instead, it was used a moist thin layer of cotton and a sheet of filter paper in the bottom. A piece of cotton soaked with 30% sugar solution was placed over the mesh to allow the insects to feed. A piece of Parafilm® paper was used to seal the top. The same method was used on an empty cup as a control sample. Three fibers were inserted for 1 h and the analysis occurred as the first trial (Fig. 1b).

Results

As general results, in all experiments using plaster of Paris there was a great contamination mainly with siloxanes preventing the chromatography reading and maybe hiding possible volatiles lowest peaks. We illustrate the results with chromatograms of first and second experiments (Fig. 2). It is noticed that after 15 and 30 min of fibers expositions, beyond siloxanes other two peaks were also identified (2-ethyl-1-hexanol and naphthalene) whereas after 6 h both peaks were hidden by siloxanes and other contaminants.

In the fourth experiment, plaster of Paris was removed of breeding pots, and the peaks of siloxanes were decreased, but still present (Fig. 2).

For all trials, we analyzed blank controls only with plaster of Paris or cotton with filter paper. Contaminant compounds were present both in treatments and in blank control. We also evaluated one empty pot and laboratory environment (Fig. 3). In both situations siloxanes disappeared and only limonene was detected.

3. Discussion

Due to their epidemiological importance, sand fly colonies are kept in laboratories to be studied in terms of their biology and vector/host/parasite interactions. It is interesting to point out that despite the high number of sand flies described in Latin America, only in *Lu. longipalpis* oviposition pheromone has been properly identified using hexane extract (Dougherty and Hamilton, 1997). For another sand fly species, *Lutzomyia renei*, it has been shown a slight behaviour response of gravid females for hexane extract of eggs, but there was no progress on the identification of possible attractive compounds (Alves et al., 2003). This lack of studies and knowledge can be due to the difficulties in colonization of sand flies in laboratory or also because problems in extraction pheromone methodologies performed, which can present negative results.

Previous behavioural experiments carried out in our laboratory have suggested the presence of oviposition pheromone in *Ny. neivai*. Hence, our primary aim was to identify the possible attractive compounds from the eggs of *Ny. neivai*. We chose to use the SPME fibers instead of hexane extract to obtain volatiles organic compounds (VOCs) and avoid constituents compounds from the eggs. In a study performed by Farine et al. (2012) with sexual pheromones, the authors used SPME fibers to find the volatiles in the headspace of an experiment container using *Drosophila melanogaster* and hexane extract to identify compounds on the insect body.

A possible criticism of using SPME to extract oviposition pheromones is the low volatility of such compounds. Studies with

alcoholic beverages have shown that SPME is a powerful method to extract volatile and semivolatile compounds of different chemical classes like alcohol, esters, carbonyl compounds and fatty acids (Demyttenaere et al., 2003; Horák et al., 2009). In both studies, dodecanoic acid, the oviposition pheromone of *Lu. longipalpis*, was detected using SPME fibers, although in small peaks (Demyttenaere et al., 2003).

Our first results showed a great contamination of siloxanes. However, the presence of siloxanes both in the column (ZB-5) and in the SPME fiber, initially was assumed that might these material be the sources of the contamination. Finally, it was realized that siloxanes are present in silica, a common contamination of quick-drying plaster used in the base of the sand fly breeding pots (Kellum and Smith, 1967; Grabbe et al., 1995; Oliveira, 1997). We also evaluated other three plaster trademarks (Linhal®; Juntalíder® and Premium®) using SPME and siloxanes were detected in all of them (data not shown).

In an attempt to remove the contamination source, the fourth trial was carried out without plaster of Paris. In spite of the decrease of siloxane amount, the presence of this compound could still be detected. The hypothesis is that the contamination is still remained because quick-dry plaster of Paris adhered to the legs of the colony insects as they were reared in pots with plaster. In blank controls (empty pots and environment) siloxanes did not appear showing that the problem is not the fiber, the pot, the chromatography column or the environment.

An alternative to solve this problem is to keep insects without plaster of Paris, but until now the attempts have been unsuccessfully. This fact was also reported by Rangel et al. (1985).

4. Conclusions

Despite being a very accurate technique and capable of detecting fatty acid, our results suggest that the use of SPME to extract volatiles compounds in environment with plaster of Paris is not feasible. Considering that for sand flies, plaster of Paris is the best base to breed them in colonies, SPME is not indicated to extract oviposition pheromones in such conditions.

Competing interests

The authors declare that they have no competing interests.

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