



Three mammal species distinction through the analysis of scats chemical composition provided by comprehensive two-dimensional gas chromatography

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ABSTRACT

In the last years, new approaches use scats analysis in ecological studies, like gas chromatographic techniques, has been developed. The aim of this study is to use VOCs (volatile organic compounds) released from scats to distinguish three mammal species: the ungulates red and fallow deer and the carnivore Eurasian otter through the application of SPME/GC × GC–ToFMS. This chromatographic technique has never been used in ecological studies. Chromatogram contour plot was effective in distinguishing between the carnivore Eurasian otter and the two ungulates. Additionally, a subset of 21 selected compounds, including hydrocarbons, ketones, aldehydes, alcohols and terpenes, allowed a distinction between Eurasian otter, red deer and fallow deer. Eurasian otter scats were characterized mainly by the presence of aldehydes that may arise from oxidative degradation of unsaturated fatty acids present in fish species, included in their diet. Red deer volatile profile is characterized by terpenes compounds, presumably related with diet, while fallow deer achieved also chemical communication markers: 2-propanone and hexane. The scat volatile profiles provide information that explains their odour and also the type of diet (herbivorous or carnivorous) and intra-specific communication through chemical signals. Being able to distinguish species using the proposed methodology is an asset to wildlife ecology and management studies.

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1. Introduction

Mammal scats has been commonly used in ecological studies, for instance to estimate population size (Kohn et al., 1999; Webbon et al., 2004), distributional patterns or species richness (Dalén et al., 2004), once they are, in most cases, abundant and easier to access (Sanz et al., 2007).

In many studies, wildlife researchers make assumption that scats are correctly assigned to the species in question, but this can be difficult based on scat morphology alone (Davison et al., 2002; Prugh and Ritland, 2005). Especially when sympatric species have similar body features, behaviour and feeding habits, the visual discrimination of scats are subjective and error-prone (Ruiz-González et al., 2008).

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Nowadays, the majority of scat analysis that provide a reliable species identification are based on genetic/molecular analysis (Hoss et al., 1992), but some problems can occur, like the discriminate power due to a lack of genetic variation in this species (Poetsch et al., 2001).

In the last years, new approaches use scats analysis in ecological studies, like gas chromatographic techniques. For instance, solid phase microextraction (SPME) followed by one-dimensional gas chromatography (1D-GC) was applied for the first time to establish a volatile profile released from scats of five canid species (Burnham et al., 2008). However, any relevant chemical information was extracted from scats composition as the species distinction was based on statistic processing of chromatographic data, without identification of the compounds detected. Such 1D-GC based methods often provide analytical results, but the co-eluted peaks, not allowing a complete analytic separation, lead to information losses of complexes matrices. In the last two decades considerable research has been dedicated to a combination of independent techniques aimed at strengthening resolving power (Kidwell and Riggs, 2004; Tranchida et al., 2004). Comprehensive two-dimensional gas chromatography (GC $\times$ GC) combined with time-of-flight mass spectrometry (ToFMS) detection represents a successful example of this combination. GC $\times$ GC employs two orthogonal mechanisms to separate the constituents of the sample within a single analysis, a technique based on the application of two GC columns coated with different stationary phases. Therefore, separation potential, sensitivity and limits of detection are greatly enhanced when compared to 1D-GC (Dalluge et al., 2003; Marriott and Shellie, 2002; Zrostlikova et al., 2003). GC $\times$ GC-ToFMS has been successfully used in several fields of analysis, revealing advantages especially for analysis of complex samples and detection of trace components (Rocha et al., 2012, 2013).

The aim of the present study is to use VOCs (volatile organic compounds) to distinguish scats of three mammal species: the ungulates red (*Cervus elaphus*) and fallow deer (*Dama dama*) and the carnivore Eurasian otter (*Lutra lutra*) through the application of SPME/GC $\times$ GC-ToFMS, an high sensitive and high throughput methodology. This chromatographic technique has never been used in ecological studies.

2. Material and methods

2.1. Samples

In order to characterize volatiles of three mammalian species through HS-SPME/GC $\times$ GC, scats from one carnivore and two ungulates were studied. Red and fallow deer are sympatric species with identical body features, behaviour and feeding habits, showing diet and habitat overlap (Azorit et al., 2012). The scats of these two species are very similar in shape, colour and nature (digested herbs) (Chapman, 2004). These factors pose a challenge to design the limits of species distribution range, especially with fine scale accuracy. The European otter was used as control species, for distinct feeding habits and morphology when comparing to ungulates. Thus, having a control species, differences in the results for carnivore and herbivorous species are expected, showing the HS-SPME/GC $\times$ GC potential to distinguish these three mammals. Scat collection was performed in four fenced natural areas, in the north and centre of Portugal (PBSL- Parque Biológico da Serra da Lousã, TNM- Tapada Nacional de Mafra, PEG- Parque Ecológico de Gouveia e PBV- Parque Biológico de Vinhais) providing different sources of individuals of the same species and individuals of the same species with different diets. Scats were collected right after deposition ensuring specie donor. The animals from the fenced natural areas were selected based on their proximity to semi-wild state, in an attempt to simulate their conditions in the wild. One sample per individual, with a total of 18 individual scats, was collected during autumn and winter of 2010: $n = 7$ from red deer, $n = 6$ from fallow deer and $n = 5$ from Eurasian otter.

The environment of the closed natural areas varied between locals, providing different secondary source of food: mainly *Pinus pinaster* in PBSL, PBV and PEG, *Quercus suber*, *Quercus faginea*, *Pinus pinea* and bushes like heather and furze in TNM.

After collection, scats were wrapped in aluminium film, avoiding the direct contact with hands and stored in individual glass vials at $-20\text{ }^{\circ}\text{C}$ until analysis. Samples stored waited for analysis a maximum of 3 months.

2.2. HS-SPME/GC $\times$ GC-ToFMS methodology

The HS-SPME experimental parameters were based on a previous study (Burnham et al., 2008). Briefly, aliquots of 0.5 g of scat were placed into a 25 mL glass vial, which was placed in a thermostatted bath adjusted to $30.0 \pm 0.1\text{ }^{\circ}\text{C}$. Then, the SPME fibre was inserted in the headspace for 10 min. Two aliquots of each animal scat were analysed. The SPME holder and fibre were purchased from Supelco (Aldrich, Bellefonte, PA, USA). The SPME device included a fused silica fibre coating partially cross-linked with 50/30 μm divinylbenzene-carboxen-poly(dimethylsiloxane), PDMS/DVB/CAR.

After the extraction/concentration step, the SPME coating fibre was manually introduced into the GC $\times$ GC-ToFMS LECO Pegasus 4D (LECO, St. Joseph, MI, USA), consisted of an Agilent GC 7890A gas chromatograph, with a dual stage jet cryogenic modulator (licensed from Zoex) and a secondary oven, and the detector is an high-speed time-of-flight mass spectrometer. The instrumental parameters were based on a previous study (Rocha et al., 2013), with exception of the ovens temperatures and modulation time. The primary oven temperature program was: initial temperature $35\text{ }^{\circ}\text{C}$ (hold 2 min), rose to $100\text{ }^{\circ}\text{C}$ ($6\text{ }^{\circ}\text{C min}^{-1}$) and then rose to $220\text{ }^{\circ}\text{C}$ ($10\text{ }^{\circ}\text{C min}^{-1}$, hold 2 min). The secondary oven temperature program was $15\text{ }^{\circ}\text{C}$ offset above the primary oven and the modulation time was 5 s. Contour plots were used to evaluate the separation general quality and for manual peak identification. The 21 components that explain the distinction between species were tentatively identified. For identification purposes, the mass spectrum of each compound detected was compared to those in mass

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