Sensors and Actuators B 257 (2018) 200–206

Contents lists available at ScienceDirect

Sensors and Actuators B: Chemical

journal homepage: www.elsevier.com/locate/snb

Laser Vaporization e-Nose method for the detection of transmitter of Chagas disease

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ARTICLE INFO

Article history:
Received 7 March 2017
Received in revised form
29 September 2017
Accepted 25 October 2017

Keywords:
Laser vaporization e-nose
Triatomine specimens
Trace of smell
Chemometrics
Chagas desease

ABSTRACT

The aim of this work is to implement a laboratory analytical procedure based on the selective detection of the volatile compound with Laser Vaporization electronic nose technique (LV e-Nose), with the purpose of distinguish between triatomines of different sex and development stage, transmitters of *Trypanosoma cruzi*, agent of the Chagas disease (ECh). A chemometric method based on Principal Component Analysis (PCA) and Linear Discriminant Analysis (LDA) was performed in order to analyze the data coming from the e-Nose. The results allowed distinguishing between male, female and nympha stages, according to their volatile emissions. The proposed methodology resulted cost-effective, has good sensitivity, acceptability and improve the effectiveness. Although a greater number of samples must be analyzed, our results provide new interesting clues as a proof of concept to advance in the operational research for the surveillance of the ECh. Therefore, it could contribute to the sustainability and coverage of control programs in scenarios of the low density of vectors (interruption of transmission step).

1. Introduction

The sustainable control of Chagas disease (ECh) is still one of the major public health challenges. In Latin America, the ECh is endemic in 21 countries, and it is estimated that worldwide there are between 7 and 8 million people infected by *Trypanosoma cruzi* (the causative parasite of ECh). Triatomine (assassin bugs) are their vectors, and prevention depends critically on maintaining the households free of these insects. With a major effort of the endemic countries, since 1990 initiatives of South America, Central America, Andean Pact and Amazonian achieved unprecedented advances in the control of transmission by domestic vectors using insecticides [1]. However, against the certification of the interruption of the vectorial transmission by *Triatoma infestans* in Uruguay, Brazil and large areas of Paraguay, Bolivia, and Argentina, persistent insect populations present new challenges to maintain over time the achievements and prevent recolonization [2–5]. In chapter 16, Emerging Chagas Disease, 2009, 156–167, of Teixeira, et al. in his conclusion says textually: . . ..*we emphasize that control of vector-transmitted* T. cruzi *infections should rely initially on an information, education and communication program, which encourages control measures by the householder. For example, the* ***identification of triatomine*** *in the proximity the household and its elimination by cleaning and spraying with insecticide, the use of screens, bed nets, and vegetation management with conservation of local fauna, should be encouraged. Also, a program for preventing the human population from close contact with triatomines should be conducted directly in communities, elementary schools, churches and social clubs, reinforced by social marketing and mass media communications* [6].

The standardized method to assess the occurrence and abundance of triatomine is a manual collection at the fixed time (hour/man) helped by the use of irritants such as flushing-out chemicals. This method is expensive (periodic visits to every household), requires trained personnel, affects the privacy of individuals, is not homogeneous between operators [7], and does not detect persistent low population density after application of insecticides [8] until the insects reproduce again generating a risk of transmission [9]. In the current scenario, the interventions against Chagas disease had been successful in decreasing the density of the insect vectors in domestic units, by the actions of the Control Programs in many countries of Latin America. However, after these interventions with insecticides, still remain populations of few insects that are below of the sensitivity threshold of the detection strate-

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https://doi.org/10.1016/j.snb.2017.10.143
0925-4005/

gies. Therefore, in most of the ECh endemic countries and localities where *Triatoma infestans* is the insect vector, the control of transmission is achieved, but due to the low densities of the bugs the usual methodology of surveillance is unable to detect these small colonies of insects, and the scattered distribution of the infested households make this strategy also unaffordable. Further, the threshold of sensitivity of these regular detection methods only allows reporting the re-settlement of triatomine colonies when the risk of transmission of the ECh is already re-installed.

Hence, we propose to develop a new methodology of detection of high sensitiveness based on the LV e-Nose technique. The samples could be managed by the same householders, for instance, a paper fixed permanently in the wall that captures the volatiles of the triatomines living in the room. This papers will be replaced, collected or send periodically to a surveillance center with a portable LV e-Nose system, where in turn the detection of just a few new bugs in the house will be reported and the control performed before the risk of ECh appears again.

The idea relies on the detection of a mixture of volatile organic compounds as a trace of smell, emitted by the insect transmitters, which function as intra and interspecific signals from the glands, feces, and cuticle [10]. As the proof of concept, we are in the step of adjusting the tools to optimize the detection, and its capability to discriminate not only *Triatoma infestans*, but the sex of the adults or other stages of development. The bugs emit this "odor signatures" all the time during their lives in closed rooms, and so in the field it is expected that the volatiles reach the capture substrate during its dispersion, but for experimental standardized purposes we fixed the period of exposition in 168 h (although the periodicity of field collection for practical reasons should be at least monthly). On the other hand, once this step is completed, the validation and standardization in semi-experimental and field actual conditions will be tested, as the cost analysis of the scaling-up strategy in large areas compared with the current detection strategies.

According to bibliographic records the study of the composition of volatile compounds emitted by triatomine from glands, feces, and cuticle, and the behavioral response that generates between stages, sexes and species is more than thirty years old. (Hack et al. [11]; Cruz Lopez et al. [12]; Sanchez et al. [13]; Figueiras and Lazzari [14]; Vitta et al. [15]). Mixtures of acetic, isobutyric, caproic, propionic, butyric, isovaleric and valeric acids and isobutyl, isoamyl and amyl alcohol, 2-phenylethanol, and traces of other carboxylic acids and esters were identified as the defensive pheromone and intraspecific communication, while from the meta-sternal glands, 3-pentanone, 2-methylbutanol, and 3-pentanol were isolated. (Juarez & Brenner [16]; Seigler & Lampman [17]; Cruz Lopez et al. [18]; Rojas et al. [19], Manrique et al. [20]). The volatile emissions of adult *T. infestans* contain 18% of aliphatic acid, and 2-methylpropanoic acid, 30% of 2- and 3-methylbutyl esters, and 22% of 2-methylpropanoate (González Audino et al. [21]). And during the coupling they emit (R, S) −2- and 3-methylbutane-1-ol in a 2: 1 ratio; short chain acids (ethanoic to nonanoic acid); long chain acids (Z) −9-octadecenoic, aliphatic aldehydes (hexanal a nonanal), benzaldehyde and dipropylsulfoxide along with traces of non-acidic components (Fontan et al. [22]). Epicuticular lipids such as a hexacosanoic acid (C26: 0) had low dose aggregation effects and a high dose repellency effect (Figueras et al. [23]). The glands of other triatomine have also been analyzed and emitted isobutyric acid, and ketones and alcohols such as 3-pentanone, (4R) −methyl-1-heptanol, 3-pentanol, (2S) −methyl-1-butanol, 3,5 2-hexanol and 6-methyl-5-hepten-2-one (Seigler et al. [17]; Vitta et al. [24]; May-Concha et al. [25]).

The procedure for determining these substances is costly in terms of analytic instrumental and long-time execution.

In the present work, it is proposed a new analytical procedure based on the Laser Vaporization electronic nose technique which allows distinguishing between sexes and different stages of development of triatomine from the analysis of the mentioned volatile secretions. This approach is to look for an odour signature instead of the characterization of each compound.The proposed method provided to operational research on the diagnosis of epidemiological scenarios could result in a more effective, inexpensive and fast response alternative to the regular surveillance strategies aimed to interrupt the Chagas disease transmission.

Table 1
Identification of samples according with the development stage and exposure time.

Sample name	Exposure time (hours)	Stage
C	0 (not exposed)	Control
N30	30	Nympha
F30		Female
M30		Male
N72	72	Nympha
F72		Female
M72		Male
N168	168	Nympha
F168		Male
M168		Female

2. Materials and methods

The developed method contains two parts. The first one consisted in the sample preparation: the trace of smell from triatomine composed of a mixture of volatiles organic compounds was collected on filter paper according to the procedure described below. The second one corresponded to the measurement protocol established to realize the measurements on these papers. It is important to emphasize that the first one determined the development of the second one. Is worth mentioning that no damage was caused to the triatomine specimens used during handling.

2.1. Sample preparation

The samples were provided by the National Institute of Tropical Medicine (INMET) of Argentina and consisted of pieces of filter paper (Whatman® qualitative filter paper, Grade 3 circles, 90 mm diameter, pack) approximately 9 cm^2 of surface. *Triatoma infestans* individuals were divided by sex (male and female) and stage of development (nympha). The bugs were placed into glass vessels during different time periods at 26 +/− 1 °C in contact with filter paper and maintaining a photoperiod of 8 h light and 16 h dark during such periods. As indicated in Table 1 all three classes remained in contact with the filter paper for different periods of time. The pieces of filter paper were stored in bags and kept at room temperature to avoid their degradation and contamination. During all the analysis, sample handling was carried out with protective gloves to prevent contaminations.

2.2. Measurements conditions

In order to establish the measurement protocol a set of essays was realized. Since the e-Nose can use environmental air, synthetic air or nitrogen as reference gas, essays were realized under these three conditions. From the obtained results it was determined that the nitrogen was the best reference gas. This is due to the negligible moisture contents (<1 ppm), and by another side, the inert character and high purity of the nitrogen allow obtaining a highly stable baseline. In this sense, it is possible to guarantee the repeatability of the measurements.

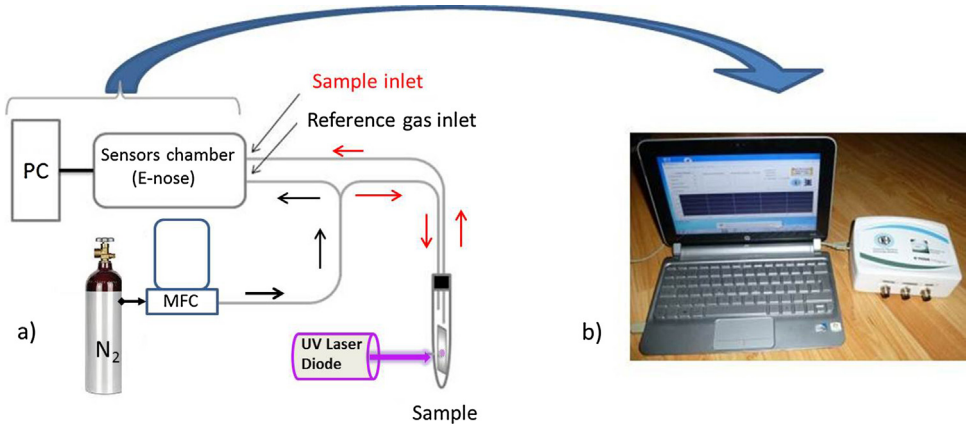

Fig. 1. a) Experimental set up. The red arrows indicate the sample flow and the black arrows, the reference gas flow, b) Model of E-nose developed in our lab. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.2.1. Set-up measurement

The system of measurement is shown in Fig. 1. As can be seen, it consists of a flow meter (5–200 cm^3/min) to control the N_2 flow, used as a carrier gas; a tube of glass Pyrex® transparent at the UV–vis laser radiation and an e-Nose with a laptop for the control and data acquisition. The system of the gas carrier is split into purge and sample channel. In this way, a correct cleaning of the detection system can be ensured and the memory effect on income of the sample channel is prevented. For each measurement, the glass tube was washed with pure N_2 during 4 min in order to avoid the mixture of the odor between different samples.

In order to realize the measurements, it was used the electronic nose (EN) "Patagonia" [26], shown in Fig. 1. Designed in our laboratories it is composed of an array of 6 SnO_2 commercial thin film sensors each of them responsive to a specific group of volatile compounds. During operation the sensors work at 380 °C, while the chamber containing them is heated to 43 °C allowing thermal stability. In this way, the humidity within the chamber can be controlled between 14 and 20%. This low content and short range of variation can improve the repeatability of measurements.

A continuous wave diode laser emitting radiation of 98 mW at 450 nm was used. According to a previous work by Rinaldi et al. [27], the irradiation at 450 nm produces not only volatilization but also a photochemical effect that probably cause a slight fragmentation of molecules.

2.2.2. Protocol measurement

Prior to the measurement, each sample was placed in a vial of 50 cm^3 and was left to stand for 1 h in order to saturate the headspace with the sample vapor. Ten different samples of each class were analyzed.

Using the electronic nose (EN) "Patagonia" described in Fig. 1 the following protocol was applied and in all cases, N_2 (99.999% from Indura) was used as a reference gas with a flow rate of 28 $cm^3\ min^{-1}$:

- The initial purge time was set in 60 s in order to clean the sensors chamber at the beginning of each set of training measurement.
- After the headspace stabilization, the closed vial was irradiated during one minute at 5 cm distance, focused on a spot of 1 mm in diameter on the filter paper. The sampling tube was moved constantly during the measurement time so that all the surface of the paper piece was irradiated in a homogeneous way. The vial headspace was subjected to a 15 s sampling to avoid the saturation of the sensor and to obtain a good signal desorption profile [26].
- The final purge time was set in 60 s in order to clean the sensors box chamber at the end of each set of measurements.

2.2.3. Multivariate data analysis

The discrimination ability of the method described above was analyzed. For the data processing, the absorption and desorption rates were taken into account in addition to the ratio of the peak value of each sensor response to the base-line value. Since different sensors have distinct desorption times, for each chemical compound, the integral of each signal between the maximum amplitude of the peak and the baseline in the desorption zone was considered. Subsequently, a group of pattern recognition algorithms was applied in order to analyze the LV e-nose response. Three methods of Multivariate Data Analysis were considered: I) Fisher Classifier, II) PCA and III) LDA. For each method, a linear classifier was implemented to separate the different areas (confidence regions) of the obtained graphs [28,29]. These methods are provided by the E-Nose-Pat version 1.01 software.

The approach adopted by Fisher Linear Discriminant is to find a linear combination of the variables that separates different classes as much as possible. That is, we seek the direction along which the different classes are best separated in some sense. The criterion proposed by Fisher is to maximize between-class separability and to minimize within-class variability [28,29].

Principal components analysis (PCA) is a projection method that permits a simple visualization of the information from a data set, often employed with gas sensor arrays. The sensor response features (response vectors) are grouped in a response matrix X that is expected to contain highly collinear variables. This collinearity means that the matrix X will have some dominating types of variability which carry most of the information. So, the aim of PCA is to express this information by a minor number of variables called principal components. The principal axes are those orthonormal axes onto which the remaining variances under projection are maximum [29,30].

Linear discriminant analysis (LDA) is a technique for dimensionality reduction. In LDA, the dimensional embeddings are reduced in such a way that the orientation of the projected data of classes on an arbitrary line or space is well-separated from each other. The method maximizes the variance between categories and minimizes the variance within categories. It is a statistical method that allows determining which group the samples belong to [29,30].

2.2.4. Cross validation

In order to validate the multivariate analysis, we have implemented cross validation with distinct combinations of algorithms: PCA, LDA, linear classifiers, K-NN classifier and neural network back

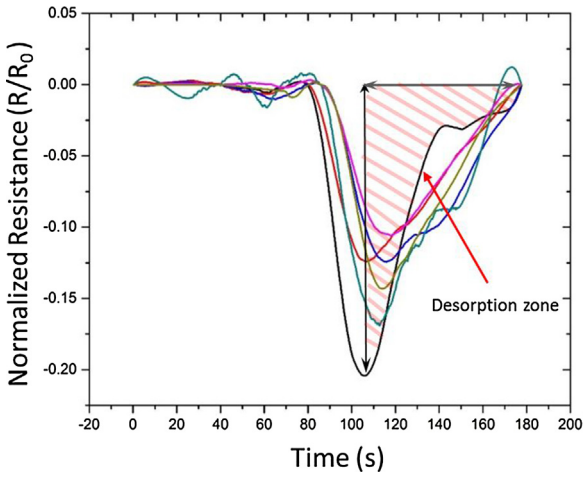

Fig. 2. A typical set of sensors signals. The dashed area indicates the desorption zone, taken for calculations.

propagation [29]. For validation purposes, we randomly left aside 20% of the measured samples. Within this restricted space, we performed different classifiers and the separate samples were tested. Then the percentage of correct classifications (CC%) was obtained. For statistical purposes, these calculations were repeated 15 times and expressed the mean value of CC%. See Table 3.

For linear classifiers, a linear discriminant function was used. It can arise through assumptions of normal distributions for the class densities, with equal covariance matrices [29]. A pattern classifier employing linear discriminant functions is termed a *linear machine*, an important special case of which is the *minimum-distance classifier*.

3. Results and discussion

Fig. 2 shows a typical set of sensors signals acquired by the device (electronic nose (EN) "Patagonia"). The signals are obtained as normalized resistance vs. time.

The desorption zone is indicated as a dashed zone. As can be observed each sensor has a different response.

3.1. Discrimination results

In order to distinguish the 3 stages (Female, Male, and Nympha) the trace of the smell of the insects as a function of the exposure time was analyzed. The result for an exposure of 72 h indicates that no discrimination of 3 stages was obtained. However, for a time of 168 h, it is possible clearly to separate the 3 groups. Fig. 3 shows the Fisher Linear Discriminant, PCA and LDA results for Female and Nympha stadium, and Fig. 4 the same analysis for Female and Male stadium samples exposed to 168 h. In these measurements, linear classifiers were used to separate the different groups. Satisfactory discrimination results were obtained with the three methods of Fisher, PCA, and LDA.

On the other hand, in order to study the capability of discrimination for each stage at different contact times of the bugs with the papers the same data processing methods than previously were applied. The results obtained can be seen in Fig. 5. in which satisfactory discrimination capability was obtained only in the case of the female specimens. This figure shows the Fisher Linear Discriminant, PCA and LDA results for Female samples exposed to 30, 72 and 168 h. According with the results obtained, the three methods give a satisfactory separation capability, being the LDA in combination with linear classifiers, the processing method that discriminates in optimal conditions as can be seen in graph III of Figs. 3–5.

Table 2
Silhouette average value for PCA and LDA methods.

Method	Average Silhouette value		
	Female, Nympha and Control groups at 168 h (Fig. 3)	Male, Female and Control groups at 168 h (Fig. 4)	Female groups at 30,72 and 168 h (Fig. 5)
PCA	0,781	0,781	0,659
LDA	0,909	0,893	0,852

Table 3
Correct classification values.

Method	Correct Classifications (CC%)		
	Control, Nympha and Female Samples at 168 h (Fig. 3)	Control, Male and Female Samples at 168 h (Fig. 4)	Female Samples (Fig. 5)
PCA and linear classifier	100%	98,8%	97,3%
LDA and linear classifier	98,6%	96,6%	94,6%
K-NN classifier (K = 1)	100%	100%	98,6%
PCA and Neural Network	98,6%	96,6%	94,6%
LDA and Neural Network	98,6%	98,8%	90,6%

In all studied cases, it can be observed that the female samples show the better discrimination capability and this behavior was obtained in different measurement days.

In order to quantify the discrimination ability of the methods the silhouette average value (from MatLab 8.0 Software) was calculated. This parameter measures the reliability of previously assigned clusters [31]. In the present case, samples are grouped into three clusters corresponding to female, male and nympha stage, and other three clusters correspond to female at 30, 72 and 168 h. The silhouette value is defined for the scoring plane as:

$$S(i) = \frac{(\min(b(i,k)) - a(i))}{\max(a(i), \min(b(i,k))}$$

Where $a(i)$ is the average distance from the ith point to the other points in this cluster, and $b(i,k)$ is the average distance from the ith point of cluster i to cluster k. The silhouette parameter ranges from −1 to +1. If the silhouette value is close to 1, it means that the sample is "well-clustered" and can thus be assigned to a particular cluster. If the silhouette value is around zero, it means that the sample can be assigned to another nearby cluster as well and that the sample lies equally far from both clusters. If the silhouette value is close to −1, it means that the sample is incorrectly classified. Table 2 show the silhouette average value for the different methods using squared Euclidean distance.

According to these results, the best discrimination method of these samples is performed by LDA.

3.2. Cross validation results

In order to establish if the different samples were correctly classified the percentage of Correct Classifications (CC%) was calculated for each of the three groups: 1) Female, Nympha, and Control at 168 h, 2) Male, Female and Control Samples at 168 h and 3) Female Samples at 30, 72 and 168 h. When the percentage of correct classifications is 100% it means that all test samples were correctly classified.The results are shown in Table 3.

The 100% CC value represents a correct classification of all test samples in all replicates performed. By analyzing Table 3, with the K-NN classifier in which K = 1, the best results were obtained, as well as with the other combinations of algorithms in all the cases good results were obtained.

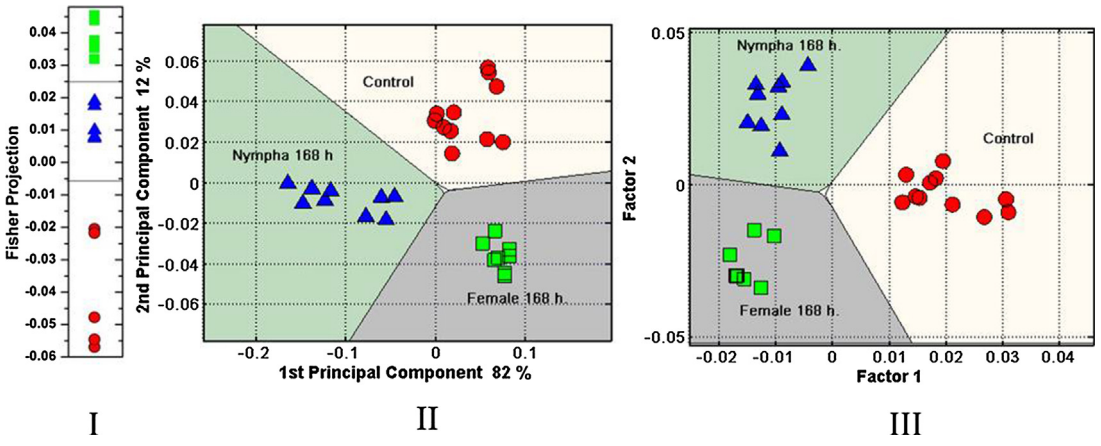

Fig. 3. Results for samples at 168 h with Fisher (I), PCA (II) and LDA (III) Samples: (●) Control, (▲) Nympha and (■) Female.

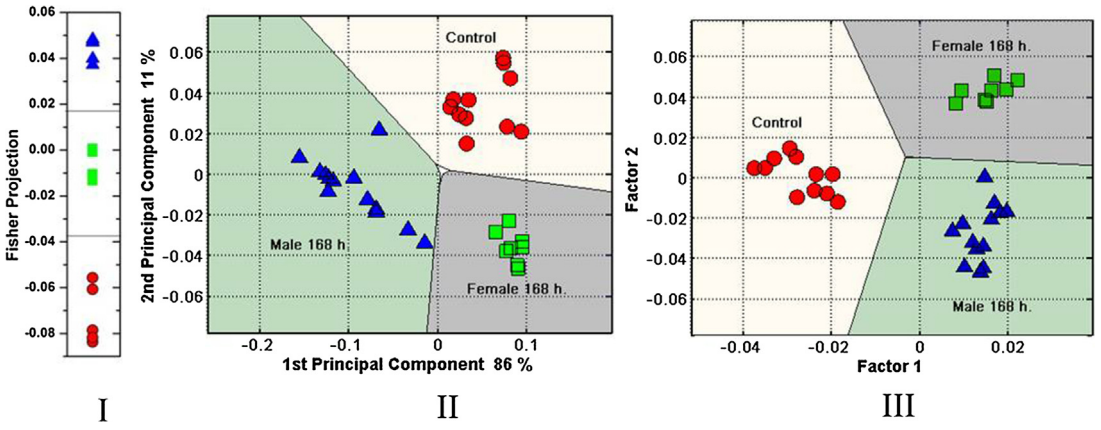

Fig. 4. Results for samples at 168 h with Fisher (I), PCA (II) and LDA (III). Samples: (●) Control, (▲) Male and (■) Female.

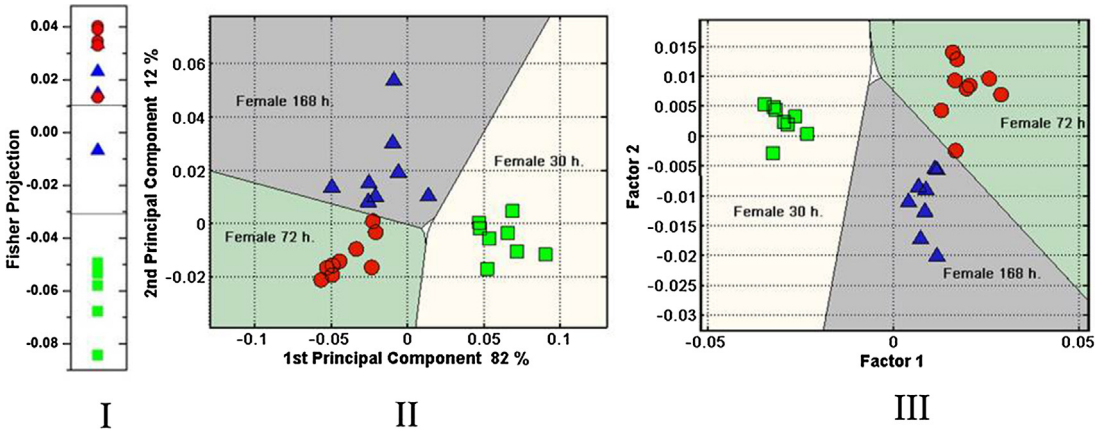

Fig. 5. Results for Female samples with Fisher (I), PCA (II) and LDA (III). Samples: exposed to (■) 30, (●) 72 and (▲) 168 h.

4. Conclusions

The LV e-Nose methodology provided to be adequate to classify nympha, male and female *Triatoma infestans* specimens for exposure times greater than 72 h. Also, the best exposure time to classify the samples was established in 168 h. For the case of female samples, it was possible to discriminate between 30, 72 and 168 h of exposure time. This fact is because of the type of the different organic compounds emitted by the female species, compared with male and nympha stage.

However, the time is fixed just for standardization experimental purposes, as in the actual use in the field it is expected to work as a passive tool that analyze the volatiles adsorbed to an exposed matrix exposed for not least than a month.

Then, in this way, a simple and low cost method of detection of the transmitter of Chagas disease was implemented using an electronic nose for the first time. This represents a potential application in non-lab conditions for health and control of disease transmitted for local and international use.

This study provides a novel look in terms of being able to investigate in the future the differences between infected or healthy individuals from a quick and low cost analysis.

In order to extend the study, a complete analysis must be performed with a big number of samples of each of the categories of individuals of the main species that are transmitters of ECh using different wavelength laser irradiation. A new set of experiments will be developed with the purpose of testing this technology under semi-field conditions and establish the validity of the method. These new experiments are needed to verify the effects of natural interferents that could give false positives.

On the other hand, once this step is completed, the validation and standardization in semi-experimental and field actual conditions will be tested, as the cost analysis of the scaling-up strategy in large areas compared with the current detection strategies.

Acknowledgements

The authors gratefully acknowledge financial support from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), the University of San Martín, Buenos Aires, and the Instituto Nacional de Medicina Tropical (INMET) from Argentina.

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