Rapid Communication

A rapid chemical odour profiling method for the identification of rhinoceros horns

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Illegal poaching causes great harm to species diversity and conservation. A vast amount of money is involved in the trade of illegal or forged animal parts worldwide. In many cases, the suspected animal part is unidentifiable and requires costly and invasive laboratory analysis such as isotopic fingerprinting or DNA testing. The lack of rapid and accurate methods to identify wildlife parts at the point of detection represents a major hindrance in the enforcement and prosecution of wildlife trafficking. The ability of wildlife detector dogs to alert to different wildlife species demonstrates that there is a detectable difference in scent profile of illegally traded animal parts. This difference was exploited to develop a rapid, non-invasive screening method for distinguishing rhinoceros horns of different species. The method involved the collection of volatile organic compounds (VOC) by headspace solid-phase microextraction (HS-SPME) and analysis by comprehensive two-dimensional gas chromatography – time-of-flight mass spectrometry (GC × GC-TOFMS). It was hypothesised that the use of the specific odour profile as a screening method could separate and differentiate geographic origin or exploit the difference in diets of different species within a family (such as white rhinoceros and black rhinoceros from the Rhinocerotidae family). Known black and white rhinoceros horn samples were analysed using HS-SPME-GC × GC-TOFMS and multivariate statistics were applied to identify groupings in the data set. The black rhinoceros horn samples were distinctly different from the white rhinoceros horn samples. This demonstrated that seized rhinoceros horn samples can be identified based on their distinct odour profiles. The chemical odour profiling method has great potential as a rapid and non-invasive screening method in order to combat and track illegal trafficking of wildlife parts.

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1. Introduction

Wildlife forensic science deals with animal abuse and illegal trade of animals and/or animal parts. Wildlife crime and trafficking has overarching economic and environmental impacts globally. Illegal poaching causes great harm to biodiversity and a significant amount of money is involved in the trade of illegal or forged animal parts [1]. In total, it has been estimated that transnational organised environmental trade in flora and fauna (excluding fisheries and timber) is valued between 7 and 23 billion USD annually [2]. In many cases, the suspected animal part is unidentifiable and requires laboratory analysis such as isotopic fingerprinting or DNA testing [1,3,4]. These tests are time consuming, destructive and

cannot be conducted on-site. The lack of rapid and accurate tests to identify illicit material at the point of detection represents a major hindrance in the enforcement and prosecution of wildlife tracking. Wildlife detector dogs are sometimes employed on-site; however, dogs can only provide an alert of the presence of an illicit material and cannot provide a species level identification once detected. Thus, there is a need for a rapid screening method that is also highly specific for identification.

The fact that dogs can be trained to alert to different wildlife species [5] suggests a difference in the odour profile of animal species. It was hypothesized that the analysis of the odour profile may therefore provide a means for separation of one unknown species from another. Analysis of volatile organic compounds has previously been successfully used for the analysis of decomposition odour by the authors [6–9]. This analysis has been conducted using both headspace solid phase microextraction (HS-SPME) and sorbent tubes for sample collection [8], with analysis by

comprehensive two-dimensional gas chromatography – time-of-flight mass spectrometry (GC×GC-TOFMS).

The specific odour profile of a species results from many factors including genetics, diet, metabolism, body secretions and environmental variables. It is hypothesised that the use of the specific odour profile as a screening method could not only separate a diverse range of trafficked species such as rhinoceros, elephant, bears, tigers, leopards, etc., but could also be used for the differentiation of different species within a family, such as white rhinoceros (*Ceratotherium simum*) and black rhinoceros (*Diceros bicornis*). The ability to separate species within a family is based on the lifestyle (including diet) and environmental characteristics. For example, white rhinoceros are known as grazers and eat grasses, whereas black rhinoceros are browsers who predominately feed on leaves and shoots. These characteristics will influence the odour profile.

The goal of this study was to develop a rapid and accurate method for the analysis of illegal wildlife parts, with a special focus on rhinoceros horn samples. Rhinoceros horns were selected as they are difficult to distinguish morphologically (and are often sold as powders, small fragments and sculptures), they are prohibited international trade as they are listed under CITES (the Convention on International Trade in Endangered Species of Wild Fauna and Flora) appendix I (and II in the case of *C. s. simum* South African and Swaziland populations), and they represent one of the most endangered mammals globally [10–12]. The added benefit of using this sample group was the availability of two different species of rhinoceros horns which enabled the investigation into the species separation within a family. This was completed through the development of a VOC analysis method via HS-SPME and GC×GC-TOFMS instrumentation. A statistical method using multivariate analysis was used to confirm the results.

This study was used as a proof-of-concept to determine the feasibility of using odour profiles to distinguish wildlife parts. It forms the basis for a larger study investigating a diverse range of trafficked wildlife samples with the long-term goal of developing a portable odour profiling device that can be used by frontline personnel to rapidly identify a species and origin at the point of detection. Odour profiling is regularly applied to other areas of forensic, medical, and environmental science, however this is the first time that it has been applied to wildlife crime.

2. Materials and methods

2.1. Rhinoceros horn samples

Eight white rhinoceros and nine black rhinoceros samples were obtained from Australian Museum and Government collections and from live rhinoceros from Australian and New Zealand zoos. Black and white rhinoceros horns were both obtained from live rhinoceros and from zoos, excluding any influence from the nature of the obtained sample. The rhinoceros horns were originally collected in the form of drilled, shaved or horn fragments for DNA identification by Australian Museum. The same samples were used in this study in order to ensure that there was no difference in the data sets based on the method of subsampling and to provide correct identifications of each sample.

2.2. SPME conditions

VOC collection was carried out via headspace sampling using a 50/30 µm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) 24 Ga Stableflex SPME fibre and manual fibre holder (Supelco, Bellefonte, PA, USA). The fibre was initially conditioned for 60 min at 270 °C before first use according to the manufacturer's recommendations. The drilled/shaved rhinoceros horns were placed in an airtight 20 mL glass SPME vial, sealed with a screw cap containing a polytetrafluoroethylene/silicone septum (Supelco, PA, USA). Prior to headspace extraction, the sample was placed in a dry bath heating block (Thermoline Scientific, Wetherill Park, NSW, Australia) and incubated for 30 min at 80 °C. The DVB/CAR/PDMS fibre was exposed to the sample headspace and the VOCs were allowed to accumulate for 60 min. The fibre was thermally conditioned and blank samples were also collected to avoid contamination of the fibre for subsequent analyses.

2.3. GC × GC-TOFMS

The SPME fibres were directly inserted into a Pegasus® 4D GC×GC-TOFMS (LECO, Australia), and thermally desorbed for 5 min at 250 °C. The first dimension (^{1}D) column was a Rxi®-624Sil MS (Restek Corporation, Australia) connected to a second dimension (^{2}D) Stabilwax® column (Restek Corporation, Australia). Helium carrier gas flow was held at a constant rate of 1.00 mL/min throughout the run. The oven parameters were as follows; an initial temperature of 35 °C held for 5 min, followed by an increase to 240 °C at a rate of 5 °C/min where it was again held for 5 min. The secondary oven temperature offset was 10 °C. The mass range examined was 29–450 amu at an acquisition rate of 100 spectra per second.

2.4. Data processing

ChromaTOF® (version 4.51.6.0; LECO) was used for data processing. Baseline smoothing was automatically conducted by the software with an 80% offset. The ^{1}D peak width was set at 25 s while the ^{2}D peak width was set at 0.15 s. A minimum signal-to-noise ratio (S/N) was set at 250 for the base peak, whereas the S/N for sub-peaks was set at 20. A minimum similarity match >800 to the 2011 National Institute of Standards and Technology (NIST) mass spectral library database was used for initial identification. The Statistical Compare software feature in ChromaTOF® was then used for peak alignment. A mass spectral match of 600 was required for peaks to be identified as the same compound across different chromatograms during alignment. Principal Component Analysis (PCA) was carried out using The Unscrambler® X (version 10.3, CAMO Software, Oslo, Norway). The data processing and interpretation methods were adapted from previous work by the authors [7,13].

3. Results and discussion

During this study, headspace samples were collected from two different rhinoceros species (black rhinoceros and white rhinoceros). All the black rhinoceros samples (n = 9) had a very distinct compound pattern based on the total ion chromatogram (TIC) (Fig. 1a). This similarity demonstrated a consistency in the odour profile of black rhinoceros samples despite the samples being from different origins. White rhinoceros samples demonstrated a larger variation in the TICs, as evident in Fig. 1b and 1c. Several samples had a large variation in the presence and abundance of the later eluting compounds. This increased complexity in the later eluting compounds was also found to be one of the major differences between the white and black rhinoceros horn samples.

In order to determine whether the two rhinoceros species could be statistically differentiated based on their odour profiles, the chromatographic results were evaluated using principal component analysis (PCA). The PCA plot (Fig. 2) demonstrated that the black rhinoceros samples were distinctly different in their volatile organic compound (VOC) composition than the white rhinoceros samples. All of the black rhinoceros samples analysed were tightly clustered and demonstrated similar VOC profiles. In contrast, the
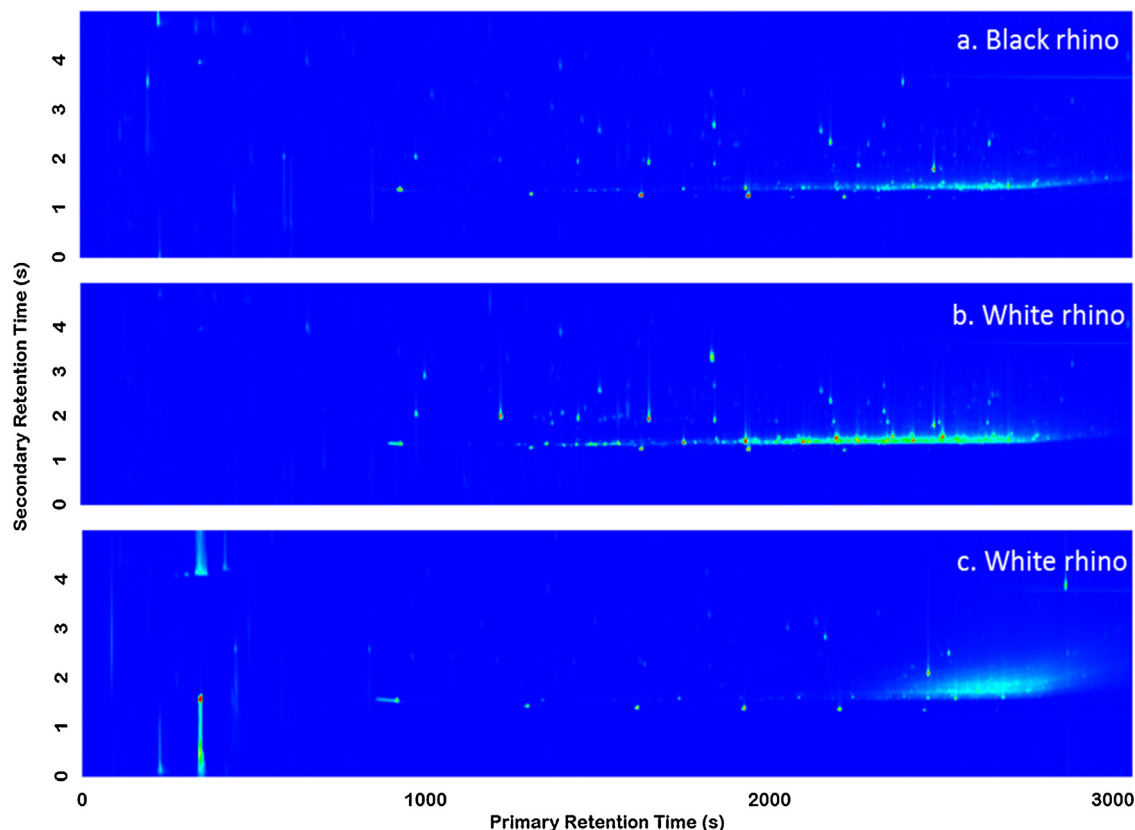
Fig. 1. GC×GC-TOFMS TIC contour plots representative of (a) the black rhinoceros samples, and (b) and (c) two different white rhinoceros samples.

white rhinoceros samples demonstrated variation in their VOC profile based on the spread of the data points. Although there was reduced grouping of the white rhinoceros samples, all samples were plotted along the positive principal component (PC-1) axis (right-hand side of the plot) when compared to the black rhinoceros samples. This suggests that the VOCs separating the white rhinoceros samples were found to a greater degree than in the black rhinoceros samples. Overall, it was determined that there was a distinct variation in the odour profile of the black and white rhinoceros horn samples.
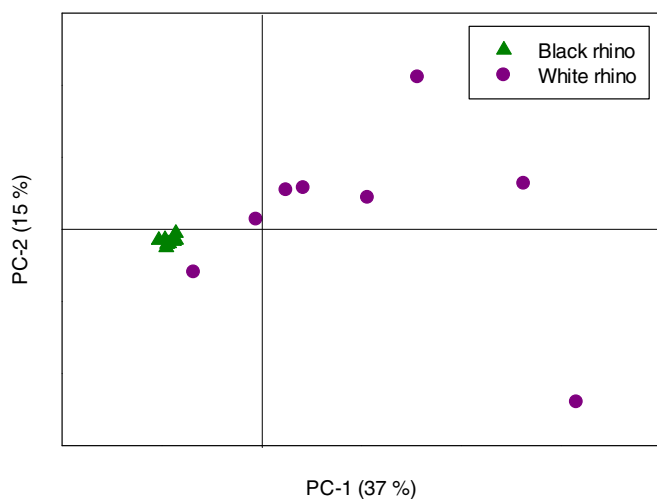

Fig. 2. Principal component analysis of the GC×GC-TOFMS peak area obtained from the Statistical Compare software showing the groupings and separation in the data from the black rhinoceros horn samples (green triangles) and the white rhinoceros horn samples (purple circles).

The ability to separate the white rhinoceros from the black rhinoceros horn samples demonstrates the potential of the odour profiling method. Through advanced data processing and statistical analysis, the method was able to separate species from two different genera within a family using their odour profile. The ability to separate species within a family suggests that it is likely that the proposed method can be used to separate animal parts from different families and genus, and thus be of significant use in identifying unknown wildlife parts trafficked illegally (such as rhinoceros horn, elephant ivory, tiger bone, etc).

This proof-of-concept study has demonstrated the potential of odour profiling as an alternative method for species identification. In this study, the HS-SPME-GC×GC-TOFMS method was applied to drilled and shaved samples based on their availability, however it can be equally applied to whole animals and animal parts illegally traded. The benefit of this method is that it is non-destructive, and faster than current methods used such as isotopic and DNA analyses. The intent is to further develop the method and create a larger database, by investigating a larger number of samples including fake samples often sold as rhinoceros horns, such as water buffalo horns and cow horns [12]. In addition, the method will be expanded to include other trafficked wildlife, their parts and the products often made from these (such as medicinal powders and jewellery). Future development of this method into a rapid and accurate screening technique for illegal wildlife products would greatly enhance border enforcement's ability to apprehend and prosecute offenders and provide greater market control over illegal wildlife products.

4. Conclusion

A rapid screening method for the differentiation of white and black rhinoceros horn samples was developed using the specific

odour profile associated with each species type. This method can be used to develop a database of odour profiles from different families, genus and species, and has the potential to be translated into a portable screening device capable of identifying suspicious animal parts based on their odour profile. Confirmation of species identification at the time of a seizure will prevent the need for laboratory analysis which can be time consuming, expensive and cannot be conducted on-site.

Acknowledgements

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