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# Determination of amitraz in canine plasma by solid-phase microextraction–gas chromatography with thermionic specific detection

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**Abstract**

A simple and rapid analytical method is presented for the determination of amitraz in canine plasma samples using solid-phase microextraction (SPME) and gas chromatography with thermionic specific detection (GC-TSD). The best conditions for the SPME procedure were: direct extraction on a polydimethlysiloxane (PDMS) fiber with 100-μm film thickness; 400 μl of sample plasma matrix modified with 4 ml sodium borate solution (0.01 mol $l^{-1}$, pH 6.5); extraction temperature 70 °C, with stirring at 2500 rpm for 45 min. The method was linear between 20 and 400 ng $ml^{-1}$ with regression coefficients corresponding to 0.998 and coefficient of the variation of the points of the calibration curve lower than 15%. The lowest limit of quantification (LOQ) for amitraz in plasma was 20 ng $ml^{-1}$. This LOQ was determined as the lowest concentration on the calibration curve in which the coefficient of variation was lower than 15%. The proposed method was applied to determine amitraz concentrations in canine plasma to look for toxicity after treatment with amitraz in a dipping bath.


*Keywords:* Solid-phase microextraction; Canine plasma; Amitraz

## 1. Introduction

Amitraz (*N′*-2,4-(dimethylphenyl)-*N*-[(2,4-dimethylphenyl)imino] methyl-*N*-methyl-methanimidamide) [33089-61-1] (Fig. 1) is a formamidine derivate widely used for the control of ticks and mange mites in animals. However, it has side effects, the commonest being transient sedation or lethargy. This occurs in about 9.2% of dogs 2–6 h after topical treatment for demodectic mange, with amitraz and it lasts for 24–72 h [1]. Transient sedation also follows amitraz dipping in 12.4% of dogs treated with amitraz for sarcoptic mange. Other side effects are bradycardia, hypothermia, hypotension, polyuria, vomiting, and hyperglycemia [1].

The mechanism of action of amitraz responsible for these effects in dogs is not completely understood; however, the adverse reactions seen in some

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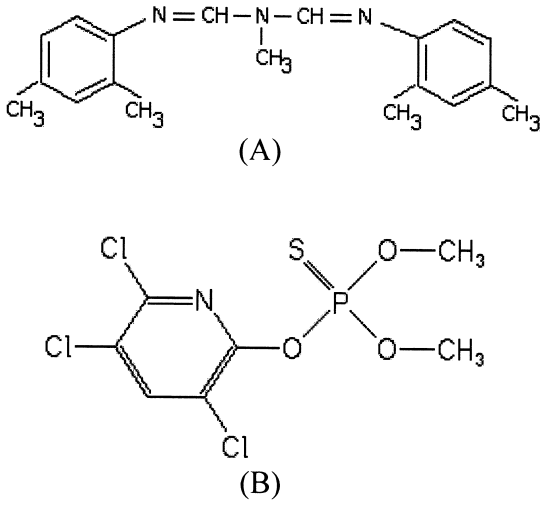


Fig. 1. Structures of amitraz (A) and chlorpyrifos (B).

dogs resemble those induced by the $\alpha_2$-adrenoreceptor agonists xylazine and clonidine [2].

Ameno et al. [3] proposed a method for quantifying amitraz in plasma for pharmacokinetic studies by gas chromatography with nitrogen–phosphorus detection after pretreatment of the samples by solid-phase extraction using extrelut-3. Hugnet et al. [4] developed a specific high-performance thin layer chromatographic method to determine plasma amitraz concentrations to evaluate the toxic effects in dogs. These methods, however, are time-consuming, require complicated procedures, and require the use of organic solvents.

Sample preparation is an important part in the analysis of veterinary compounds in body fluids. The aim of the sample preparation method is to eliminate interfering compounds from the matrix to produce a reproducible methodology involving a minimum number of steps. Solid-phase microextraction (SPME) has been evaluated as a suitable sampling technique for a wide range of applications, and has been shown to be a very effective and highly sensitive solvent-free sampling technique [5]. Although this technique was introduced for the extraction of organic compounds from environmental samples [5,6], since 1995 it has also been applied to various biological matrices such as urine, plasma and hair [7–10].

In this study, we established the optimum conditions for the determination of amitraz in animal plasma samples by SPME–GC-TSD. Additionally, the proposed methodology was validated by investigating the limit of quantitation, linearity, and precision, after which it was applied to the analysis of canine plasma samples from animals treated with amitraz dipping bath.

## 2. Experimental

### 2.1. Reagents and analytical standards

Amitraz and chlorpyrifos (internal standard, Fig. 1) analytical standards were purchased from Supelco (São Paulo, Brazil), with purity greater than 97%. The working analytical standard solutions were prepared by diluting the stock solutions of these compounds (1 mg $ml^{-1}$ in acetone) with acetone. They were stored at −20 °C. Drug-free plasma was collected from healthy experimental animals and stored at −20 °C.

Sodium chloride analytical grade (Merck, Rio de Janeiro, Brazil) was used after purification by heating at 300 °C overnight; acetone and *n*-hexane HPLC grade were purchased from Merck (Darmstadt, Germany), sodium tetraborate anhydrous was purchased from Sigma (Steinheim, Germany).

### 2.2. SPME equipment

The SPME fiber holder and 100-μm film thickness polydimethly siloxane (PDMS) fibers were from Supelco (São Paulo, Brazil). The fibers were conditioned by heating in the injection port of the chromatographic system for 1 h at 250 °C, according to the manufacturer's recommendation.

### 2.3. Instrumentation

GC analysis were performed on a Varian Star 3400 Cx gas chromatograph equipped with thermionic specific detector. The compounds were separated on a DB 1 (methyl siloxane) column from J and W Scientific (Varian, São Paulo, Brazil) (30 m×0.25 mm I.D.×0.1 μm film). The injector port and detector temperatures were 250 and 300 °C, respectively. The column temperature was held at 90 °C for 4 min increased to 205 °C at 20 °C $min^{-1}$, main-

tained at 205 °C to 2 min, increased to 260 °C at 20 °C $min^{-1}$, and then to 300 °C at 6 °C $min^{-1}$, with the final temperature held for 1 min. Nitrogen (White Martins, Ribeirão Preto, Brazil) was used as the carrier gas, the column head pressure being at 12 p.s.i. Injections were made in the split mode at a split ratio of 20:1, and a septum purge rate of 2 ml $min^{-1}$.

### 2.4. Optimization of the SPME conditions

The first step was to evaluate the pH influence on the extraction recovery. For that purpose, sodium borate solution (0.01 mol $l^{-1}$) at four different pH values (pH 4.8, 6.5, 7.5, 9.0) were investigated. In a conical glass tube (5 ml), 100 μl internal standard (1 μg $ml^{-1}$ chlorpyrifos in acetone) and 1.2 ml of borate solution (0.01 *M*) were added to 400 μl of plasma spiked with the working analytical standard resulting in 1 μg $ml^{-1}$ amitraz; the sample was vortexed for 10 s. The fiber was immersed in the sample under stirring at 40 °C for 15 min, and then was directly transferred to the hot injector and desorbed for 4 min. Triplicate analyses were performed for all experiments.

The effect of ionic strength of the sample solution (addition of 0, 2.5, and 5.0% NaCl w/v), the extraction temperature (50, 70, 80 °C) and the equilibrium time (15, 30, 45, 60 min) on the extraction efficiency were also investigated. The variables, equilibrium time and temperature were simultaneously optimized. The desorption conditions were evaluated by testing the main variables involved: injection temperature, desorption time and depth of fiber into the injector liner.

## 3. Results and discussion

### 3.1. Optimization of the SPME conditions

The optimum SPME desorption conditions were found to be 250 °C (injection temperature) and 4 min (desorption time). Those parameters were optimized not only aiming a quantitative desorption (maximum detector response), but also to cause a minimum of carryover. Increasing the desorption time from 4 to 10 min at 250 °C was not found to produce significant differences in the obtained results.

The effect of fiber depth into the liner showed that the deeper the fiber was exposed to the hottest part of the injector (closer to the column entrance), the higher the peak areas obtained.

The best pH value for the matrix using sodium borate solution was obtained at pH 6.5; this value showed the best results (extraction efficiency). Adjusting the pH of the sample can improve the sensitivity of the method for basic and acid analytes. This is related to the fact that, unless ion-exchange coatings are used, SPME can extract only neutral (non-ionic) species from sample [5]. As a function of the results obtained in this work, a pH of 6.5 was selected to continue the SPME optimization (Fig. 2A).

The effect of ionic strength of the sample solution was investigated in the range 0, 2.5, and 5.0% (w/v) NaCl concentration at pH 6.5. The results demonstrate that there was an inverse relationship between the extracted amount and NaCl concentration (Fig. 2B). The salt may itself interact with the analyte in solution, possibly either electrostatically or ion-pairing interactions, reducing the ability of the drug to move into the fiber coating, thus reducing the amount extracted. The equilibrium time and temperature were optimized simultaneously (Fig. 2C). At 45 min the amounts extracted increased with temperature up to 70 °C. After this value the extraction efficiency decreased. The increase in extraction temperature enhanced the drug diffusion, which shortens the equilibrium time but decreases the drug distribution between the fiber coating and the extraction mixture [5]. At 50 °C the extraction equilibrium was not reached. So the extraction time of 45 min and temperature 70 °C were selected as best conditions. Stirring using a magnetic bar was performed to achieve rapid mass transport.

In order to investigate the influence of proteins on the extraction yield, the plasma was diluted with the borate solution. The dilution factors varied from 1:1 to 1:10 v/v. The extraction yield increased with increasing the dilution factor (Fig. 2D).

Since drugs are usually compounds having relatively high boiling points, with molecular masses in the region of 200–400, they adsorb slowly onto SPME fibers. The biological matrices usually have

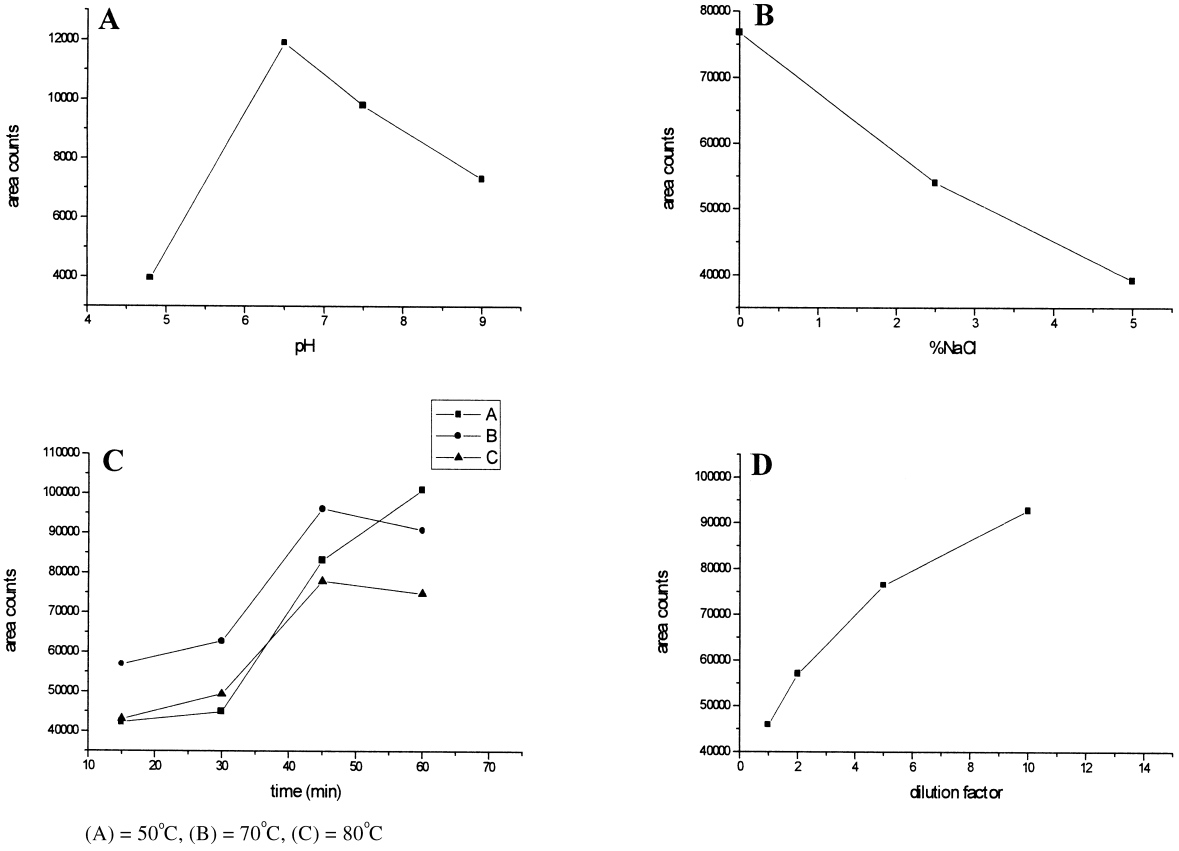


Fig. 2. Effect of the experimental variables on the SPME efficiency. (A) pH, (B) NaCl concentration, (C) temperature ''versus'' extraction time, (D) dilution factor. These values represent the average of triplicate experimental points.

high viscosity, compared to water and sorption of drugs onto the fiber is significantly reduced in viscous samples [11].

Compared to water, diffusion coefficients are smaller in more viscous protein solutions. Therefore, the flux of drug into the fiber and the recovery at a distinct extraction time are also decreased. However, according to the well-agitated model, viscosity of the analyte solution has no influence on the extraction time and may be of minor influence also in real stirred solutions [12].

Bermejo et al. [13] also diluted the plasma solution (1:4 v/v with buffer) in order to optimize the SPME conditions for the determination of methadone in plasma.

The amitraz extracted from plasma diluted with borate solution showed smaller areas (amount extracted) than the values obtained when the extraction was performed in water. The recovery from plasma is just 50–60% of the recovery from water, showing the influence of the protein in the SPME procedure.

When the proteins in the plasma samples were precipitated and the supernatant was spiked with amitraz (remaining aqueous phase from plasma), the peak areas obtained were comparable to those values obtained by spiking pure water. Protein precipitation has been used to release drugs from the plasma protein binding prior to sampling by SPME [11,14,15].

Thus, we concluded that the best experimental conditions for the SPME procedure were as follows: direct extraction with PDMS fiber (100-μm film thickness); 400 μl of sample plasma diluted with 4 ml sodium borate solution (pH 6.5); extraction temperature at 70 °C with stirring at a rate of 2500 rpm for 45 min.

### 3.2. Validation of the method

The linearity of the assays was determined using drug-free plasma spiked with the amitraz and internal standard. The interval evaluated (20–400 ng $ml^{-1}$) was linear with the correlation coefficient of 0.998 and linear regression equation ($y=-0.014444+0.00165x$) with a slope standard deviation=0.01311 and intercept standard deviation=0.00006. The coefficients of variation (C.V.s) of the five data points used to prepare the calibration curve were all below 15%.

The precision was determined by the percentage coefficient variation of within and between day variations at three different concentrations (Table 1). The precision ranged from 4.7 to 12.5% for within-day measurement, and for between-day variation was in the range 4.5–13.2%.

Amitraz and chlorpyrifos (I.S.) have similar behaviors during extraction by SPME, and the recovery of both by SPME under the best conditions, in spiked plasma samples, was low, 12.5–17.5 and 21.4–26.8%, respectively.

The limit of quantification (LOQ) of amitraz in plasma samples was 20 ng $ml^{-1}$. This LOQ was determined as the lowest concentration on the calibration curve in which the C.V. was lower than 15%.

The selectivity of the method was demonstrated by satisfactory separation of the compounds with no interfering peaks in the chromatograms of drug-free plasma from healthy dogs (Fig. 3A).

### 3.3. Determination of amitraz in canine plasma samples

The calibration graphs for the determination of amitraz in plasma were based on peak area ratios (area amitraz/area chlorpyrifos) versus the concentration of amitraz with spiked plasma calibrants in linear interval from 20 to 400 ng $ml^{-1}$.

SPME methodology described here was applied to the determination of amitraz in plasma samples from two dogs that were both dipped in an amitraz bath, at a concentration of 292 mg $kg^{-1}$ of body weight (Table 2). The difference in the amitraz absorption by the dogs is attributed to the fact that dog no.1 had shorter hair while dog no. 2 had longer hair. Clinical signs of toxicosis were observed in both dogs after

Table 1
Precision within and between days of the procedure with plasma sample spiked with amitraz

| Concentration added (ng $ml^{-1}$) | Measured concentration (mean±SD[a]) | C.V.s[b] (%) |
|---|---|---|
| Within-day ($n$=6) | | |
| 20.00 | 18.57±2.32 | 12.5 |
| 50.00 | 47.95±4.09 | 8.53 |
| 80.00 | 79.53±3.73 | 4.7 |
| Between-day ($n$=6) | | |
| 20.00 | 18.88±2.49 | 13.2 |
| 50.00 | 49.56±3.84 | 7.75 |
| 80.00 | 79.89±3.59 | 4.5 |

[a] SD, standard deviation.
[b] C.V., coefficient of variation.

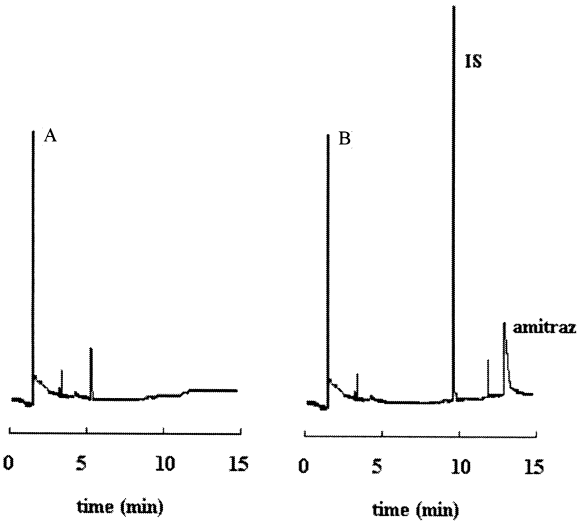


Fig. 3. Capillary GC-TSD chromatogram for the SPME extracts of (A) blank plasma from healthy dog; (B) plasma from dog that was dipped in a bath of amitraz, 292 mg $kg^{-1}$ of body weight, resulting in plasma levels of 31.37 ng $ml^{-1}$. Internal standard (I.S.)=chlorpyrifos.

Table 2
Determination of amitraz in plasma samples from two dogs in treatment, both dipped in an amitraz solution of concentration 292 mg $kg^{-1}$ body weight

| Collect time (h) | Plasma amitraz concentration (ng $ml^{-1}$) | |
|---|---|---|
| | Dog no. 1 | Dog no. 2 |
| 0.5 | 52.78 | 21.04 |
| 3.0 | 36.24 | 26.40 |
| 7.0 | 26.32 | 31.37 |

0.5 h, including sedation and hypothermia, related to the alpha 2-agonist activity of amitraz. Those effects were reversed by the intravenous administration of low doses of yohimbine, a potent alpha 2-antagonist. Fig. 3 shows the capillary GC-TSD chromatogram of the SPME extract of plasma from (Fig. 3B) a dog, resulting in plasma levels of 31.37 ng $ml^{-1}$, collected 7 h after amitraz dipping.

In short, the proposed application showed that SPME–GC-TSD for the determination of amitraz in canine plasma samples has many advantages compared with articles reported in the literature [3,4] such as simplicity (all steps of sample preparation are integrated in one step and in one device), low cost, compatibility with analytical systems, automation, the absence of extraction solvent, and a relatively short extraction time. Based on the analytical validation, the new method described has been applied to studies of toxicity of the amitraz in dogs after dipping baths.

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## References

[1] D.D. Schaffer, W.H. Hsu, D.L. Hopper, Toxicol. Appl. Pharmacol. 104 (1990) 543.
[2] W.H. Hsu, F.B. Hembrough, Toxicol. Appl. Pharmacol. 84 (1986) 418.
[3] K. Ameno, C. Fuke, S. Ameno, T. Shinohara, I. Ijiri, J. Anal. Toxicol. 15 (1991) 116.
[4] C. Hugnet, F. Buronrosse, X. Pineau, J.L. Cadore, P.J. Berny, Am. J. Vet. Res. 57 (1996) 1506.
[5] J. Pawliszyn, Applications of Solid-Phase Microextraction, Royal Society of Chemistry, Cambridge, 1999.
[6] M.E.C. Queiroz, S.M. Silva, D. Carvalho, F.M. Lanças, J. Environ. Sci. Health 36 (2001) 517.
[7] G. Theodoridis, E.H. Koster, G.J. Jong, J. Chromatogr. B 745 (2000) 49.
[8] M.E.C. Queiroz, D. Carvalho, F.M. Lanças, J. Chromatogr. Sci. 40 (2002) 219.
[9] M.E.C. Queiroz, S.M. Silva, D. Carvalho, F.M. Lanças, J. Sep. Sci. 25 (2002) 91.
[10] S. Ulrich, J. Chromatogr. A 902 (2000) 167.
[11] M. Krogh, H. Grefslie, K.E. Rasmussen, J. Chromatogr. 689 (1997) 357.
[12] S. Ulrich, J. Martens, J. Chromatogr. B 689 (1997) 217.
[13] A.M. Bermejo, R. Seara, A.C. Santos, M.J. Tabernerd, R. Marsili, J. Anal. Toxicol. 24 (2000) 66.
[14] E.H.M. Koster, C. Wemes, J.B. Morsink, G.J. Jong, J. Chromatogr. B 739 (2000) 175.
[15] K.J. Reubsaet, N.H. Ragnar, P. Hemmersbach, K.E. Rasmussen, J. Pharm. Biomed. Anal. 18 (1998) 667.