Journal of Chromatography B, 875 (2008) 392–398

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Headspace solid phase microextraction/gas chromatography–mass spectrometry combined to chemometric analysis for volatile organic compounds determination in canine hair: A new tool to detect dog contamination by visceral leishmaniasis

Lidia S. de Oliveira[a], Frederico de M. Rodrigues[a,b], Fabio S. de Oliveira[d], Paulo R.R. Mesquita[a], Danielle C. Leal[c], Adriano C. Alcântara[c], Barbara M. Souza[c], Carlos R. Franke[c], Pedro. A. de P. Pereira[a], Jailson B. de Andrade[a,*]

[a] *Instituto de Química, Universidade Federal da Bahia, Campus Universitário de Ondina, 40170-290 Salvador, BA, Brazil*
[b] *Empresa Baiana de Desenvolvimento Agrícola S.A. (EBDA), Salvador, BA, Brazil*
[c] *Escola de Veterinária, Universidade Federal da Bahia, Campus Universitário de Ondina, Salvador, BA, Brazil*
[d] *Centro de Ciências da Saúde, Universidade Federal do Recôncavo da Bahia, Cajueiro, Santo Antonio de Jesus, BA, Brazil*

ARTICLE INFO

Article history:
Received 18 March 2008
Accepted 19 September 2008
Available online 2 October 2008

Keywords:
Canine hair
VOCs
HS-SPME/GC–MS
Multivariate optimization
PCA
Diagnosis
Visceral leishmaniasis

ABSTRACT

A new analytical methodology using HS-SPME/GC–MS was optimized in order to attain maximum sensitivity, using multivariate strategies. The proposed method was employed to evaluate the VOC profile exhaled from canine hair samples collected from 8 healthy dogs and from 16 dogs infected by *Leishmania infantum*. 274 VOCs were detected, which could be identified as aldehydes, ketones and hydrocarbons. After application of the Soft Independent Modeling of Class Analogy (SIMCA) and Principal Component Analysis (PCA) healthy and infected dogs, with similar VOCs profiles, could be separately grouped, based on compounds such as 2-hexanone, benzaldehyde, and 2,4-nonadienal. The proposed method is non-invasive, painless, readily accepted by dog owners and could be useful to identify several biomarkers with applications in the diagnosis of diseases.

1. Introduction

Visceral leishmaniosis (VL) represents a serious problem of public health, especially due to the gravity of the clinical presentation of the illness and to the raised lethality registered in the absence of treatment [1]. The illness occurs in 65 countries, and about 90% of the human casuistry concentrated in the agricultural and suburban zones of Bangladesh, India, Nepal, Sudan and Brazil [2]. Presently, it is becoming enlarged in great urban centers [3–5].

Dogs play an important role in the illness cycle, and they are considered the main source of infection for the *Lutzomyia longipalpis*, vector of disease, in the domiciliary and peridomiciliary cycle [6,7]. It has been observed that in the case of VL, the vectors are more attracted to feed in infected animals than in healthy animals [8,9]. As the main orientation mechanism for insects is based on the recognition of odors [8], this preference is possibly related to the different odors exhaled by the infected animals.

It is well known that illnesses can modify odors exhaled by individuals [10]. Thus, techniques for detection and identification of volatile compounds have been used in the diagnosis of illness, with the advantage to be less invasive and painless [11]. The applications of these techniques have shown useful for several pathologies, such as breast cancer [11], pulmonary cancer [12] and diabetes [13]. In this context, VOCs emitted by canine hair can be representative of the compounds exhaled through dog skin [14]. Thus, this type of sample is easy to collect and therefore acceptable to dog owners.

VOCs potentially related to canine odors are expected to be present at trace levels. To improve the sensitivity and precision in the determination of these compounds, samples must be concentrated before gas chromatography/mass spectrometry analysis. Different analytical methods have been developed to extract the VOC, such as solvent extraction, distillation [15,16], dynamic headspace and static headspace [17,18]. However, some of them are laborious, time-consuming and expensive and may lead to erroneous conclusions about the VOC profile. Therefore, an ideal sample preparation technique should be simple, fast, solvent-free, inexpensive and compatible with a wide range of analytical instruments [19].

* Corresponding author.
E-mail address: jailsong@ufba.br (J.B. de Andrade).

1570-0232/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2008.09.028

Headspace solid phase microextraction (HS-SPME) seems to be one that meets most requisites for sample preparation steps to analyze VOCs in dog hair samples [20,21], although, conditions such as type of fiber employed, the extraction time and temperature, the amount of sample and the desorption time and temperature [22,23] must be optimized in order to attain maximum sensitivity [24,25]. On the other hand, multivariate analysis by PCA has been applied successfully, as an approach to detect tendencies and relationships [22]. Thus, the combination of HS-SPME and PCA can be a powerful tool to differentiate healthy dogs from those ones infected.

Soft Independent Modeling of Class Analogy (SIMCA) [26–30] is a well known multivariate supervised pattern recognition method based on Principal Component Analysis (PCA) [31,32] that allows data classification. This method requires a training data in which several distinct measurements (i.e. chromatographic areas of different peaks, absorbance at different wavelength) were carried out for samples belonging to each evaluated class. Once classification rules based on training sets for each evaluated class are obtained the classification of unknown samples could be accomplished.

For a given class the mean distance of training data samples from hyper-plane defined by PCA is compared with the distance of unknown samples and the critical value to evaluate if these unknown samples belong to this particular class is based on *F*-distribution usually at 95% confidence level. Mathematical details of SIMCA could be found elsewhere [26,27].

Then, the aim of this study was to develop an analytical methodology based on HS-SPME/GC–MS to identify and evaluate the profile of volatile compounds in canine hair. This methodology was then applied together with PCA and SIMCA, in order to evaluate the grouping tendencies of healthy and infected dogs with similar VOCs profiles, allowing the identification the substances which could be used as indicative of infection by visceral leishmaniasis.

2. Materials and methods

2.1. Diagnostic of visceral leishmaniasis

A total of 41 dogs in the range of 1–8 years old were studied. Blood (3 ml) was collected from each dog, through venopunction of cephalic or jugular veins. Of this, 2 ml was separated into a tube without EDTA, and the serum obtained by centrifugation was stored under refrigeration −20 °C for ELISA test [33]. The volume remaining was put into a tube containing EDTA for subsequent extraction of DNA and implementation of the PCR [34]. Based on these tests, two groups could be separate: a first one with 18 healthy dogs and a second one with 23 dogs infected.

2.2. Sample collection

The hair sample's length ranged from 1 to 3 cm. The samples were collected by cutting hair, close to the skin of dogs that had not been washed for at least 15 days before sampling. The hair samples were stored in sealed plastic bags under refrigeration.

2.3. Reagents

Standard solution of hydrocarbons, C_9–C_{24} (DRH-008S-R2-PAK, AccuStandard, New Haven) and carbonyl compounds (2-hexanone, 2-octanone, 2-nonanone, 2-decanone, benzaldehyde, hexanal, heptanal, octanal, nonanal, decanal, trans-2-heptenal, trans-2-octenal and trans-trans-2,4-nonadienal) were acquired from Sigma–Aldrich, USA.

2.4. Solid phase microextraction

A mass of hair samples, ranging from 30 mg to 130 mg, was put in sealed 20 ml glass vials for VOC headspace microextraction. The vial was then inserted into an aluminum heating block (4 cm in height by 14 cm in diameter) placed onto a temperature controlled hot plate. The SPME extractions were carried out using polydimethylsiloxane divinylbenzene fiber (PDMS-DVB 65 μm, Supelco, Bellefonte) with a manual holder. This fiber presents an intermediate polarity and when compared to the polydimethylsiloxane (PDMS) and the polyacrilate (PA), exhibited a higher efficiency to extract VOC from canine hair. Following VOC extraction and pre-concentration, the fiber was then inserted directly into the GC injector.

2.5. GC–MS analysis

The analyses were done in a GC–MS system (Shimadzu GC-2010/QP-2010 high performance quadrupole, Japan) under the following instrumental conditions:

- Column: HP-5 MS (30 m × 0.25 mm i.d. × 1.00 μm, Agilent, Palo Alto).
- He flow rate: 0.69 ml min^{-1}.
- Oven temperature program: 40 °C (1 min); 4 °C min^{-1} up to 140 °C; 140 °C for 3 min; 8 °C min^{-1} up to 240 °C; 240 °C for 3.5 min.
- Injector mode and temperature: split, 240 °C.
- Split ratio: 1:5.
- Source temperature: 250 °C.
- Transfer line temperature: 250 °C.
- Energy of impact: 70 eV.

2.6. HS–SPME multivariate optimization

Headspace solid phase microextraction efficiency can be related with parameters such as fiber material, temperature, extraction time interval, sample amount, desorption time and headspace equilibrium time. In this way optimization of microextraction condition is a multiparameter evaluation task that can be overcome by multivariate techniques.

In order to identify relevant parameters that contribute to the sensibility of the proposed method a factorial screening design was carried out. In an initial step, using canine hair of healthy dogs, a screening 2^{5-2} fractional factorial design, using linear multivariate regression model [21–23] was applied to evaluate significant variables involved in HS-SPME. Three replications were performed in the central point of the factorial design in order to quantify the experimental error and then 11 experiments were carried out. The variables evaluated by screening the experimental design were canine hair mass, extraction temperature, equilibrium time, extraction time, and desorption time. The levels employed in these experiments are listed in Table 1 and were chosen after preliminary

Table 1
Experimental levels employed in the screening design to evaluate the 1 extraction method applied to selected VOC in canine hair.

Variable	Coded variable		
	(−1)	(0)	(+1)
Extraction temperature, °C	40	60	80
Extraction time, min	15	30	45
Desorption time, min	1	3	5
Hair mass, mg	30	50	70
Equilibrium time, min	10	20	30

Table 2
Experimental levels employed in the central composite design to evaluate the extraction method applied to selected VOC in canine hair.

Variable	Coded variable		
	(−1)	(0)	(+1)
Extraction temperature, °C	70	80	90
Extraction time, min	25	40	55
Hair mass, mg	90	110	130

experimental studies. The responses evaluated during the experiments were the total sum of peak areas obtained in the GC–MS analysis.

After the significant variables related to these compounds were retained, a central composite design was built using the experimental levels of extraction time and extraction temperature that yielded the best response in factorial design as the central point. Five replicates were performed at the central point to estimate the experimental error and detect lack of fit resulting in 19 experiments. The levels employed for central composite design are described in Table 2. The response surface methodology was applied to locate the optimum values of significant variables.

A quadratic multivariate regression model with interaction terms was built based on this central composite design and it did not showed lack of fit by ANOVA at 95% confidence level.

The statistical experimental designs and optimization calculations were carried out using the Statistica 7.0 software (Statsoft, USA).

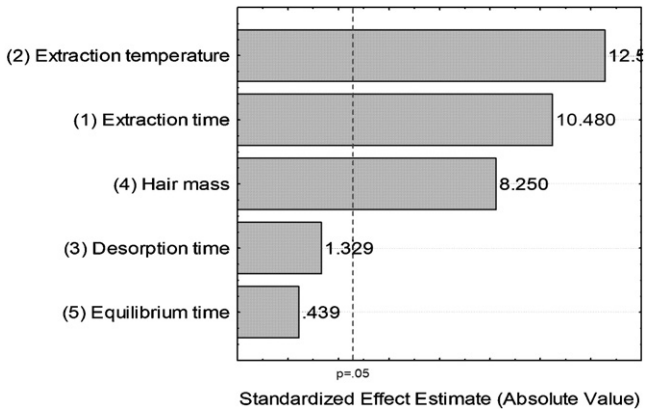

Fig. 1. Pareto chart of effects for fractional factorial design for the evaluation of selected VOC in canine hair.

2.7. Chemometrics analysis

An initial screening analysis was accomplished by PCA using a set of 23 samples from dogs positively diagnosed as infected by leishmaniasis and 18 samples from dogs negatively diagnosed as infected by leishmaniasis to allow visual inspection of this data set. Data preprocessing was preceded by autoscaling [32].

In this work, the employment of SIMCA [26–30] to identify if dogs were infected with leishmaniosis, was proposed based on the areas of 24 chromatographic peaks of volatile organic compounds

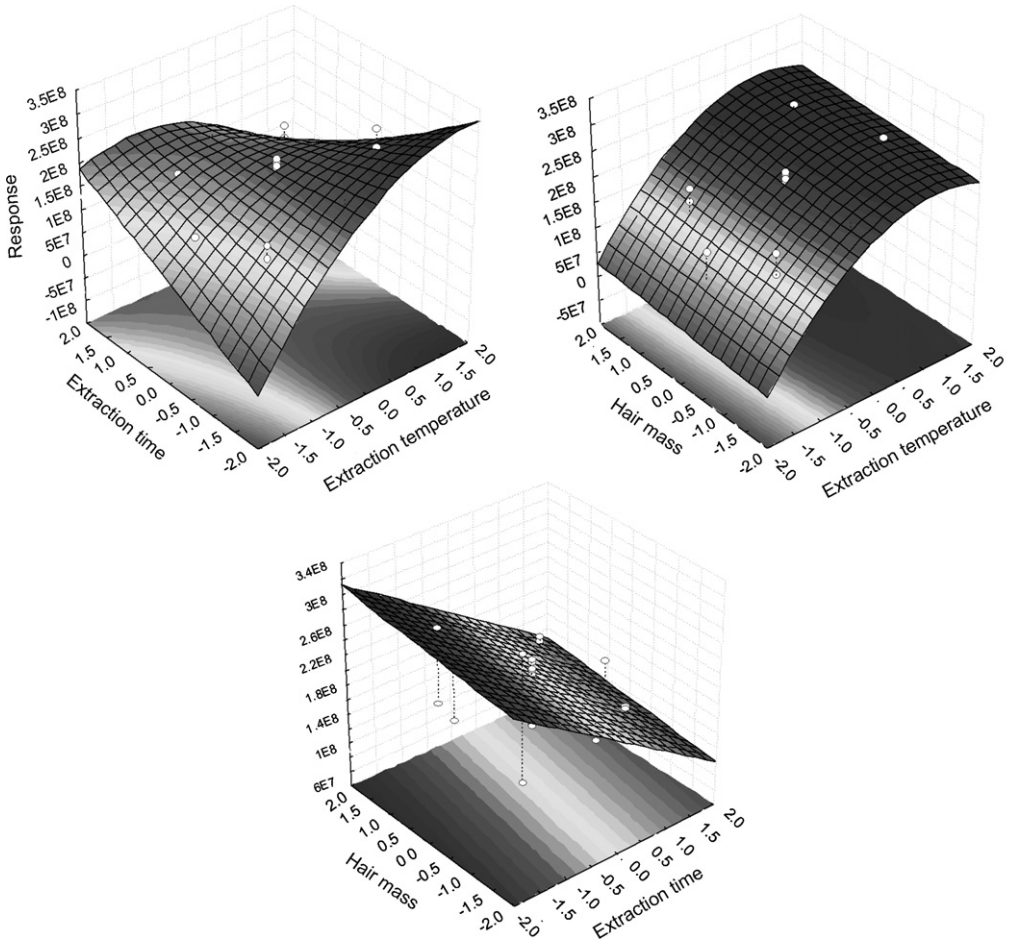

Fig. 2. Response surface obtained by central composite for the evaluation of VOC in canine hair.

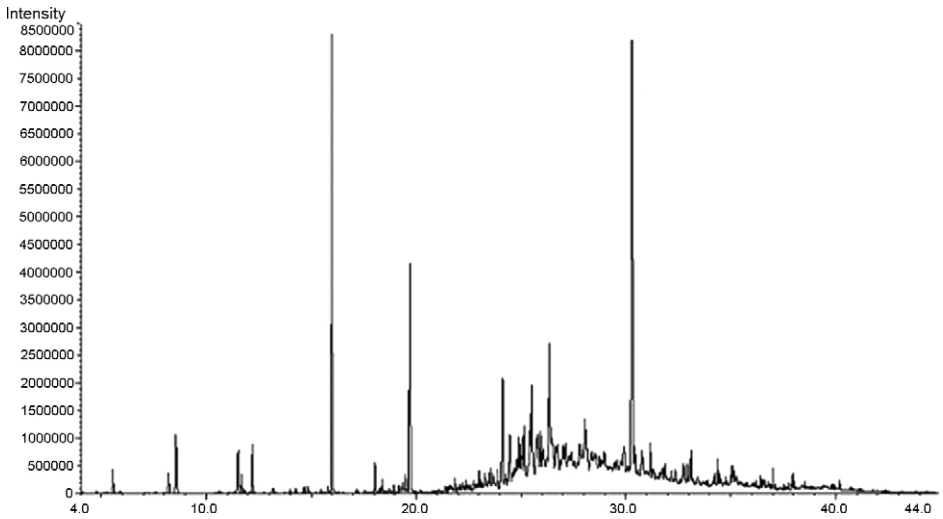

Fig. 3. Chromatograms obtained by HS-SPME/GC–MS analysis of canine hair samples, for all VOCs. The experimental conditions are presented in Section 2.

detected in the dogs hair. The sample set was randomly splitted in 14 infected dogs and 11 negative infected dogs for training sets, while 9 infected and 7 non-infected dogs were taken for an independent test set. For the independent test set three replicates were recorded, and thus a total of 27 data point related to infected dogs and 21 data point related to non-infected dogs were processed.

3. Results and discussion

3.1. Optimization of the SPME conditions

The results obtained from the evaluation of significant parameters by screening factorial design are summarized in the Pareto chart of effects depicted in Fig. 1. As can be seen, the hair mass, extraction time and temperature were significant at a 95% confidence level. Once relevant variables were detected by the factorial design, further experiments were carried out keeping non-significant variables such as desorption time at 3 min and equilibrium time at 30 min.

A response surface obtained by central composite design is illustrated in Fig. 2. It must be highlighted that the graphs showed in that figure describe part of the overall four-dimensional response surface. The levels of hair mass in the central composite design were higher than in previous screening design (Table 1) due to its lower effect value pointed out in Pareto chart of effects in comparison with other retained variable (Fig. 1). The results obtained with this central composite design indicated that greater hair mass and extraction temperature and lower extraction time would lead to an increase in the analytical response. Since a saddle shaped response surface was obtained, a maximum point was not located and the optimized conditions were elected as those ones that lead to a greater overall sensitivity (extraction temperature = 90 °C; extraction time = 18 min; hair mass = 130 mg). The quadratic equation obtained using coded values for the variable is given by $R = (2.08 \pm 0.07)10^8 + (2.2 \pm 0.6)10^7 T - (2.3 \pm 0.6)10^7 T^2 - (1.2 \pm 0.6)10^7 t + (6.7 \pm 0.6)10^6 m - (3.4 \pm 0.8)10^7 T \times t$, where R means the response, T the extraction temperature, t the extraction time, m the canine hair mass, and $T \times t$ is the interaction between desorption temperature and time.

Fig. 3 illustrates a chromatogram obtained by HS-SPME/GC–MS under optimized conditions for the evaluation of all VOC in canine hair samples. These conditions allowed the detection of 274 chromatographic peaks of VOC from canine hair samples. Of these, 24 were unequivocally identified, based on comparisons made with mass spectra from NIST electronic library and retention times of standards as follow: pentadecane, hexadecane, 2-hexanone, 2-decanone, dodecane, 2-octanone, 2-nonanone, eicosane, 2-octenal, benzaldeide, tridecane, 2-heptenal, heneicosane, nonadecane, heptanal, 2,4-nonadienal, pentadecane, nonanal, decanal, octadecane, heptadecane, tetradecane, hexanal and octanal.

3.2. Leishmaniasis identification by multivariate analysis

The PCA was performed in order to identify grouping tendencies of leishmaniasis infected and non-infected dogs. The results of PCA for the whole data set is depicted in the score graph of first and third principal components (Fig. 4) and this pair of PCs was chosen due to better visual grouping tendencies. Nine principal components explained 91, 34% of variance for this data set. The score graph (PC1 × PC3) illustrated in Fig. 4 evidence group tendencies between leishmaniasis infected dogs and non-infected dogs.

For SIMCA classification, one distinct PCA model was built for each dog group (leishmaniasis infected and non-infected) using the chromatographic peak areas of the 24 volatile organic compounds

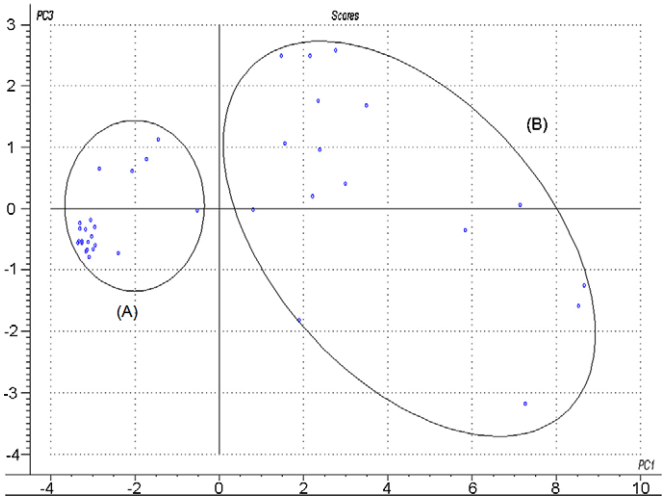

Fig. 4. PCA scores graph based on chromatographic peak areas of VOC of the hair of leishmaniasis infected (A) and non-infected dogs (B).

Table 3
Performance of PCA models used by SIMCA for identification of leishmaniasis infected and non-infected dogs using VOC chromatographic profiles.

PCA model	Number of PCs	Explaned variance (%)
Leishmaniasis infected	10	92.5
Non-infected	8	91.8

previously identified, as described in Section 3.1 above. The parameters that describe the performance of each of these PCA models are listed in Table 3.

The results obtained by SIMCA classification analysis for the test set samples can be visualized by sample to model distance ratio versus leverage graph (Fig. 5) [26,27,32] where each data point represent a particular dog sample. The upper limits for sample to model distance and for leverage are represented by horizontal and vertical lines, respectively.

Fig. 5A refers to SIMCA analysis using infected dogs classification model while Fig. 5B is related to non-infected dogs classification model. SIMCA classification rules points out that each data points contained into the lower left quadrant of model defined by sample to distance ratio and leverage upper limits lines was classified to belong to the class in evaluation at 95% confidence level. In other words, for Fig. 5A all data points containded into this lower left quadrant were defined by SIMCA as infected dog samples. In a

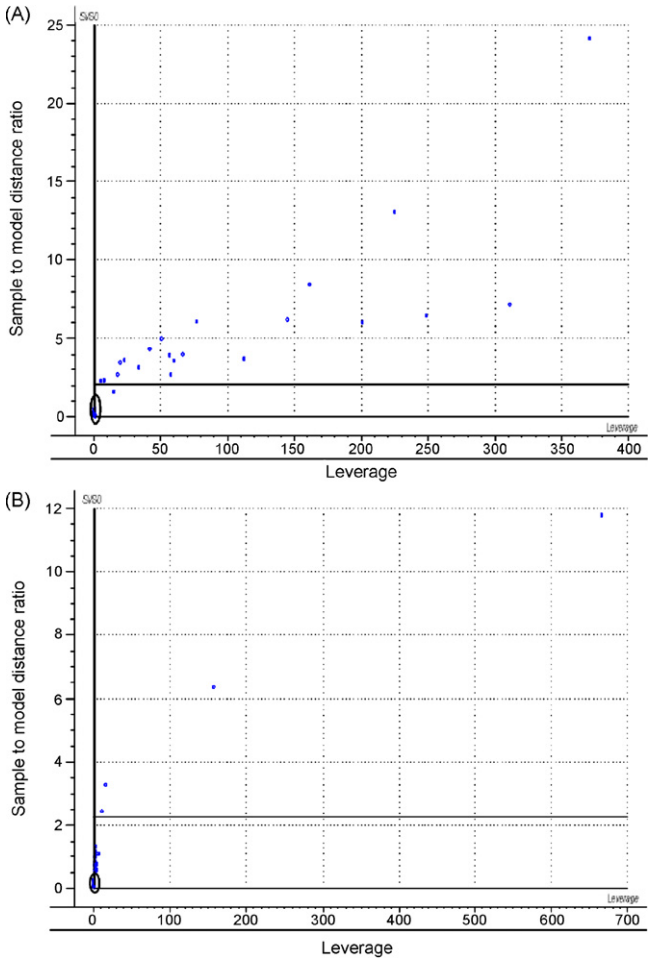

Fig. 5. SIMCA classification results of model distance ratio vs. leverage for (A) of leishmaniasis infected class and (B) non-infected dogs for the independent data set. Data points that were circled represent leishmaniasis infected dogs (A) and non-infected dogs (B).

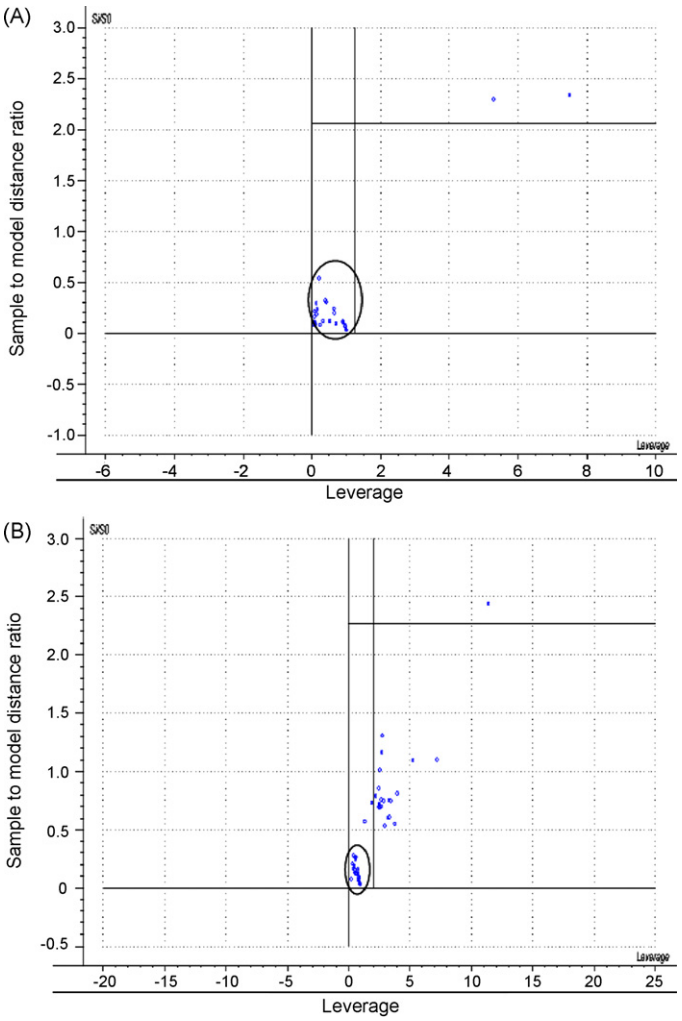

Fig. 6. Zoom of Fig. 5. Data points that were circled represent leishmaniasis infected dogs (A) and non-infected dogs (B).

similar way, for Fig. 5B all samples contained into this lower left quadrant were discriminated as non-infected dogs. Fig. 5 shows excellent performance of SIMCA associated with chromatographic data of canine hair VOC to identify leishmaniasis infected and non-infected dogs, since all infected dogs samples (highlited by a circle in Fig. 5A) were correctely classified as well as non-infected dogs samples (highlited by a circle in Fig. 5B). A closer view of Fig. 5 is depicted in Fig. 6 where the limits for sample to model distance ratio and leverage at 95% confidence level can be better visualized.

The SIMCA errors in classification could be of two types: type I (object not included in its own class) and type II (object included in a wrong class). Table 4 summarizes the error in classification of dogs' samples of the independent test set for proposed SIMCA model. As can be verified in Table 4 type I error (object not included in its own class) was not observed but few cases of type II error (sample classified in the wrong class) were recorded for leishmaniasis infected

Table 4
Percentile error in classification by SIMCA for test set.

	Error (%)	
	Type I	Type II
Leishmaniasis infected	0	4.2
Non-infected	0	0

Table 5
Volatile organic compounds evaluated by multivariate analysis.

Number	Compound	Number	Compound
1	2-Hexanone	13	Dodecane
2	Hexanal	14	Decanal
3	Heptanal	15	2,4-Nonadienal
4	2-Heptenal, (E)-	16	Tridecane
5	Benzaldehyde	17	Tetradecane
6	2-Octanone	18	Pentadecane
7	Octanal	19	Hexadecane
8	2-Octenal, (E)-	20	Heptadecane
9	2-Nonanone	21	Octadecane
10	Undecane	22	Nonadecane
11	Nonanal	23	Eicosane
12	2-Decanone	24	Heneicosane

class. In other words, two samples (i.e. 4.2% of test set samples) of non-infected dogs test set were wrongly pointed out as leishmaniasis infected by SIMCA, as can be seen in Fig. 6. A closer examination revealed that these wrongly classified data points were related with single replicates of two distinct dogs' samples but two other replicates of these samples were correctly classified. In this way, SIMCA classification errors for this independent test set seen to be tolerable.

The SIMCA classification model also provides parameter that could identify the volatile organic compounds that presented higher contribution to discriminate leishmaniasis infected and non-infected dogs. The 24 volatile organic compounds employed for SIMCA classification model are listed in Table 5. Discrimination power [26] plot (Fig. 7) shows how much chromatographic peak areas of each evaluated volatile organic compound contributed to separate leishmaniasis infected and non-infected dogs classes. Since discrimination power values higher than 3 indicates an important variable [26], all evaluated VOC were significant to discrimination process. The three VOC with the greatest discrimination power values were benzaldehyde, 2-hexanone and 2,4-nonadienal (Fig. 7) but the other ones are also significant to discriminate leishmaniasis infected and non-infected dogs.

VOC emissions were already related to odor modifications due to infections [35,36]. The odor modification in a host, infected by parasites transmitted by hematophagous mosquitoes, could be related to the parasite survive strategy [4,9]. It was reported that dogs and hamsters infected by VL seen to be more attractive to diseases transmitter vectors [4,9].

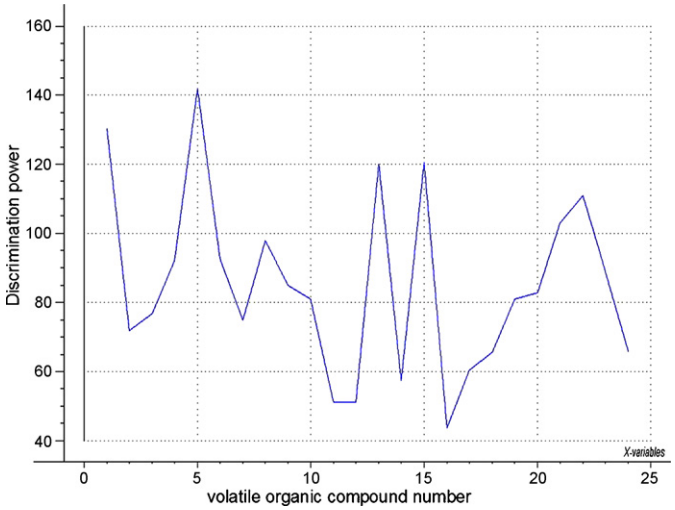

Fig. 7. Discrimination power values for 24 VOC evaluated in canine hair samples obtained by SIMCA.

Some of the compounds identified in the present study were already recognized as attractive to hematophagous mosquitoes. Tetradecane is one of the main exhaled compounds from chicken and it is a potential attractive to *Culex annulirostris*, a vector of diseases such as Ross River fever, Barman forest virus, Kunjin virus and Murray Valley encephalitis [37]. Nonanal elucidated the electrophysiologic and behavior responses of *Culex quinquefasciatus*, human philariosys vector [38], while 2-hexanone, benzaldehyde and hexanal were reported as important compounds in *L. longipalpis* orientation [39] and tridecane to present repellent proprieties to *Anoplolepis longipalpis*, *Sitotroga cerealella* and *C. quinquefasciatus* [40].

4. Conclusions

The strategy of multivariate experimental design allowed the identification of the most significant parameters relating to the overall sensitivity and the optimization of experimental conditions for the proposed headspace SPME sampling method with a small number of experiments. The HS-SPME method, coupled with GC–MS analysis, successfully separated more than 250 volatile organic compounds in canine hair, according to the following parameters: polydimethylsiloxane divinylbenzene fiber (PDMS-DVB 65 μm, Supelco, Bellefonte); extraction time 18 min; extraction temperature 90 °C; hair mass 130 mg; desorption temperature 240 °C; and desorption time 1 min. SIMCA and PCA provided visual differences of VOC profiles from dogs infected with *Leishmania infantum* and healthy dogs.

The proposed method is non-invasive, painless, readily accepted by dog owners and could be useful to identify several biomarkers with applications such as in the diagnosis of diseases. This methodology is now in use in our research group in order to study leishmaniosis biomarkers.

Finally, these results provide a new field in diagnosis research, investigations about VOC that are potential attractive to diseases vectors, development of synthetic odorants that are attractive to vectors, development of non-invasive diagnostic techniques and to improve entomologic traps and other instruments that contributes to combat and control visceral leishmaniasis.

Acknowledgements

The present work was supported by Brazilian National Research Council (CNPq), FAPESB, PRONEX, FINEP and CAPES. Alexander von Humboldt Foundation provided fellowship support for Carlos Roberto Franke (III-ERSX-BRA/1067633).

References

[1] Brasil, Manual de vigilância e controle da leishmaniose visceral, 2003.
[2] P. Dejeux, Nat. Rev. Microbiol. 9 (2004) 692.
[3] E.S. Silva, C.M.F. Gontijo, R.S. Pacheco, V.O.P. Fiuza, R.P. Brazil, Mem. Inst. Oswaldo Cruz 96 (2001) 285.
[4] F.S. Julião, B.M.P.S. Souza, D.S. Freitas, L.S. Oliveira, D.A.F. Larangeira, A.G. Dias-Lima, V.M.M. Souza, S.M. Barrouin-Melo, E.D. Moreira Jr., B.J.A. Paule, Pesq. Vet. Bras. 27 (2007) 319.
[5] D.C.P.M. Barbosa, C.M.B. Gomes Neto, D.C. Leal, D.V.V. Bittencourt, B.M.P.S. Souza, L.S. Oliveira, F.S. Julião, V.M.M. Souza, C.R. Franke, Rev. Bras. Saúde Prod. An. 7 (2006) 152.
[6] I.A. Sherlock, Mem. Inst. Oswaldo Cruz 91 (1996) 671.
[7] P.T. Guerin, P. Olliaro, S. Sundar, M. Boelaert, S.L. Croft, P. Desjeux, M.K. Wasunna, A.D.M. Bryceson, Lancet Infect. Dis. 2 (2002) 494.
[8] B.G.J. Knols, J. Meijerink, Sci. Med. 4 (1997) 56.
[9] B. O'Shea, E. Rebollar-Tellez, R.D. Ward, J.G.C. Hamilton, D. El Naiem, A. Polwart, Trans. R. Soc. Trop. Med. Hyg. 96 (2002) 117.
[10] D. Penn, W.K. Potts, Trends Ecol. Evol. 13 (1998) 391.
[11] M. Phillips, R.N. Cataneo, B.A. Ditkoff, P. Fisher, J. Greenberg, R. Gunawardena, C.S.K. Won, F. Rahbari-Oskoui, C. Wong, Breast J. 9 (2003) 184.

[12] C. Di Natale, A. Macagnano, E. Martinelli, R. Paolesse, G. D'arcangelo, C. Roscioni, A. Finazzi-Agro, A. D'Amico, Biosens. Bioelectron. 18 (2003) 1209.
[13] P. Dalton, A. Gelperin, G. Preti, Diabetes Technol. Ther. 6 (2004) 534.
[14] U.R. Bernier, M.M. Booth, R.A. Yost, Anal. Chem. 71 (1999) 1.
[15] R.L. Chitwood, R.M. Pangborn, W. Jenning, Food Chem. 11 (1983) 201.
[16] N. Kocsis, M. Amtmann, Z. Mednyanszky, K. Korany, J. Food Compos. Anal. 15 (2002) 195.
[17] K.E. Murray, F.B. Whitfield, J. Sci. Food Agric. 26 (1975) 973.
[18] T. Jagella, W. Grosh, Eur. Food Res. Technol. 209 (1999) 22.
[19] O. Sabzevari, Kh. Abdi, M. Amini, A. Shafiee, Anal. Bioanal. Chem. 379 (2004) 120.
[20] J. Kenji, H. Seiichi Kashimura, M. Kashiwagi, M. Kageura, J. Chromatogr. B 758 (2001) 95.
[21] F.V. Parreira, Z.L. Cardeal, Quim Nova 28 (2005) 646.
[22] E.T. Sousa, F.M. Rodrigues, C.C. Martins, F.S. Oliveira, P.A.P. Pereira, J.B. de Andrade, Microchem. J. 82 (2006) 142.
[23] S.L.C. Ferreira, R.E. Bruns, E.G.P. da Silva, W.N.L. dos Santos, C.M. Quintella, J.M. David, J.B. de Andrade, M.C. Breitkreitz, I.C.S.F. Jardim, B.B. Neto, J. Chromatogr. A 1158 (2007) 2.
[24] D.L. Massart, B.G.M. Vandeginste, L.M.C. Buydens, S. De Jong, J. Lewi, J. Smeyer-Verbeke, Handbook of Chemometrics and Qualimetrics Part A, Elsevier, Amsterdam, 1997.
[25] T.L. Lundstedt, E. Seifert, L. Abramo, B. Thelin, A. Nystro, J. Pettersen, R. Bergman, Chemom. Intell. Lab. Syst. 2 (1998) 3.
[26] S. Wold, M. Sjostrom, B.R. Kowaslki (Eds.), Chemometrics: Theory and Application, American Chemical Society, Washington, 1973, p. 243.
[27] F.S. Oliveira, L.S.G. Teixeira, M.C.U. Araujo, M. Korn, Fuel 83 (2004) 917.
[28] M.D. Derde, D.L. Massart, Anal. Chim. Acta 191 (1986) 1.
[29] M. Sjostrom, B.R. Kowalski, Anal. Chim. Acta 112 (1979) 11.
[30] R. De Maesschalck, A. Candolfi, D.L. Massart, S. Heuerding, ChemoLab 47 (1999) 65.
[31] S. Wold, K. Esbensen, P. Geladi, ChemoLab 2 (1987) 37.
[32] K.H. Esbensen, Multivariate Data Analysis in Practice: An Introduction to Multivariate Data Analysis and Experimental Designing, Camo Process AS, Norway, 1994.
[33] F.S. Julião, B.M.P.S. Souza, D.S. Freitas, L.S. Oliveira, D.A.F. Larangeira, A.G. Dias-Lima, V.M.M. Souza, S.M. Barrouin-Melo, E.D. Moreira Jr., B.J.A. Paule, Pesq. Vet. Brás. 27 (2007) 319.
[34] A.C. Alcântara, ELISA indireto e mkDNA PCR-RFLP para o diagnóstico e avaliação da infecção por *Leishmania* sp. em reservatórios domésticos (cães) e silvestres (marsupiais) em Barra do Pojuca, Camaçari, Bahia, Dissertação (Mestrado em Ciência Animal nos Trópicos), 2006.
[35] D. Smith, P. Spanel, J.M. Thompson, B. Rajan, J. Cocker, P. Rolfe, Appl. Occup. Environ. Hyg. 13 (1998) 817.
[36] M.A.H. Braks, R.A. Anderson, B.G.J. Knols, Parasitol. Today 15 (1999) 409.
[37] C.R. Williams, J.K. Michael, B.P. Smith, J. Chem. Ecol. 29 (2003) 1889.
[38] S.N. Puri, M.J. Mendki, D. Sukumaran, K. Ganesan, S. Prakash, K. Sekhar, J. Med. Entomol. 43 (2006) 207.
[39] M.J. Dougherty, P.M. Guerin, R.D. Ward, J.G.C. Hamilton, Physiol. Entomol. 24 (1999) 251.
[40] E. Gunawardena, H.M.W.K.B. Herath, J. Chem. Ecol. 17 (1991) 2449.