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Case Report - Veterinary Medicine

Evaluation of amitraz and derivatives in poisoned cat fur: a new screening method via Headspace/Gas Chromatography-Mass Spectrometry – case report

[*Avaliação de amitraz e derivados em pelo de gato intoxicado: um novo método de triagem via Headspace/Cromatografia Gasosa-Espectrometria de Massa – relato de caso*]

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ABSTRACT

Amitraz is a toxic pesticide that causes severe poisoning in animals; however, few reports of poisoning in cats can be found. Current diagnostic techniques mostly use invasive sampling and require a long time. A new noninvasive technique presented in this report provides accurate diagnosis. Here, we report a case of a 5-year-old male Persian cat with no prior history of illness, presenting vomiting, decreased consciousness, ataxia, and prostration following topical amitraz application for flea control. When admitted to the emergency service, 0.1% of intravenous yohimbine hydrochloride solution was administered to reverse the α-2 adrenergic agonist effects caused by the pesticide. Clinical tests indicated a slight increase in the alanine transaminase (ALT). Furthermore, samples from fur were collected during hospitalization to confirm and follow up the amitraz diagnosis using headspace/gas chromatography-mass spectrometry (HS/GC-MS) analysis. The presence of amitraz in the fur was detected by HS/GC-MS through its derivative products 2,4-dimethylaniline (DMA) and 2,4-Dimethylphenylformamide (DMF). After 24 hours, the patient was released with recommendations to avoid amitraz use and a recommendation for a bath and clipping. Fast aid and antidote administration were decisive factors in the successful treatment. Fur HS/GC-MS stands out as a definitive diagnosis of amitraz poisoning using non-invasive samples.

Keywords: feline, pesticide, poisoning, hair, HS/GC-MS

RESUMO

O amitraz é um pesticida tóxico que causa intoxicação grave em animais, mas há poucos relatos de casos de intoxicação em gatos. As técnicas de diagnóstico atuais utilizam sobretudo amostras invasivas e requerem muito tempo. Uma nova técnica não invasiva, apresentada neste relato, permite um diagnóstico preciso. Aqui, relatau-se um caso de um gato persa, macho, de cinco anos de idade, sem doenças anteriores, que apresentou vômitos, diminuição da consciência, ataxia e prostração após a aplicação tópica de amitraz, durante um banho para controlar a infestação por pulgas. Quando deu entrada no serviço de emergência, foi administrada uma solução intravenosa de cloridrato de ioimbina a 0,1% para reverter os efeitos agonistas α-2 adrenérgicos causados pelo pesticida. Os testes clínicos indicaram um discreto aumento da alanina transaminase (ALT). Além disso, foram recolhidas amostras de pelo durante a hospitalização para confirmar e acompanhar o diagnóstico de amitraz, utilizando-se a análise de headspace/cromatografia gasosa acoplada à espectrometria de massas (HS/GC-MS). A presença do amitraz no pelo por HS/GC-MS foi detectada por meio dos seus produtos derivados 2,4-dimetilanilina (DMA) e 2,4-dimetilfenilformamida (DMF). Após 24 horas, o paciente recebeu alta, com recomendações para evitar o uso de amitraz e indicação para banho e tosa. O auxílio rápido e a administração do antídoto foram fatores decisivos para o sucesso do tratamento. A HS/GC-MS do pelo destaca-se como um diagnóstico definitivo da intoxicação por amitraz utilizando-se amostras não invasivas.

Palavras-chave: felino, pesticida, intoxicação, cabelo, HS/GC-MS

INTRODUCTION

Amitraz is a readily available commercial pesticide responsible for poisoning animals and humans (del Pino *et al*., 2015), however, data concerning this poisoning are incomplete, especially regarding cats. Amitraz (IUPAC name: N'-(2,4-dimethylphenyl)-N-[(2,4-dimethylphenyl)iminomethyl]-N-methylmethanimidamide) is a low-cost and easily acquired formamidine pesticide widely

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used (Jorens *et al*., 1997; del Pino *et al*., 2015). In veterinary, it is readily applied to control mites, lice, and ticks in cattle, pigs, sheep, and pets (Marafon *et al*., 2010). Still, its overuse might lead to poisoning. Moreover, amitraz is contraindicated for horses (Duarte *et al*., 2003), and manufacturers do not declare any recommendations for cats.

In vertebrates, it primarily acts as an agonist to the central α_2-adrenergic receptor (Jorens *et al*., 1997). It also stimulates α2 and α1 peripheral receptors and inhibits monoamine oxidase and prostaglandin E2 synthesis (del Pino *et al*., 2015). Poisoning is uncommon in both humans (Jorens *et al*., 1997) and animals, but previous reports include dogs (Andrade and Sakate, 2003), horses, and cats (Andrade *et al*., 2006).

Diagnosis of amitraz poisoning is often based only on clinical history and case evolution. However, chromatographic analysis can establish a definitive diagnosis. Previous methodologies included high-performance liquid chromatography (HPLC) (Iken *et al*., 2020) and gas chromatography (GC) (Marafon *et al*., 2010), using blood samples and other tissues (Yu *et al.*, 2010). This report describes a rare case of amitraz poisoning in a feline with a successful clinical intervention and a novel confirmation tool for amitraz presence methodology using Headspace/Gas Chromatography-Mass Spectrometry (HS/GC-MS) with non-invasive samples (fur).

CASE SERIES DESCRIPTION

A 5-year-old Persian cat, a spayed male with a body weight of 4kg, was referred to the emergency service after the owner bathed the animal with a solution prepared with a commercial amitraz, at a concentration of 12.5% indicated for dogs and cattle, aiming to control a flea infestation. The owner did not declare the dilution percentage. Immediately after bathing, the animal began to experience vomiting, ataxia, and apathy, and at the time of attendance, approximately 2 hours later, presented a decreased consciousness, a score of 14 by the Glasgow Scale Modified for Small Animals (Ash *et al*., 2018). On initial physical examination, vital parameters of heart rate, respiratory rate, temperature, and blood pressure were within normal limits. However, at abdominal palpation, the patient showed signs of pain.

A single dose of yohimbine was administered intravenously (yohimbine hydrochloride 0.1mg kg^{-1}, Reset®, Botupharma, Brasil), promoting consciousness improvement in twenty minutes. After stabilization, blood samples were collected for blood count and serum biochemistry (ALT-alanine aminotransferase and creatinine). A slight increase in liver enzyme ALT 103UI L^{-1} was observed. An electrocardiogram was also performed, showing no alterations.

The animal was kept under observation for 24 hours and during hospitalization, received maintenance fluid therapy with ringer lactate at a rate of 1mL h^{-1}, ondansetron 1% (Emedron®, ondansetron hydrochloride, Agener União Saúde Animal, Brazil) at a dose of 0.1mL kg^{-1}, IV, twice daily (BID), and tramadol hydrochloride 50mg mL^{-1} (Tramal®, tramadol hydrochloride, Grünenthal Health, Germany) at a dose of 0.08mL kg^{-1}, subcutaneous (SC), BID. Vomit and ataxia improved significantly, as the animal reached 18 on the Glasgow scale. The patient was released 24 hours after care with a recommendation of a complete clipping and a warm bath. These procedures were not performed at the veterinary hospital due to the patient’s reactiveness.

Fur samples were collected from the dorsal region in two rounds, one at the patient's admission at the emergency care and the other during discharge. The sampling was made with sterilized metal scissors, stored in airtight bags, kept from sunlight exposure, and frozen at -20ºC for 7 days until analysis.

For the HS/GC-MS analysis, 38.7±1.1 mg of the collected fur samples were weighed directly into 20mL headspace (HS) vials without any sample preparation steps. The HS/GC-MS analysis for diagnostic purposes that our work was based on was developed by Shorkry *et al*., 2017. 0.1µL of the 3-methylcyclohexanone (Sigma-Aldrich®, EUA) was used for the chromatography runs as the internal standard (IS). The HS/GC-MS analysis of the amitraz commercial formulation was performed using the same formulation as the owner reported.

The static HS extraction was conducted in a Shimadzu AOC-5000 (Shimadzu®), supplied with a VT32-20 tray and a 2500µL gas-tight syringe. The HS module holds a preheating LHS0 Combi Pal with heating time and temperature control (PAL System®), coupled with a glue-free high-dynamic and high-temperature syringe (Halmiton®) at 150^{o}C. The module agitation was configured at 500rpm at 160^{o}C (incubation temperature) and 60min (agitation time). The injection volume was 2500μL, with a fill and injection speed of 100μ s^{-1} and 1mL s^{-1}, respectively.

The GC instrument used was a Shimadzu GCMS-QP2010 Ultra System (Shimadzu®). Sample injection was carried out at 250^{o}C in the splitless mode, and the chromatography elution was performed in an NST-100ms capillary column (25m×0.25mm i.d.×0.3μm film thickness; NST, São Paulo, Brazil) with a polyethylene glycol stationary phase. High-purity helium (99.999%—5.0, White Martins®) was used as the carrier gas with a linear velocity of 45.8cm s^{-1} and a constant flow of 1.36mL min^{-1}. MS was performed in the electron ionization (EI) mode at 70 eV with a scan speed and time of 1600u s^{-1} and 0.3s, respectively. The chromatographic run was performed as previously described by Shorkry *et al*., 2017. Volatile derivative products of amitraz were confirmed by comparing MS fragments with those in the NIST17s mass spectral libraries (National Institute of Standards and Technology®). Eluted compounds with the same base peak and at least 80% fragmentation similarity to NIST17s library standards were marked as putative chemical compounds.

Two MS data acquisition approaches were used to confirm amitraz residues in the samples: i) the data was acquired in full-scan mode from 40 to 500 *m/z*; ii) selected ion monitoring (SIM) mode, setting as the monitored ions 162, 293, 121, and 132 *m/z* for the amitraz, and the 121, 120, 106, and 77 *m/z* for its derivative products, such as 2,4-dimethylaniline (DMA), 2,4-Dimethylphenylformamide (DMF), and N'-(2,4-Dimethylphenyl)-N-methylimidoformamide (DMPF). We monitored molecular and base peak ions for IS (m/z 112 and 69, respectively) for confirmation purposes regarding the retention time. All the potential amitraz derivative products in the samples were also confirmed by the relative retention time (rRT) to the IS and the MS fragmentation pattern comparison with the analysis of 0.1μL of amitraz commercial formulation (Triatox$^{®}$, amitraz, MSD saúde animal, Brazil).

Albeit amitraz is an unstable molecule that easily suffers degradation reaction, the process ushers to the form of stable chemical products such as DMA, DMF, and DMPF (Picó *et al*., 2008). Thus, it is crucial to correctly detect amitraz's presence in the organism due to the similarity of clinical signs to organophosphate poisoning, which frequently leads to misdiagnosis and incorrect management of amitraz overexposure (Dhooria; Agarwal, 2016).

The HS/GC-MS analysis of the fur sample at the admission led to the annotation of 39 volatile organic compounds from different organic classes (Table 1). Across the annotated compounds, two exogenous chemicals (*i.e*., not products of intrinsic metabolism and potential chemical compounds of exposure events) linked to the amitraz molecule were observed: DMA (VOM 9, Table 1) and DMF (VOM 13), indicating a suspect animal intoxication by amitraz.

We detected DMA and DMF in the fur samples of the poisoned cat, which are significant indicators of the presence of amitraz. The absence of DMPF in the fur samples could be explained by the amitraz fragmentation pathway (Fig. 1), where DMPF acts as an intermediate before converting into DMF (chemical formula: C9H11NO$^{\bullet+}$). Detection of DMA, the primary product of amitraz, is often used to indicate pesticide residues in the tissues of food-producing animals (Yu *et al*., 2010). DMF, with a molecular ion at m/z 149 (chemical formula: C9H11NO$^{\bullet+}$), serves as the main degradation product for detecting amitraz residues (Özcan & Akman, 2019). DMA, with a base peak at m/z 121 (C8H11N$^{\bullet+}$), forms through a neutral loss of 28 Da (CO) from DMF. As observed in Figure 1, the suspected peaks of amitraz derivatives (DMA and DMF) were the major ones in terms of abundance in the total ion chromatograms (TIC), which presented high (>90%) similarity with DMA and DMF in NIST17s libraries (98% and 96%, respectively). To confirm the presence of the derivatives, we ran an analysis of the amitraz commercial formula in the same full-scan mode. The expected MS structures and fragments are presented in Fig. 1. Figure 2 shows the TIC profile of the commercial formulate amitraz, which indicates the derivative DMA, DMF, and DMPF.

Table 1. Volatile organic derivatives annotated in the fur analysis of the poisoned cat, the retention time information, respective match similarities according to NIST17s libraries, major peak (m/z), and CAS No. Exogenous volatile organic metabolites are labeled in bold. The remaining ones represent endogenous compounds present in the biological matrix.

Nº	Volatile organic metabolites	Retention time		Similarity (%)	Major peak (m/z)	CAS No
		Absolute	Relative			
1	Ethanol	1.234	50	96	45	64-17-5
2	Acetone	1.384	56	90	43	67-64-1
3	Methoxyacetone	1.399	57	89	43	5878-19-3
4	2-Methylpropanal	1.896	77	90	43	78-84-2
5	n-Hexane	2.384	97	89	57	110-54-3
6	3-Methylbutanal	3.540	144	91	44	590-86-3
7	Cyclohexane	3.720	152	90	56	110-82-7
8	2-Octanone	26.345	1.075	96	43	928-68-7
9	2,4-Dimethylaniline (DMA)	47.869	1.952	98	121	95-68-1
10	Dodecane	64.540	2.632	92	57	112-40-3
11	Dodecanal	64.780	2.642	93	43	112-54-9
12	1-Dodecanol	67.087	2.736	96	55	112-53-8
13	2,4-Dimethylphenylformamide (DMF)	67.300	2.745	95	120	60397-77-5
14	Tetradecanal	68.192	2.781	94	57	124-25-4
15	1-Tetradecanol	69.892	2.851	86	55	112-72-1
16	Diethyltoluamide	70.123	2.860	87	119	134-62-3
17	6,10-Dimethyl-2-undecanone	70.519	2.876	80	58	1604-34-8
18	Pentadecane	70.593	2.879	92	57	629-62-9
19	Pentadecanal	70.873	2.891	95	57	2765-11-9
20	2,6-Dimethyl-7-octen-2-ol	71.333	2.910	85	59	18479-58-8
21	1-Pentadecanol	72.351	2.951	94	43	629-76-5
22	2-Undecanone	72.866	2.972	80	43	0112-12-9
23	Ethylene glycol monododecyl ether	73.236	2.987	93	57	4536-30-5
24	1-Hexadecanol	73.562	3.000	89	43	36653-82-4
25	Tetradecanoic acid	74.160	3.025	89	73	544-63-8
26	N-Butylbenzenesulfonamide	74.921	3.056	93	77	3622-84-2
27	Isopropyl myristate	75.761	3.090	91	43	110-27-0
28	1-Cyclododecylethanone	77.344	3.155	85	43	28925-00-0
29	1-Heptadecanol	77.403	3.157	92	55	1454-85-9
30	2-Heptadecanone	78.127	3.187	93	58	2922-51-2
31	1-Octadecanol	79.237	3.232	95	55	112-92-5
32	Hexadecanoic acid	79.987	3.263	96	43	57-10-3
33	Diethylene glycol mono-dodecyl ether	81.187	3.311	88	57	3055-93-4
34	Isopropyl palmitate	81.894	3.340	94	43	142-91-6
35	1-Nonadecanol	83.513	3.406	95	43	1454-84-8
36	1-Heneicosanol	84.861	3.461	96	55	15594-90-8
37	n-Butyl palmitate	85.970	3.507	96	56	0111-06-08
38	n-Butyl stearate	90.422	3.688	93	56	123-95-5
39	Squalene	93.848	3.828	95	69	0111-02-04

Although the DMPF was not observed in analyses, the DMA and DMF were confirmed by similar ion ratio patterns in fur samples, commercial amitraz, and NIST17s libraries (Table 2), which confirmed the indication of amitraz's presence in the patient. DMA presented a proportion of ion ratios of ~10:9:8 (*m/z*: 121.10, 120.10, and 106.05), while the DMF showed ion ratios of ~10:7:6 (*m/z*: 120.10, 149.10, and 106.05).

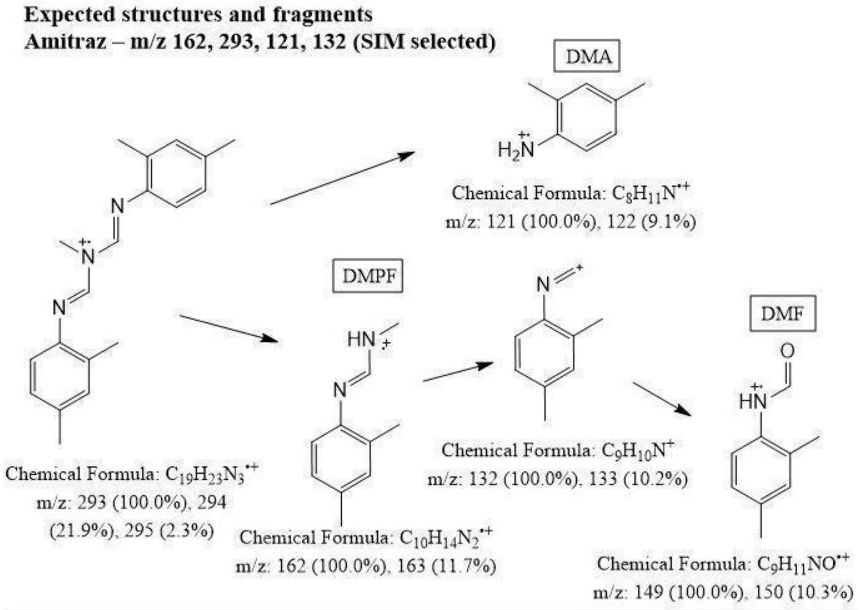

Figure 1. Amitraz's fragmentation route (electron ionization mode) shows the formation of its stable derivatives (DMPF, DMF, and DMA). This pathway does not illustrate the resonance structures of the fragmentation.

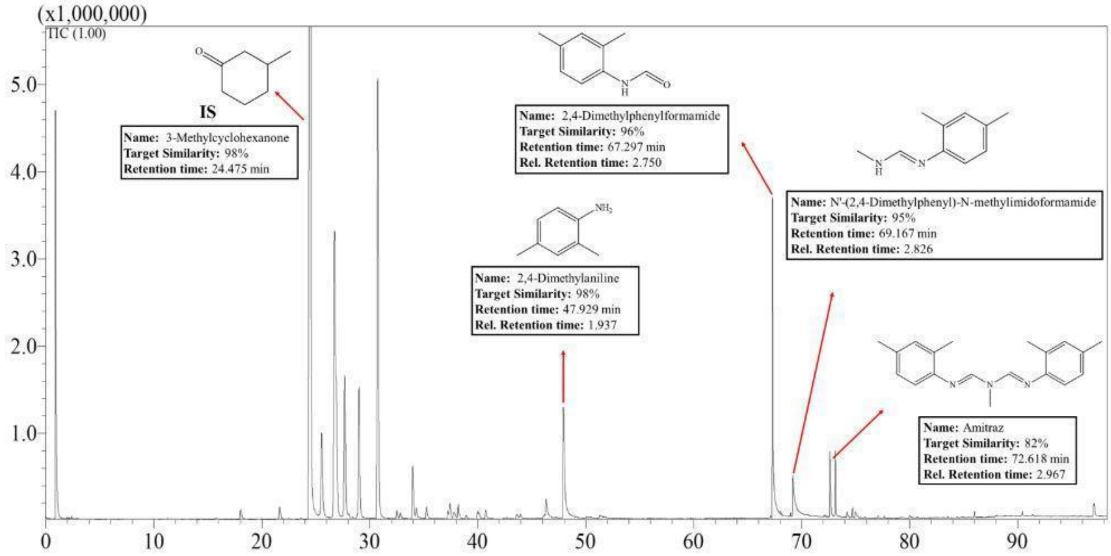

Figure 2. HS/GC-MS Total ion chromatograms (TIC) profile of the commercial Amitraz showing its major detected compounds: 2,4-Dimethylaniline (Retention time - rt: 47.929min), 2,4-Dimethylphenylformamide (rt: 67.297min), N'-(2,4-Dimethylphenyl)-N-methylimidoformamide (rt: 69.167min).

Table 2. The mass table of the major m/z ions for DMF and DMA was detected in the fur samples compared to their respective mass table in NIST17s libraries and the commercial Amitraz

2,4-Dimethylphenylformamide (DMF)		2,4-Dimethylaniline (DMA)	
Target in the fur samples			
m/z	Average Relative intensity	m/z	Average Relative intensity
120.10	100.00	121.10	100.00
149.10	73.13	120.10	90.37
106.05	56.81	106.05	76.08
NISTs.lib			
m/z	Absolute Intensity	m/z	Absolute Intensity
120.10	100.00	121.10	100.00
149.10	91.19	120.10	92.49
106.05	51.75	106.05	84.38
Commercial Amitraz			
m/z	Absolute Intensity	m/z	Absolute Intensity
120.10	100.00	121.10	100.00
149.10	74.54	120.10	90.72
106.05	57.10	106.05	76.18

The absolute and relative retention time of the DMA (47.869min and 1.952, respectively) and DMF (67.300min and 2.745) also matched with the observed in the commercial amitraz (DMA: 47.929min and 1.937 / DMF: 67.297min and 2.750). Fur analyses of the patient after treatment indicated a decrease in the abundance of DMA and DMF, in a factor of ~3.5 and 3.2, respectively (Figure 3).

Using non-invasive samples, we report a detailed description of the successful treatment of amitraz poisoning in a cat, including history, clinical evolution, exams, and toxicological diagnosis.

Amitraz poisoning is considered rare, and toxicity derives from the inhibition of MAO and agonist effects of adrenergic receptors. Poisoning occurs through topic and oral exposure, causing severe neurological signs. In this case, the most prominent symptoms were consistent with previous findings, especially reduced consciousness (del Pino *et al*., 2015). It is essential to highlight that the case was considered moderate. Yohimbine was successfully used as an antidote, as it was the only reversible agent available in emergency care. However, previous research has defined atipamezole as the safest antidote due to its fewer adverse effects (Andrade *et al*., 2006).

Among the advantages of using non-invasive biological matrices is the ease of the process since the analysis is carried out with the sample *in natura*, avoiding laborious work in sample preparation and pre-concentration steps. Furthermore, this analytical workflow associated with conventional clinical approaches may be a powerful tool for decision-making in veterinary medicine regarding treatment follow-up, as already described in other pesticide outbreaks (Barbosa *et al*., 2020). The main limitation of our approach is the qualitative approach, which only makes it possible to indicate the amitraz presence in the animal but not estimate its concentration. However, since our methodology does not require sample preparation, and the result can be reached in a relatively short time, it can be used as a quick screening to point out the amitraz presence. In case of a positive result - and when the pesticide concentration is needed for better veterinary medical intervention - amitraz levels can be determined by an auxiliary plasma analysis, requiring an invasive sampling (Marafon *et al*., 2010).

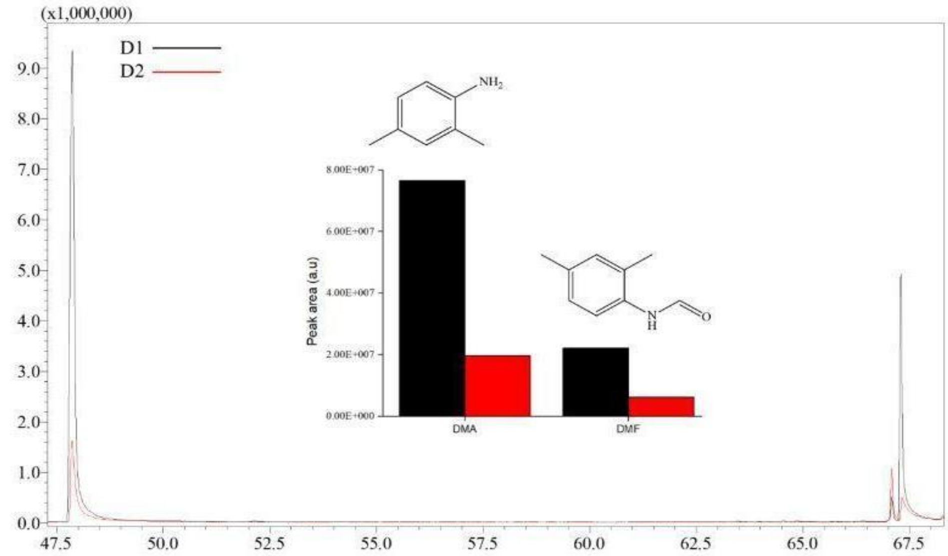

Figure 3. Peak abundance of the Amitraz derivatives - 2,4-dimethylaniline (DMA, retention time: 47.869min) and 2,4-dimethylphenylformamide (DMF, retention time: 67.297min) – of the fur samples collected during the check-in (D1) and check-out (D2) of the patient.

CONCLUSIONS

We describe a rare case of amitraz poisoning in a cat with successful treatment and an alternative approach using fur analysis by HS/GC-MS to detect, confirm, and follow up on amitraz presence in the patient. As far as the authors are aware, this is the first approach showing the identification of amitraz using fur as a non-invasive biological sample.

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