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Short communication

# Earwax: A clue to discover fluoroacetate intoxication in cattle

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ABSTRACT

An innovative method was developed to detect fluoroacetate poisoning in cattle by headspace/gas chromatographic analysis of earwax samples of intoxicated cattle. Samples were collected from 2 groups of cattle subjected to induced fluoroacetate intoxication, each group receiving a different dose of acetamide (antidote). Monofluoroacetic acid was detected in samples of intoxicated cattle in concentrations inversely proportional to the dose of acetamide. Thus, earwax analysis represents a successful approach for detection and monitoring of fluoroacetate poisoning.



Plant poisoning is one of three major causes of death in livestock in Brazil, ranked as one of the top producers of beef in the world (Penrith et al., 2015). In Brazil, *Palicourea marcgravii* known as “cafezinho” or “erva de rato” is one of the most important plants related to sudden death syndrome in cattle. This is due to its good palatability, wide geographic distribution and cumulative effects (Tokarnia et al., 2012). The toxic principle of *P. marcgravii* is monofluoroacetic acid (MFA), also referred to as fluoroacetate or compound 1080 (Lee et al., 2012). It is absorbed into the body by inhalation (Kulling et al., 1992), through the skin (Kusch et al., 1990), and orally by ingestion which is most toxic route (EPA, 1995). The MFA toxicity develops from its chemical similarity to acetate which enables it to combine with acetyl Co-A. This results in disruption of the citric acid cycle, impairment of oxidative metabolism, and adenosine triphosphate (ATP) production (Proudfoot et al., 2006). The diagnosis of fluoroacetate poisoning is usually done only at lethal doses, on the basis of history, clinical signs, anatomopathological lesions and chemical analyses. Clinical signs include excessive salivation, lethargy, apparent blindness, disoriented running, tachycardia, dyspnea, muscle spasms, tremors, coma, and terminal tonic convulsions, ending with sudden death within 22 and 96 h while anatomopathological lesions, when present are represented principally by hydropic degeneration of epithelial cells of the distal convoluted kidney tubules. As for chemical analyses, it is much less common due to poor stability and short half-life of the substance in biological samples (Nogueira et al., 2011). The problem in using pathology for verifying a tentative diagnosis for sudden death is that, it is difficult to predict the cause of death, because fluoroacetate poisoning can be confused for several possible differentials as blood anthrax, snake bite, cyanogenic plants and other plants causing sudden death (Nogueira et al., 2011). That is why a clue that can guide the investigation may solve this problem. For the above reasons, it would be favorable to develop an easy, rapid, inexpensive sampling, readily available to collect, and analyze with no or simple pretreatment for monitoring MFA either in survivors or postmortem samples. The conditions were satisfied in earwax sampling, overcoming problems faced with other biological fluids (Nogueira et al., 2010).

Chemical standards for fluoroacetic acid (95%) and 3-methyl cyclohexanone (internal standard) (IS) were purchased from

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Sigma-Aldrich (Riedel de Haën, Germany) and Polyscience Corporation (Illinois, USA), respectively. For the experiment, earwax samples were collected from 7 cattle, all males, aged 6–8 months (live weight of 110–170 kg) included in the study randomly divided into two groups (T1 and T2), consisting of 4 and 3 cattle, respectively. The intoxication protocol consisted of an adaption period in which the animals were confined in individual pens for 14 days, receiving hay and commercial ration. Before intoxication, the animals were fasted for a period of 24 h. The animals were then experimentally intoxicated with *P. marcgravii* at a dose of 1,8 g/kg by forced ingestion of the dried and ground plant (Tokarnia and Döbereiner, 1986; Tokarnia et al., 1990), 3 h after oral administration of single doses of acetamide of 1,0 g/kg (group T1) and 2,0 g/kg (group T2) (Peixoto et al., 2012). Then, after the consumption of the plant, the animals were able to drink water and eat normally. ad libitum. The animals presented clinical signs evident of intoxication, however discrete or of short duration, that started about an average of 15 h after intoxication. The main signs observed were apathy, reluctance to movement, muscle tremors, positive venous pulse, and ataxia. Some other signs were also observed such as mydriasis, sternal decubitus, and loss of balance. Earwax samples were collected 30 days after the intoxication, using a metal curette, transferred in eppendorf tubes, immediately stored in a freezer at −20 °C, and analyzed within 7 days of freezing. All procedures performed were in strict accordance with the ethical standards of the ethical committee at the Federal University of Goiás (Protocol number 027/16). Of each sample, 20 mg were accurately weighed, transferred into 20-mL GC vials, and 0.2 μL of IS was added. The vials were tightly sealed with gas tight polytetrafluoroethylene (PTFE)-lined rubber septum caps. For calibration samples, a standard solution was prepared in water at a concentration of 10 μg/mL. Blank earwax samples (20 mg) from healthy cattle were pooled, spiked with 5 μl of various dilutions of the standard solution and prepared in the same way described above to prepare a six-point calibration curve over the concentration range equivalent to (12.5–625 pg/mg earwax). Samples of *P. marcgravii* used in intoxication, were collected from dense forests located in Santo Antônio de Goiás, subjected to dehydration process for a period of two weeks, ground in a Wiley mill (Model EDB-5®), and stored at 22 °C. HS-GC/MS was applied in the analysis of earwax samples using high purity helium (99.999%) as a carrier gas. The system was composed of Shimadzu GCMS-QP2010 Ultra equipped with AO5-5000 headspace analyzer (Shimadzu, Japan), a 2500 μL gas-tight syringe, a VT32-20 tray for 20-mL standard vials and an additional pre-heating module LHS0 Combi PAL Liquid for six vials (PAL System, Switzerland). The parameters were set at: agitation speed:

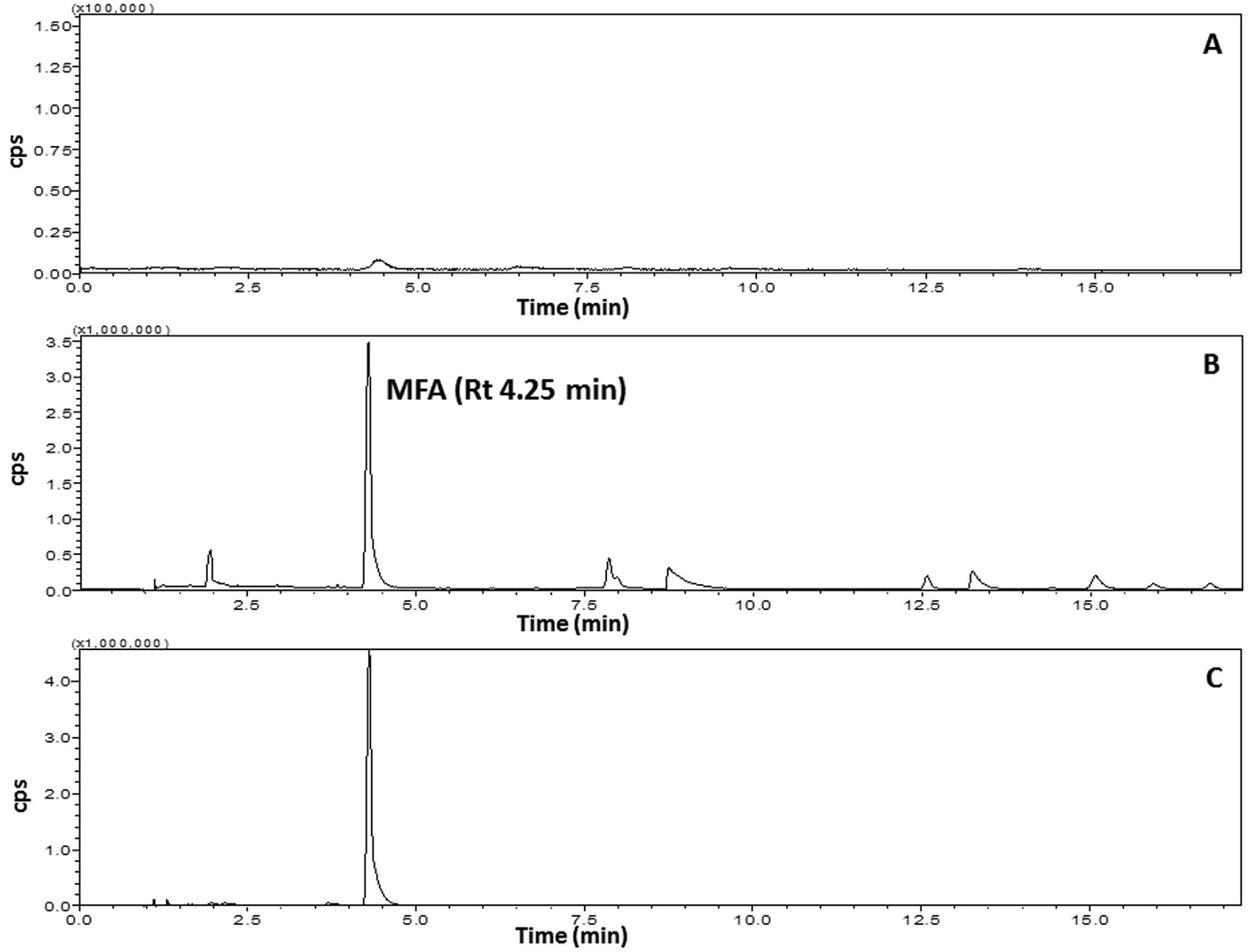


**Fig. 1.** GC-MS chromatogram of (A) earwax sample of healthy control not subjected to intoxication with *P. marcgravii* (B) *P. marcgravii* sample (C) earwax sample of cattle experimentally intoxicated with *P. marcgravii*, all showing the same peak of monofluoroacetic acid (MFA).

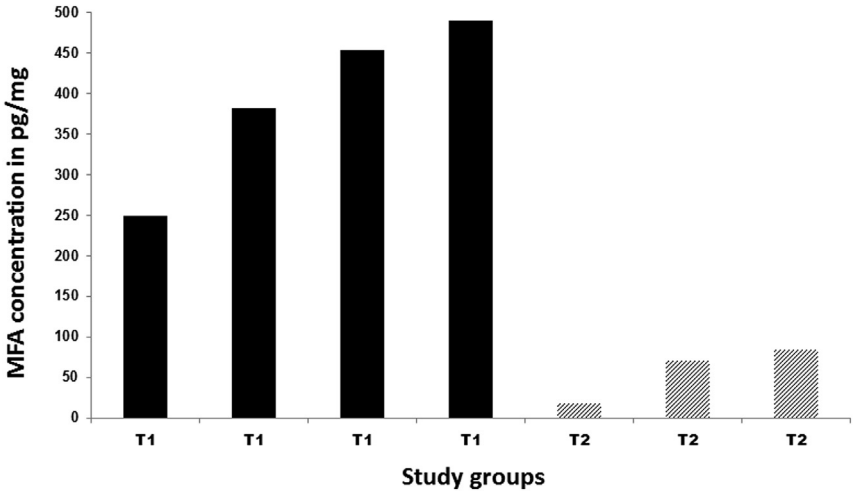


**Fig. 2.** Concentrations of monofluoroacetic acid detected by HS/GC-MS analysis of earwax samples obtained from T1 and T2 study groups experimentally intoxicated with 1,8 g/kg *P. marcgravii* and treated with acetamide at a dose of 1,0 and 2,0 g/kg, respectively.

500 rpm, incubation temperature: 195 °C, and injection volume: 2500 μL. For the chromatography, an analytical capillary column NST-100 (25 m × 0.25 mm x 0.3 μm) (NST, Brazil) with a polyethylene glycol based high-polarity stationary phase was used. The GC temperature program starts with 30 °C (held for 5 min), increased by 2 °C/min to 45 °C (held for 5 min) then by the same rate to 50 °C (held for 5 min) then to 120 °C, followed by 6 °C/min to 200 °C (held for 5 min), and finally by 5 °C/min to 250 °C (held for 10 min). The MS was operated in electron ionization (EI) mode at 70 eV. Initial data acquisition was performed in the full SCAN mode from m/z = 33–400 with a scan time of 0.3 s. MFA peak was initially annotated by comparing its mass spectrum against NIST11 mass spectral library (99% match probability) and the identification was further confirmed by comparison and retention time matching against a reference MFA standard. Monitoring was then performed in the SIM mode to increase the sensitivity and selectivity. Quantification was based on peak area ratios versus the IS. The monitored ions for quantitation were m/z 78 for MFA and m/z 112 for the IS. HS was performed at a temperature 195 °C based on the fact that MFA becomes volatile at a temperature >165 °C at the atmospheric pressure and after trying different temperatures between 165 °C and 200 °C (Ellis et al., 2002). MFA which was absent in the earwax samples obtained from healthy cattle (Fig. 1A), was detected in the samples of intoxicated cattle of both groups (Fig. 1B) as well as in the plant samples (Fig. 1C). The peak corresponding to MFA eluted at a retention time of 4.25 min. The levels of MFA encountered in groups (T1) receiving a lower dose of acetamide were higher than group (T2) receiving a higher dose of acetamide, with average MFA concentrations of 394 and 57 pg/mg, respectively, in spite that the same dose of MFA was administered (Fig. 2) indicating that the amount of MFA detected in earwax is inversely proportional to the dose of acetamide which is the antidote. The importance of this study not only lies in its ability to confirm a rapid and easy diagnosis for sudden death in cattle without the need for autopsy but also, in survivors, it can detect exposure to sub-lethal intoxication with MFA (Pacheco and Carneiro, 1932). This could be useful, especially that it was reported that cumulative subsequent exposure to small doses can cause damage to the heart and other organs (Clarke, 1991; Gooneratne et al., 2008). The method could be more preferred to blood because of being noninvasive, detecting past exposure to MFA, where it was detected in earwax of intoxicated cattle 1 month after exposure, meaning that it accumulated in earwax as it was secreted in the ear of the cattle, unlike blood where it is rapidly eliminated with an elimination half-life of 2 h (Kulling et al., 1992). These aspects are not only important for the animal welfare but also due to the risk of secondary intoxication attributed to unmetabolized MFA itself. Secondary intoxication was previously confirmed in dogs feeding on carcasses of poisoned animals showing MFA accumulation in animal tissues for a long time after being poisoned, which pose a huge risk to other animals and humans (Eisler, 1995; Meenken and Booth, 1997). This work offers further evidence for the diagnostic ability of earwax, that was used in forensics for monitoring of drugs of abuse/drug facilitated crimes (Shokry et al., 2017a) and in diagnosis of diabetes in humans (Shokry et al., 2017b) in recent studies by the authors.

## Ethical statement

All procedures performed in this study were in strict accordance with the ethical standards of the ethical committee at the Federal University of Goiás, where the study was approved and conducted (Protocol number 027/16).

## Conflict of interest

None declared.

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## Transparency document

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