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Original Research

Reptile volatilome profiling optimisation: A pathway towards forensic applications

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ABSTRACT

Reptiles are the most trafficked live taxa in the illegal wildlife trade, in part due to their popularity as an exotic pet. Current methods used to detect these illegally trafficked animals are limited. This study optimised the collection and analysis parameters associated with volatilome collection that will set the foundation for targeted odourant detection methods. This study determined that the dual sorbent type (Tenax® TA and Carbograph 5DT) in combination with 20-min sampling times and 15-min sampling intervals collected the most reproducible reptile volatilome profiles. It was also determined that desorption methods with mid-range desorption flows (20 ml/min), trap temperatures (-15 °C), and mid-range trap desorption (25 ml/min) were most effective in retrieving collected reptile volatilomes. Two-dimensional gas chromatography coupled with time-of-flight mass spectrometry was used for analysis, where combined Rxi-624 Sil MS (mid-polar) first dimension and Stabilwax® (polar) second dimension column sets were selected as the most effective columns for analysing reptile volatilomes. The resultant data collected and analysed using these parameters demonstrated that individual volatilomes from three reptile species were distinct using principal component analysis. In addition, this work highlighted the need for more rigorous statistical methods to determine reptile biomarkers and which compounds most significantly influence volatilome profiles between species.

1. Introduction

The most illegally trafficked class of live animals are reptiles [1,2]. The demand for these animals is driven by interests in keeping them as an exotic pet, or for farming them for their products (*e.g.* leather, meat) [3]. Reptiles carry a lower risk profile for traffickers due to the relative ease of capture (*e.g.* docile nature, limited population monitoring, resources required for obtaining), concealment (*e.g.* non-vocal, low bone density) coupled with the survivorship success in long distance transit as compared to other taxa [4,5]. Resources allocated to mitigate this illegal trade have been limited as most reptile species are not protected by the Convention of International Trade in Endangered Species (CITES) trade regulations [6]. The illegal trade of live reptiles is generally initiated through online platforms [6] and specimens are trafficked through postal and commercial airline services [7,8]. The most common methods used for detecting wildlife contraband in transit are visual examination by customs personnel, inspection through X-ray machines and olfactory detection by wildlife detection dogs. Traffickers can attempt to circumvent these detection methods by limiting the animal's movement and vocalisation through binding [9], masking the animal from X-ray detection through wrapping in aluminium foil or placing in tins [8] and/or by masking the animal's scent through the use of adulterant potent odours, such as pepper spray or garlic [10,11]. Additionally, training programs administered to customs staff in many major airports often fail to adequately address the detection of these enhanced concealment methods [8], which enables further trade activity to occur. Due to these limitations, there are concerns that the monitoring practices and frameworks are ineffective at detecting illegally trafficked reptiles in transit.

The advancement of odourant detection may be the most relevant way to address the current limitations in live reptile detection. As with most animals, reptiles rely on chemical signatures to identify individuals, mark territories and explore their environment [12]. This reliance indicates that reptiles are constantly producing and releasing odours. Furthermore, these odours are persistent [13] and are generally able to penetrate most vented or semi-porous materials in which animals are traditionally packaged [11]. Detection dogs are currently the most effective odourant detection tool due to their remarkable ability to

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detect trace odours [14,15]. The most common use of detection dogs is related to the discovery of more high-profile contrabands, such as explosives, narcotics [16] and items related to biosecurity (*e.g.* seeds, meats, vegetables [17]). The resources needed to train detection dogs generally prevents their use for singular detection purposes, such as solely detecting wildlife contraband [18]. Additionally, the resources required to train and maintain detection dog efficiency limits their implementation in every air or seaport [19] or requires sharing between multiple locations [20]. Even when detection dogs are available, they are restricted in their ability to reduce illegal wildlife trade activity by their biological and behavioural constraints such as fatigue, handler bias and motivation [21]. These dogs are further limited by their training capacity (*i.e.* number of items/species that they can identify) and their handler's knowledge of what they are detecting (*i.e.* total odour profile or odour biomarkers [22]). The development of an automated, electronic technology that is programmed to selectively detect target odour biomarkers that are specific to animal species may be an appropriate alternative to detection dogs. This method may be less susceptible to adulterating odours as it can selectively detect the presence of biomarkers that are independent of additional odours. This technology could serve as a cost effective, reliable and quantitatively measurable solution to law enforcement agencies and communities globally.

Odours are comprised of volatile organic compounds (VOCs) that are carbon based, have low molecular weights and are able to sublimate, evaporate or dissolve into surrounding environments at room temperature. VOCs belong to various chemical classes which include sulphur and nitrogen-based compounds, hydrocarbons, acids, esters, alcohols, ketones and aldehydes [23–25]. These compounds can be monitored singularly (*i.e.* biomarkers) or collectively as a whole profile (*i.e.* volatilomes). Biological volatilomes are produced naturally through a combination of metabolic, microbiomic (*e.g.* skin or gut) and genetic processes [26–28]. Volatilome profiles can be further divided into stable biologic "primary" elements (*e.g.* genetic), unstable biologic "secondary" elements (*e.g.*, diet, immune, metabolic or hormonal state) and unstable abiotic "tertiary" elements (*e.g.* external applications unrelated to organism) [29–31]. It is suspected that the "primary" elements of biological volatilomes are stable over time, despite changes in immune status, dietary profile or levels of exposure to seasonal and genetic influences [32,33]. The apparent specificity of volatilomes indicates a potential avenue of research in wildlife forensics sciences. For example, targeting elements of the primary volatilome may aid in the developments of detection technology as where elements of secondary volatilome elements may be beneficial for post-confiscation (*e.g.* captive versus wild) applications.

The analysis of wildlife contraband using volatilome profiling has demonstrated the potential applications of these methods to wildlife forensic science [34,35]. However, volatilome profiling has not yet been conducted for live animals and the potential influences of biological activities on the total volatilome profiles are yet to be assessed. To ensure that live animal volatilome profiling meets the standards of forensic applications, the methods for collection and analysis need to be optimised, standardised and validated. Optimising volatilomes from biological sources can be challenging due to the range of compounds and chemical classes that may be present within each sample. These compounds can have differing volatilities, polarities and quantities which may complicate reproducibility. Furthermore, the entire volatilome may not be informative for species detection, species identification, captive versus wild applications, or geographic origin. The disparate characteristics of volatilome constituents may impact the selection of both sampling and analytical methods when building a reference database. For example, the sorbent material used in the collection apparatus, the amount of time selected for the animal's acclimation period and the volume of headspace collected in the sample must all be optimised from the context of the analytical quality and the welfare of the animal. Additionally, the sample desorption (*e.g.* optimisation of sample desorption temperatures, flow rates) and separation techniques (*e.g.* column selection, ramping temperatures) must also be optimised.

VOCs can be collected either passively through solid phase micro extraction (SPME) fibres or actively through stainless steel tubes that are internally coated with various sorbent types. SPME fibres facilitate the passive collection of VOCs through the selective and competitive sorption properties of the sorbents used. However, SPME fibres have limited collection surface area, which makes this method susceptible to oversampling [36]. Additionally, most SPME fibre sorbent types cannot accommodate VOCs of a high molecular weight [37] that may be present in reptile volatilomes. The limitations in available sorbent types and the increased sampling time associated with the passive sampling aspect of SPME fibres make this type of sampling method unsuitable for live animals. As a contrast to SPME fibres, sorbent tubes function by pre-concentrating VOCs onto packed particulate sorbent materials [36]. The packed sorbents generate larger collection surface areas which reduces the likelihood of compound displacement due to overexposure [38]. Sorbent tubes can also facilitate the use of pumps in order to collect VOCs actively at a constant rate in order to significantly reduce sampling times. Furthermore, sorbent tubes have been routinely used in field applications [39], making them more suitable for live animal volatilome collection.

The selection of collection sorbents is critical to ensuring the retrieval of whole volatilomes for analysis. Wildlife volatilome analysis is primarily non-targeted with the expectation of a wide range of VOCs. Each animal's volatilome profile may be impacted by its sex, age, disease status and relative level of metabolic activity [40,41]. Thus, it is important to select sorbents that can absorb a wide range of compounds in order to collect the most robust profiles. To ensure that VOCs of both high and low molecular weights are collected, the selection of tubes with multiple sorbent types may be advantageous. The added capacity of additional sorbent types will also ensure that a sufficient array of chemical classes is captured during each sampling period. Furthermore, sorbent strength should also be considered throughout collection and analysis as the selected sorbents should be strong enough to adsorb target analytes while being weak enough to release the target compounds during thermal desorption [42].

VOCs are often analysed through analytical instrumentation, such as gas-chromatography coupled with mass spectrometry (GC–MS) or two-dimensional gas chromatography (GC×GC) coupled with time-of-flight mass spectrometry (ToFMS). Both methods rely on thermal desorption methods for sample injection and use inert high purity gas to transport the compounds through the column(s) for separation. The primary difference between these two instrumentations is the addition of a secondary column in GC×GC which enables further separation of compounds with similar sizes and chemical properties. Due to the complexity of volatilome samples and the potential of co-elution of certain compounds, GC×GC is better suited for volatilome analyses [43,44].

The optimisation of volatilome analysis is generally completed through the selection of the most appropriate collection sorbent. However, more recent work has demonstrated that the optimisation of other analysis parameters, such as type of column used, also affects volatilome analysis and reproducibility [45]. Additionally, a majority of volatilome analysis is confined to inert, or non-living specimens. Thus, the impacts of biological changes (*e.g.* response to stimulus, internal factors) to the reproducibility of volatilome collection and analysis have not been adequately assessed. In order to reduce the influence of biological responses to volatilome profiling, both acclimation time and headspace collection time must be optimised in order to collect VOCs in a reproducible manner. It is the aim of this work to develop sampling and analysis methods that are optimised for reptile volatilome collection for forensic purposes. Parameters with regards to volatilome sampling, desorption and analysis were optimised using GC×GC-ToFMS. For this study, volatilome collection was optimised in relation to the retrieval of VOCs associated with primary and secondary factors (*i.e.* diet), while limiting the retrieval of VOCs associated with tertiary factors (*e.g.*

defaecation, marking behaviour). This work was structured to be untargeted, while selective for volatilomic compounds that could be utilised for pre- and post-confiscation purposes to help combat the illegal trade in live reptiles.

2. Method development

2.1. Animal information

All sampling was conducted on living, captive and human-habituated reptiles from the Featherdale Wildlife Park collection in Sydney, Australia. Two captive Shingleback Lizards (*Tiliqua rugosa*; one male, one female), one Children's Python (*Antaresia childreni*; male) and one Eastern Blue-Tongue Lizard (*Tiliqua scincoides*, male) were used for this study.

2.2. Sampling method

A 60 cm (length)×50 cm (width)×50 cm (height) aluminium sampling container with a polycarbonate viewing window, side vent and dorsal sampling port was constructed for reptile volatilome sampling (Fig. 1; Supplementary Fig. 1).

The size of the container was selected to allow for adequate space for the reptile to move inside the container and to accommodate the varying sizes of reptiles. Prior to sampling live reptiles, two pure organic solvents were compared for potential use (high purity acetone and isopropyl alcohol) during the study. These solvents were required to serve as both sterilization and VOC mitigation agents for the container between sampling periods. Each solvent was used to wash the inside of the sampling container and was followed by a one-min period where the container was vented with a hair dryer. Sample periods were tested over durations of 10 min, 15 min and 20 min. The pure acetone was less persistent and was selected as the cleaning and conditioning agent for the remainder of the study (data not shown).

2.3. Sorbent tube selection

Two commercially available sorbent tubes were selected for comparison of reptile volatilome collection capabilities. The first was a Tenax® TA sorbent tube (Markes International Ltd, UK; parts number C1-AAXX-5003), which is a a general-purpose weak sorbent which can collect a range of volatiles (*e.g.* aromatics, polar and nonpolar) and semi-volatiles from the carbon range C7-C30. Additionally, a dual sorbent tube which combines Tenax® TA with a medium-to-strong sorbent type (Carbograph 5DT; *i.e.* "dual", Markes International Ltd, UK; parts number C2-AAXX-5149) was also tested. This sorbent combination allows for the additional collection of highly volatile compounds which extends the carbon collection range from C4-C32. Prior to sampling, both sorbent tubes were conditioned as per the manufacturer's instructions.

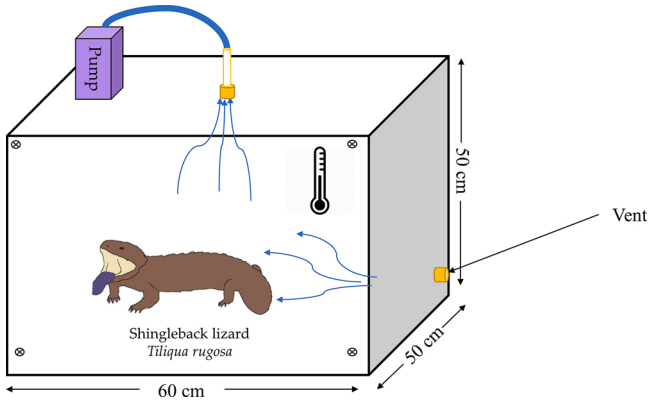

Fig. 1. Sampling diagram of sampling container used for reptile volatilome collection.

For the sorbent selection aspect of the study, one male Shingleback Lizard was acclimated for 20 min and three replicates of three different sampling times (10 min, 15 min and 20 min) at a rate of 100 ml/min were taken. If the animal crawled persistently on the window (*i.e.* escape behaviour) at any time during the acclimation or sampling period, sampling was abandoned and the animal was returned to its enclosure. Prior to each acclimation period, a blank sample of the container was taken for each of the sampling times in order to identify VOCs related to the empty container for downstream analysis. All sorbent tubes were sealed using brass caps after collection and wrapped in aluminium foil. All samples were analysed no later than one day following the day sampling date. Where storage was required, the capped tubes were stored at 4 °C for 12−20 hours.

2.4. Acclimation and sampling time

Three acclimation periods and two sampling intervals were tested. The 20-min sampling interval was abandoned after escape behaviour was documented during the sorbent selection trials. The Shingleback Lizards were individually acclimated in the sampling container for 15 min, 20 min and 25 min, respectively, prior to sampling. During the acclimation period, the biological activity (*e.g.* movement, secretions) and the temperature of the container were monitored to determine if these factors influenced the animal's biological profile. After each acclimation period, the individual Shingleback Lizard was sampled in triplicate for 10-min and 15-min intervals using an ACTI-VOC pump (Markes International Ltd., UK) at a rate of 100 ml/min. All sorbent tubes were sealed using brass caps after collection and were wrapped in aluminium foil after sampling. Between each sampling interval, and between each individual, the container was washed with acetone and vented for one min with a hair dryer and a container blank was taken. To help reduce VOC variability associated with diet, health or environmental factors, the sampling associated with each acclimation period was conducted on the same day.

2.5. Thermal desorption method

All samples were thermally desorbed using a Markes Unity 2 Thermal Desorber and Series 2 ULTRA multi-tube autosampler (Markes International Ltd, UK) with a Markes General Purpose Carbon C4/5-C30/32 cold trap (parts number U-T11GPC-2S). Seven thermal desorption methods were employed for sorbent tube desorption. The first two thermal desorption (TD) methods were optimised using Shingleback Lizard skin sheds (data not shown) to determine whether a split was required. No split was implemented due to the trace nature of the volatiles collected. The pre-desorption pre-purge time (2 min), the flow path temperature (150 °C), the tube desorption time (3 min), the desorption temperature (300 °C), the maximum trap temperature (300 °C) and the trap hold (4 min) for the TD method comparison were held constant for all methods. The tube desorption flow, trap flow and minimum trap temperature parameters were optimised for this study (Table 1). These parameters were optimised due to the differing strength of sorbents and the unknown boiling points of the compounds present in the samples. One male Shingleback Lizard was sampled in triplicate for each TD method using optimised acclimation and sampling periods. To

Table 1
Sample flow, trap flow, and minimum trap temperatures for the five tested TD methods (V1-V5).

	V1	V2	V3	V4	V5
Sample Flow (mL/min)	25	20	20	20	30
Trap Flow (mL/min)	25	20	25	20	30
Minimum Trap Temperature (C)	−17	−15	−15	−10	−15

reduce the variability between the TD methods, the Shingleback Lizard was sampled for five consecutive days. Each sample was analysed on the same day it was collected.

2.6. GC×GC separation method

All samples were analysed through comprehensive two-dimensional gas chromatography coupled with time-of-flight mass spectrometry using a Pegasus 4D GC×GC-ToFMS (LECO Australia Pty Ltd, Australia). Prior to injection, each sorbent tube was injected with 0.2 µl of d_5-chlorobenzene (CAS number 3114-55-4; Merck, AUS) which served as an internal standard. High purity helium was used as the carrier gas with a flow rate of 1 ml/min for the duration of the run. The first-dimension oven temperature was initially set to 35 °C with a hold of two min. The temperature was ramped at 7 °C/min to a target temperature of 80 °C with no hold. The temperature was then ramped at 10 °C/min to a target temperature of 240 °C with a 5 min hold. The secondary oven had a 10 °C offset relative to the GC oven temperature and the modulator had a 30 °C offset from the secondary oven temperature. The modulation period was 5 s with a hot pulse time of 1 s and a cool time of 1.5 s between stages. The transfer line between the secondary oven and the ion source was held at 250 °C. The acquisition rate was 100 spectra/s with a target mass range of 29–550 amu. The ion source was held at 200 °C with an electron ionisation energy at 70 eV. The detector voltage was programmed to have a 200 V offset to the optimised detector voltage.

2.7. GC × GC column selection

Individual representatives of three different reptile species (*i.e.* Shingleback Lizard, Eastern Blue Tongue Lizard and the Children's Python) were sampled in quintuplicate using the optimised parameters (*i.e.* sampling and acclimation period, TD method and separation method) for column selection. The three individuals were sampled over four consecutive days to reduce variation associated with biological status. Four different column sets were employed (Table 2) that all used a 1 m uncoated silica transfer line (Markes International Ltd, UK) which was joined to the primary column through the use of an Ultimate Union (Agilent Technologies, Australia). All secondary columns were attached to primary columns through the use of a SilTite µ-Union connector (SGE Analytical Science, Australia).

2.8. Data processing

The resulting chromatograms were analysed and aligned using ChromaTOF (version 4.51.6.0; LECO). For alignment, a signal-to-noise ratio (SNR) of 150 with a 0.8 baseline offset was used. Peak identification required a first-dimension peak width of 30 s and a second-dimension peak width of 0.15 s. Sub-peaks were identified using a minimum S/N of 6. The National Institute of Standards and Technology (NIST) Mass Spectral Library was used for compound identification with a required a minimum similarity match of 75 %. The Statistical Compare function of ChromaTOF was used to align peaks between samples using a match threshold of 60 %. After samples were aligned and all analyte peak areas were normalised to the corresponding internal standard peak areas. The resulting data was exported to Microsoft Excel (2010), where the data was manually filtered to remove contaminants (*i.e.* column bleeds, internal standard and solvents). All unidentified analytes were included in this analysis. The compounds related to the blank containers were removed using 50 % threshold values, adapted from Forbes et al. [39].

Principal component analysis (PCA) was conducted on data generated from the different column sets as a quality check using Unscrambler X® (version 10.5; CAMO Software, Norway). Prior to this analysis, the data was mean centred and scaled by standard deviations and normalised through unit vector normalisation.

Table 2
Specifications of each column set used for column selection.

	Primary Column	Secondary Column
Column set 1	Rxi-624 Sil MS (30 m×0.25 mm inner diameter (ID), 1.40 µm film thickness)	Stabilwax® (2 m×0.25 mm ID, 0.50 µm film thickness)
Column set 2	BPX5 (30 m×0.25 mm inner diameter (ID), 0.25 µm film thickness)	Stabilwax® (2 m×0.25 mm ID, 0.50 µm film thickness)
Column set 3	BPX5 (30 m×0.25 mm inner diameter (ID), 0.25 µm film thickness)	BPX90 (2 m×0.25 mm ID, 0.25 µm film thickness)
Column set 4	Rxi-624 Sil MS (30 m×0.25 mm inner diameter (ID), 1.40 µm film thickness)	BPX90 (2 m×0.25 mm ID, 0.25 µm film thickness)

3. Results and discussion

3.1. Sorbent selection

During the 20 min sampling intervals for both Tenax® TA and dual sorbents, the Shingleback Lizard expressed escape behaviours. For these reasons, triplicate samples were not taken and further analysis related to the 20 min sampling interval was abandoned. The 10 min sampling interval showed high variability between each triplicate sample for both sorbents (Supplementary Table 1). For both Tenax® TA and dual sorbent tubes, each replicate contained more volatiles than the one prior, with the last replicate retrieving two-to-three times more compounds than the first replicate. This may indicate that this sampling interval does not collect the entire volatilome during the first two replicates, reducing the reproducibility and reliability of the results. The 15 min sampling intervals had the lowest standard deviation between replicates for both sorbents, making this sampling duration the most suitable for volatilome profile collection (Table 3). Both sorbents were suitable for collecting compounds likely associated with the animal's diet, including fruit and meat products (Table 4). However, after the blanks were removed, the Tenax® TA sorbent indicated that compounds associated with plastics, acrylic polymers, acrylic stabilisers and metals were present in the volatilome profile (Table 4).

Given the association with synthetic materials, it is likely that these compounds originated from the sampling container rather than the Shingleback Lizard. This may suggest a differential uptake ability of the Tenax® TA sorbent towards these compounds during each sampling intervals as compared to the dual sorbent, as these compounds were removed during the data processing stage for the dual sorbents. These sampling irregularities produced by Tenax® TA may inappropriately indicate that these compounds are relevant to the animal's profile, which may complicate or prevent the accurate development of reptile volatilomes to be used in a screening context. The Tenax® TA sorbent also appeared to be more sensitive to secondary elements of the volatilome profile, such as behavioural changes, that do not exhibit utility for screening purposes. For example, both pheromones (*e.g.* 1-iodo-2-methylundecane [59]) and compounds related to femoral secretions from reptiles (*e.g.* dotriacontane, heptadecane, nonanal, eicosane, nonadecane, [60]) were detected more predominantly in the Tenax® TA

Table 3
Average amount of compounds collected from Tenax® TA and dual sorbents (n = 3 per sorbent).

Sampling Method	Average Number of Compounds Retrieved
Tenax® TA 20 min acclimation, 10 min sampling	152 ± 40
Tenax® TA 20 min acclimation, 15 min sampling	237 ± 46
Dual 20 min acclimation, 10 min sampling	130 ± 75
Dual 20 min acclimation, 15 min sampling	123 ± 36

Table 4
Compounds retrieved from Shingleback Lizard collection using both sorbent types.

Odour association	Compound name	Sorbent	Reference
Fruit	trichloromonofluoromethane, toluene, methylcyclohexane, cyclohexane, o-cymene, methyl propionate, 3-methylheptyl acetate	Tenax® TA, Dual	[46,47,48, 49]
Meat Products	2-ethyl-1-hexanol, 9-octadecenal, pentadecane, heptanal, pentanal, 4,8-dimethyl-tridecane, 2,8-dimethyl-undecane	Tenax® TA, Dual	[50,51,52]
Compounds likely originating from container	4-methyl-2-heptene, cyclopropanemethanol, 2,2,3,3-tetramethyl-, α-methylstyrene, butyl 2-ethylhexanoate, 2-ethylhexyl acrylate, methyl methacrylate, 1-propyldecylbenzene, 1-propyloctylbenzene, 1-butylheptylbenzene, 1-butylnonylbenzene	Tenax® TA	[53,54,55, 56,57,58]

sorbents as compared to the dual sorbents. Some of these compounds were also retrieved by the dual sorbents (*e.g.* heptadecane, eicosane, nonadecane) but not to the extent retrieved by Tenax® TA sorbents. Although the dual sorbent collected less compounds, it appeared to be less susceptible to compounds released from the container and biological changes within the animal. For these reasons, the dual sorbent was selected as the more suitable collection material for forensic volatilome profiling of reptiles.

3.2. Acclimation and sampling time interval selection

Throughout all acclimation periods and sampling times, the temperature of the container ranged from 17−24 °C. The 20 min sampling period was not tested further due to the escape behaviour expressed by the animals as detailed in Section 3.1. After the compounds originating from the blanks were removed, the 10 min sampling interval retrieved the lowest quantity of compounds with the largest amount of variation despite increased acclimation periods (Table 5). The high variability suggests that the entire volatilome is not collected within the first two replicates. Alternatively, it could be that the 10 min sampling interval is susceptible to changes in external environmental or internal biological conditions between samples. The 15 min sampling intervals were then compared across all acclimation periods (Table 5). The 20 min acclimation and 15 min sampling intervals exhibited the lowest variance between sampling replicates (For information regarding the change in quantities of compounds from each chemical class, please see Supplementary Table 2). This indicated that these acclimation and sampling intervals retrieve the most consistent and reproducible volatilome profile for both male and female Shingleback Lizards. For these reasons, the 20 min acclimation period and 15 min sampling time were selected as the optimised sample collection intervals.

Table 5
Number of compounds retrieved using 15, 20, and 25 min acclimation times with 10 and 15 min sampling intervals (n = 3 per animal). X represents data that was not taken.

	Sampling interval (min)		Sampling interval (min)	
Acclimation period (min)	10 (Male)	10 (Female)	15 (Male)	15 (Female)
15	32 ± 9	99 ± 20	177 ± 61	X
20	144 ± 7	242 ± 82	131 ± 31	133 ± 16
25	133 ± 90	181 ± 35	215 ± 50	193 ± 59

3.3. Thermal desorption

The total number of compounds retrieved from each thermal desorption method are displayed in Fig. 2. Both the V2 and V4 methods were not as effective at retrieving aromatics, esters, ketones and aldehydes as compared to the other TD methods (Fig. 2). V2 was also ineffective at retrieving terpenes. Both of these methods (V2 and V4) had a sample desorption and trap flow rate of 20 mL/min. The flow rate from the trap may have been too low to allow for the full retrieval of sample compounds for these methods. Thermal desorption methods V1, V3, and V5 were very similar in compound retrieval and reproducibility success (Fig. 2). Of these three methods, the V3 TD method was selected as it was able to retrieve ketones, terpenes and esters, which are known to be odourous compound classes [61]. Additionally, the esters retrieved are likely related to diet (Table 4) which can be informative for wildlife crime investigations.

3.4. Column selection

The optimal column combination for this study was selected based on the separation ability and sensitivity of each column set. The separation abilities were assessed through visual examination of total ion chromatograms (TIC; (Fig. 3)). The sensitivities were assessed through averaging the number of compounds identified per compound class (Table 6 and 7) and through the summation of the normalised mass areas for each represented chemical class (Supplementary Table 3). Column set 4 was immediately disregarded due its poor second dimension separation ability (Fig. 3D). Column set 2 was not selected due to the lack of sensitivity to the Children's Python's volatilome profiles (only tentatively identifying an average of 15 compounds after blank removal (Supplementary Table 4)). Between the remaining column sets, column set 1 had the highest sensitivity in terms of number of compounds retrieved per chemical class across all reptile volatilomes (Table 6 and 7). Additionally, this column set combination retrieved more compounds with larger total summed average mass areas in comparison to column set 3 (Supplementary Table 3). This indicates that this column set may be able to differentiate and detect more compounds with lower relative abundances than the other tested column sets. The improved sensitivity of column set 1 is likely due to the increased film thickness of the first dimension Rxi-624 Sil MS column and the second dimension Stabilwax® column, which is known to increase the resolution of compounds with a low retention factor, such as VOCs [62].

The total volatilome profile generated per species was then compared to assess the influence of column set 1 and 3 to volatilome composition (Fig. 4). The data generated from column set 1 showed a clear distinction in volatilome profiles between reptile species, both in total number of compounds (Table 6 and 7) and based on the summed area of each compound class (Fig. 4A-C). However, using data generated by column set 3, the differences of volatilome profiles became less distinct (Fig. 4D-F). The disparity in total profile composition retrieved using each column set highlights the importance of trace volatiles to total profiles. Without rigorous selection criteria of column sets, the volatilome information generated per individual may be misrepresented. To further determine the influence of the volatilome profile differences between each column set, principal component analysis was conducted. The PCA for column set 1 (Fig. 5A) demonstrated that the volatilome between the three individuals of each species was reasonably different. The compounds responsible for the separation between the Shingleback Lizard and the Children's Python for PC-2 included a suite of terpenes, ketones and esters (*e.g.* α-terpineol [63], allyl nonyl oxalate [64], acetophenone [65], calamenene [66], diethyl carbonate [67]) and additional compounds that could be related to diet, or metabolic waste processes, (*e.g.* 2-pentyl-furan [68,79]), benzylaldehyde [65]. Additionally, a suite of benzene compounds also contributed to this separation. This included 1,2,3-trimethylbenzene and 1-ethyl-2-methylbenzene potentially related to biological activity [69]

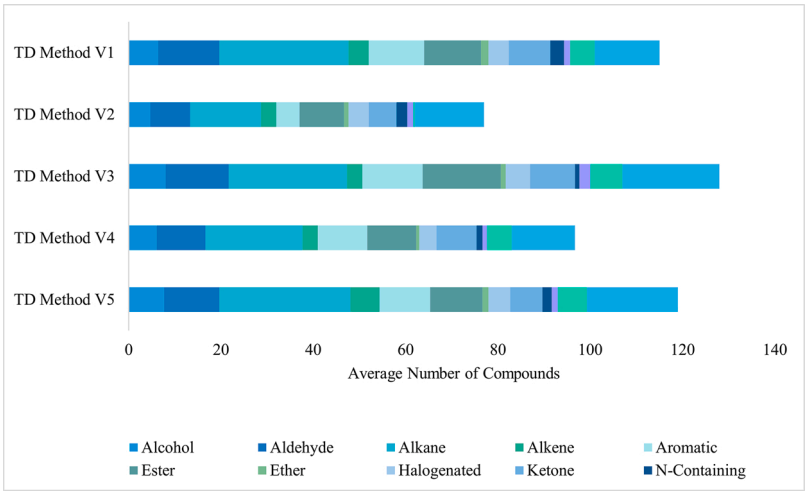

Fig. 2. Average number of compounds retrieved per chemical class using each TD method (n = 3).

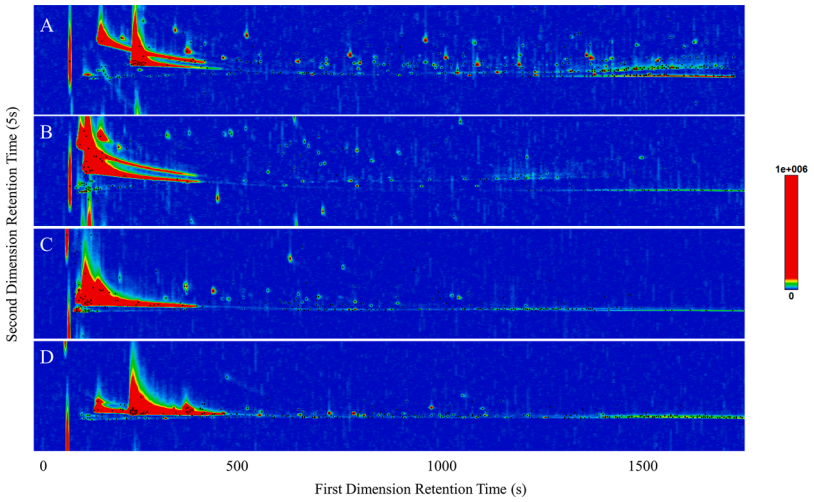

Fig. 3. Comparison of chromatograms generated from Shingleback Lizard volatilomes using column set 1 (A), column set 2 (B), column set 3 (C) column set 4 (D).

and 1-methyldecylbenzene, 1-pentylheptylbenzene and 1-ethyl-3-methylbenzene potentially related to diet [70,71]. The Shingleback Lizard clustering in PC-2 (Fig. 5A) was attributed more to hydrocarbons, potentially associated with the Shingleback Lizard's marking behaviour or diet such as dodecane, nonadecane, tetradecane, heptadecane, and dodecanoic acid, methyl ester [60,72].

In the PCA produced for column set 3 (Fig. 5B), only three compounds were responsible for the clustering of the Shingleback Lizard and Children's Python in PC-1 (one unidentified analyte, o-xylene, and 2,4-dimethyl-2-pentene). Some of the clustering seen in PC-2 between the Eastern Blue Tongue Lizard, Shingleback Lizard, and Children's Python was associated with dietary compounds (*e.g.* limonene, acetophenone [65], 6-methyl-5-hepten-2-one [73], undec-10-enyl pentanoate [74]). A suite of benzene compounds was responsible for the clustering of the two Shingleback Lizard samples and the Children's Python sample in PC-2. These included as previously seen compounds related to metabolism (*i.e.* 1,2,3,4-tetramethylbenzene, 1-ethyl-2-methylbenzene, 1,2,3,5-tetramethylbenzene, 1-ethyl-2,3-dimethylbenzene [69]). The increased influence of these compounds to the column set 3 PCA may be associated to the lowered sensitivity of this column set to other compounds. Thus, the combination of Rxi-624 Sil MS as a first-dimension column and Stabilwax® as a second-dimension column was selected as the most robust column set to distinguish reptile volatilome profiles, both in terms of number and abundance of VOCs. However, due to the high dimensionality of the data, and the log-scale differences in relative abundance between compounds, more robust statistical analysis is required to determine the influence of each compound to the total profile.

4. Conclusions

This work presents a novel workflow that was optimised to collect and analyse volatilomes from live animals, an area that has thus far been lacking in stringent and complete method optimisation. This study specifically targeted mid-sized reptiles for the development of a standardised workflow that can be applied for untargeted and reproducible volatilome assessment. It is recommended that future work directed towards the optimisation of volatilome collection and analysis from other species of live animals follow this workflow. The aim of this work was to standardise a volatilome collection method which has limited influences from secondary or tertiary factors that may reflect the biological status of the animal (*e.g.* metabolism, health status). Previous literature has demonstrated the utility of Tenax® TA sorbents for monitoring metabolic changes or chemical signalling within organisms [75,76]. However, this study aimed to develop methods to collect data that may be relevant for further wildlife crime investigations, such as determining whether an individual is wild caught or captive bred. As a result, the dual sorbent was determined to be the most appropriate

Table 6
The average number of compounds per class retrieved per species using column set 1.

		Alcohol	Aldehyde	Alkane	Alkene	Alkyne	Anhydride	Aromatic	Ester	Ether	Halogenated	Ketone	N-Containing	S-Containing	Terpene	Unknown
Shingleback Lizard	**Average number of compounds**	12 ± 2	13 ± 4	44 ± 7	9 ± 2	–	–	28 ± 3	15 ± 3	3 ± 0	8 ± 1	11 ± 2	5 ± 1	1 ± 0	3 ± 0	35 ± 6
Eastern Blue Tongue Lizard	**Average number of compounds**	5 ± 3	7 ± 1	15 ± 6	6 ± 2	–	0 ± 1	4 ± 2	6 ± 2	–	4 ± 1	2 ± 2	3 ± 1	1 ± 0	–	24 ± 4
Children's Python	**Average number of compounds**	10 ± 1	13 ± 2	23 ± 5	6 ± 2	–	1 ± 1	22 ± 5	15 ± 2	1 ± 1	5 ± 1	13 ± 2	4 ± 1	1 ± 0	2 ± 1	27 ± 6

Table 7
The average number of compounds per class retrieved per species using column set 3.

		Alcohol	Aldehyde	Alkane	Alkene	Alkyne	Anhydride	Aromatic	Ester	Ether	Halogenated	Ketone	N-Containing	S-Containing	Terpene	Unknown
Shingleback Lizard	**Average number of compounds**	6 ± 2	7 ± 3	18 ± 8	5 ± 2	3 ± 1	–	23 ± 6	9 ± 4	–	3 ± 2	3 ± 3	6 ± 3	1 ± 1	5 ± 0	22 ± 6
Eastern Blue Tongue Lizard	**Average number of compounds**	6 ± 1	9 ± 1	16 ± 1	3 ± 1	3 ± 1	–	13 ± 2	15 ± 1	–	4 ± 2	6 ± 1	8 ± 2	1 ± 0	6 ± 0	20 ± 4
Children's Python	**Average number of compounds**	5 ± 2	7 ± 2	17 ± 5	5 ± 1	2 ± 1	–	21 ± 7	12 ± 4	–	3 ± 1	4 ± 2	7 ± 2	1 ± 0	4 ± 1	20 ± 3

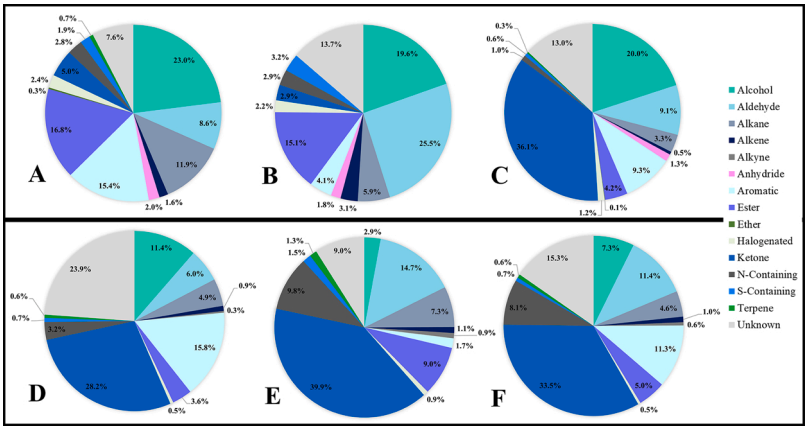

Fig. 4. Total volatilome profiles of sampled reptiles using average summed areas per compound classes. The total profiles determined through column set 1 include the Shingleback Lizard (A), Eastern Blue Tongue Lizard (B), and Children's Python (C). The total profiles determined using column set 3 included the Shingleback Lizard (D), Eastern Blue Tongue Lizard (E) and Children's Python (F).

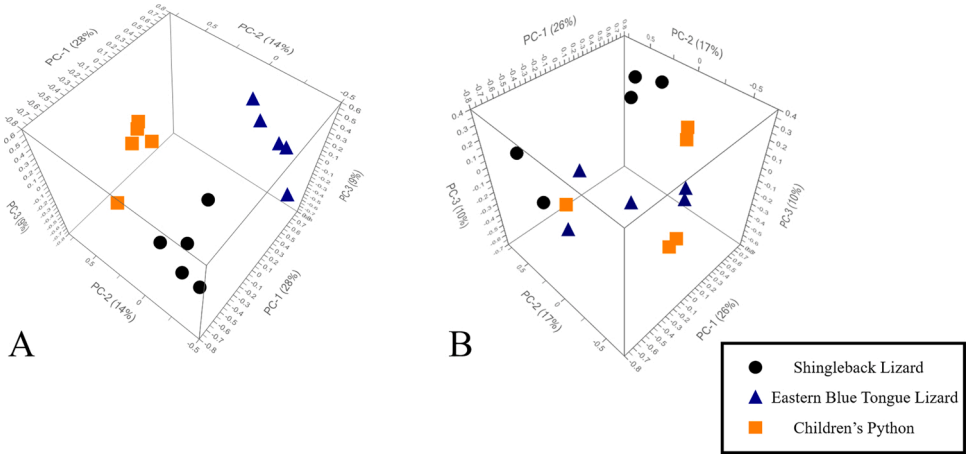

Fig. 5. Three-dimensional principal component score plots generated from the volatilomes of a Shingleback Lizard, Eastern Blue Tongue Lizard, and Children's Python analysed using column set 1 (A) and column set 3 (B).

collection material for forensic purposes. This study also determined that the acclimation and sampling time periods of 20 min and 15 min, respectively, were optimal for the most effective volatilome collection criteria for medium sized reptiles. The importance of column selection for non-targeted volatilome assessment was also highlighted in this study. Columns that are specifically suited for VOC separation, with increased film thickness should be strongly considered when working with trace volatile compounds. The potential loss of information associated with poor column selection may severely impact the assessment of volatilome profiles. Through this work, it was determined that the combination of Rxi-624 Sil MS and Stabilwax® column sets were optimal for separating VOCs related to Shingleback Lizards and similar reptile species.

Only adult, well-handled animals with no known diseases were sampled during this study. Due to the limitations of acquiring representative animals from all classes (*e.g.* sex, immune/disease status, age class), this study optimised sample collection to reduce variability in association with metabolic and environmental changes. Given the influence of compound presence and relative abundance to volatilome profiles, a much larger sample size will need to be created for next order validation processes, such as biomarker selections and determination of method sensitivity. Furthermore, due to the high dimensionality of the generated data, more rigorous statistical methods should be employed for volatilome analysis, including determining compounds of interest relating to species separation, diet, or reptile specific biomarkers.

In this study, the volatilomes profiles of the sampled animals did not appear to be influenced by the temperature range of the sampling container. It appeared instead that compounds potentially associated with diet were responsible for the separation of these species during this preliminary investigation. The presence and analysis of potential dietary compounds may provide research opportunities for determining whether VOC profiling can be utilised to determine the captive history of confiscated animals in transit. For example, unique dietary compounds from wild caught animals may differentiate from those that have a regularly supplemented or controlled diet. This is especially pertinent for reptiles as it is suspected that 50 % of individuals, and 90 % of reptile species, found in the illegal wildlife trade are wild-caught [6] and the laundering of wild-caught reptiles as captive bred is increasing in the illegal wildlife trade [77]. The influence of dietary compounds opens an additional research avenue to determine whether volatilome profiles can be utilised to determine the geographic origin of confiscated individuals. This type of research will require extensive datasets that are generated from captive bred or held representatives, as well as datasets have been built from wild populations across the species range. The data generated from volatilome profiling is of high dimensionality and can have scalar differences between the relative abundances of compounds. For this reason, more rigorous statistical methods will need to be employed to determine whether it is the presence or absence of certain compounds, or their relative abundances, that are responsible for volatilome separations.

Due to the limitations of detecting wildlife contraband in transit, priorities have been placed on detecting illegal wildlife contraband in both destination and origin countries [8]. Both enforcement and regulatory bodies are ill equipped with validated technologies that can aid in reducing the illegal wildlife trade [78]. The use of VOC profiling, and developing a portable electronic device to detect selected reptile biomarkers may aid in detecting illegally trafficked live reptiles. The development of such devices relies on processes that are optimised and validated for volatilome collection and analysis, so that relevant biomarkers can be selected. This work provides the basis for volatilome database establishment for medium sized reptiles and will further be developed in order to determine method sensitivities and biomarker selection.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.fsiae.2021.100024.

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