Reducing variation in decomposition odour profiling using comprehensive two-dimensional gas chromatography

Running title: Decomposition odour profiling using GC×GC

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Nonstandard Abbreviations:

1D: First dimension, primary; 2D: Second dimension, secondary; DMS: Dimethyl sulphide; EPA: Environmental Protection Agency; FR: Fisher ratio; GC-MS: Gas chromatography – mass spectrometry; GC×GC-TOFMS: comprehensive two-dimensional gas chromatography – time of flight mass spectrometry; HPLC: High-performance liquid chromatography; PCA: Principal component analysis; PTFE: Polytetrafluoroethylene; TD: thermal desorption; VOCs: Volatile organic compounds

Keywords: Decomposition odour profiling, Forensic chemistry, GC×GC-TOFMS, Non-target data handling, Volatile Organic Compounds

Abstract

Challenges in decomposition odour profiling have led to variation in the documented odour profile by different research groups worldwide. Background subtraction and use of controls are important considerations given the variation introduced by decomposition studies conducted in different geographical environments. The collection of volatile organic compounds (VOCs) from soil beneath decomposing remains is challenging due to the high levels of inherent soil VOCs, further confounded by the use of highly sensitive instrumentation. This study presents a method that provides suitable chromatographic resolution for profiling decomposition odour in soil by comprehensive two-dimensional gas chromatography – time of flight mass spectrometry (GC×GC-TOFMS) using appropriate controls and field blanks. Logarithmic transformation and t-testing of compounds permitted the generation of a compound list of decomposition VOCs in soil. Principal component analysis demonstrated the improved discrimination between experimental and control soil, verifying the value of the data handling method. Data handling procedures have not been well documented in this field and standardisation would thereby reduce misidentification of VOCs present in the surrounding environment as decomposition by-products. Uniformity of data handling and instrumental procedures will reduce analytical variation, increasing confidence in the future when investigating the effect of taphonomic variables on the decomposition VOC profile.

1 Introduction

Characterisation of the odour produced during mammalian decomposition has been ongoing for the past decade, presenting many analytical challenges. A comprehensive and accurate chemical representation of this odour is imperative for understanding carrion insect semiochemistry, mammalian thanatochemistry, and for the search and recovery of human remains. A suitable method for the analysis of decomposition volatile organic compounds (VOCs) is essential for advancing this knowledge base.

Numerous publications have resulted from the investigation of the decomposition VOC profile [1–21]. Initial studies examined the chemical classes and trends that existed over the various stages of decomposition. Nonetheless, the overall decomposition VOC profile is still poorly understood due to the wide variation of VOCs reported. The detection of core decomposition VOCs has been proven to be reproducible in different geographical regions [14,20,22]. Although the taphonomic variables related to the decomposition process (i.e. biomass, climate, season, etc.) can affect the VOC profile, differences in analytical parameters can also be a source of variation. Uniformity among the analytical methods employed throughout such studies would improve the ability to subsequently discriminate variation resulting from taphonomic variables.

The matrix analysed, the collection of VOCs from the matrix, and the instrumentation chosen are all variable factors that can have substantial effects on the analytical results obtained. Variation introduced by matrix and VOC collection techniques have recently been conducted [20,21]. Sorbent tubes are now widely used to collect decomposition VOCs due to analytical advantages such as sample pre-concentration, lack of chromatographic artefacts, stability and ease of use in the field [15,17–21,23]. While there is a move towards collection method standardisation, variation in instrumentation usage has yet to be addressed.

Due to the widespread use and advantages associated with collection of VOCs using sorbent tubes, sample introduction onto chromatographic instrumentation is well established using thermal desorption (TD) [15,17–21,23]. Early studies used conventional gas chromatography coupled with mass spectrometry (GC-MS) [1–12,16], while recent investigations have moved towards the use of comprehensive two-dimensional gas chromatography – time of flight mass spectrometry (GC×GC-TOFMS) [13–15,18,19]. The complexity of the decomposition VOC profile noted in early literature has caused this shift for benefits of providing higher peak capacity, enhanced sensitivity, improved characterisation of dynamic range as well as producing structured chromatograms. The benefits of GC×GC-TOFMS cannot be disputed and are now well documented [13–15,18,19,22]. Although more sophisticated analytical instrumentation can be advantageous, new challenges are also inevitable. The use of two columns in tandem with GC×GC presents many potential combinations, few of which have been used for the analysis of decomposition VOCs. Higher sensitivity increases the background VOC profile present and does not selectively target compounds of interest. Control sample complexity is therefore increased and challenges discrimination of background VOCs from carrion VOCs.

Presence of a complex background VOC profile is especially challenging when sampling is performed from soil. The collection of decomposition VOCs from soil can be beneficial due to a wider characterisation of VOCs resulting from microbial metabolism [20,21]. Many VOCs identified from mammalian decomposition are found in common with background VOCs from soil. Sample replicates, appropriate control measures and proper data handling are necessary to distinguish between VOCs found naturally in soil and those that are contributed by decomposition. Most studies have not specified measures for attributing compound identification to the decomposition process rather than background VOC reference. As such, establishing background VOC profiles has likely been performed manually, producing variation across studies and creating potential for compound misidentification as resulting from the decomposition process.

The objective of this study was to present a method for the analysis of decomposition VOCs in soil by GC×GC-TOFMS with appropriate control measures for establishing removal of background VOCs. Soil is a complex matrix, exhibiting its own inherent VOC profile that overlaps in many aspects with the VOC profile of decomposition. During active decomposition, the influx of decomposition by-products into the soil causes the decomposition VOC profile to be readily detectable above background VOCs. As time progresses, the influx reduces as post-mortem period increases or if the remains are removed by animal scavenging. Distinguishing background VOCs from decomposition VOCs is more difficult under these circumstances. Hence, developing a suitable analytical method and data handling approach is necessary for the detection of trace decomposition VOCs from soil due to the challenging nature of this matrix.

2 Experimental

2.1 Sample collection

An outdoor research trial was performed using four pig (*Sus scrofa domesticus*) carcasses weighing approximately 70 kg each deposited on the soil surface. The facility was located at an open eucalypt woodland site with sandy clay topsoil on the outskirts of Sydney, Australia. A 3-month period of carrion decomposition was performed (January-April 2013) allowing sufficient influx of decomposition by-products into the surrounding soil. A 30 cm VOC-Mole™ Soil Probe (Markes International Ltd., UK) was inserted into the ground near the torso of each carcass at each experimental site (and at the centre of each control site) to collect VOCs from the soil. A Tenax TA/Carbograph 5TD dual sorbent tube (Markes International Ltd.) was connected to the external side of the VOC-Mole™ soil probe cap and soil gas was collected using an ACTI-VOC air pump (Markes International Ltd.) for 15 minutes at 100 mL/min. Details of the setup used in this study have been previously reported [20]. Sorbent tubes were sealed with brass storage caps, wrapped in aluminium foil, and transported to the laboratory in an airtight glass jar. VOC samples were collected after 3 months of decomposition (t0) when the remains were skeletal. To mimic the animal scavenging process, remains were manually removed from each site after 3 months (t0). VOC samples were collected at t0 immediately following removal of the remains and again after 3 months (t1) and 7 months (t2) to investigate the trace decomposition odour profile in the soil.

2.2 Control and blank collection

Control samples were collected at t0, t1 and t2 in the previously described manner from four control sites (approximately 20 m from carrion sites) containing no carcasses in order to represent the natural variation in the background soil VOC profile. Two field blank samples were also collected on each sampling day: one prior to sample collection and one after sample collection. A blank sorbent tube was opened for 10 seconds in an area adjacent to the control and experimental sites. Field blanks are commonly used as a control measure for environmental air monitoring in accordance with the Environmental Protection Agency (EPA) Compendium Method TO-17 [24] to characterise artefact levels from capping and uncapping sorbent tubes in the field and laboratory. Field blank tubes were transported and stored with sample tubes to account for contamination and artefacts that were not associated with the sample.

2.3 GC×GC-TOFMS analysis

Each sorbent tube was injected with 2 µL of 150 ppm bromobenzene (GC grade, Sigma Aldrich, Australia) in methanol (HPLC grade, Sigma Aldrich) using an eVol® XR hand-held automated analytical syringe (SGE Analytical Science, Australia) to allow for internal standard normalisation. Blank tubes containing internal standard (i.e. ‘internal standard blanks’) were analysed in order to identify low-level artefacts associated with internal standard injection. Compounds collected on sorbent tubes were thermally desorbed using a Unity 2 Thermal Desorber equipped with a Series 2 ULTRA™ multi-tube autosampler (Markes International Ltd.). The Unity 2™ was connected to the Pegasus® 4D GC×GC-TOFMS (LECO, Australia) using a 1 m uncoated fused silica heated transfer line (Markes International). Sorbent tubes underwent desorption for 4 minutes at 300°C onto a general purpose cold trap (Tenax TA/Carbograph 1TD) at -10°C followed by trap desorption for 3 minutes at 300°C. A split flow of 20 mL/min was used at the cold trap.

In the first dimension (1D) an Rxi®-624Sil MS (30 m × 0.250 mm ID, 1.40 µm film thickness) column was used and in the second dimension (2D) a Stabilwax® (2 m × 0.250 mm ID, 0.50 µm film thickness) column was used (Restek Corporation, Australia). The 1D column was affixed to the TD transfer line using an Ultimate Union kit (Agilent Technologies, Australia) and to the 2D column using a SilTite™ µ-Union (SGE Analytical Science, Australia). Helium (high purity, BOC, Australia) was used as the carrier gas with a constant flow rate of 1.0 mL/min. The primary GC oven was held initially at 35°C for 5 minutes, followed by an increase to 240°C at 5°C/min, and was held at 240°C for 5 minutes. The modulator and secondary oven temperature offsets were 5°C and 15°C, respectively. The modulation period was 5 seconds with a hot pulse of 1 second. The MS transfer line was held at 250°C and mass acquisition was performed with a range between 29-450 amu at 100 spectra/second. The ion source temperature was 200°C and the electron ionization energy was -70 eV. A 200 V offset above the optimized detector voltage was used.

2.4 Column suitability

As this column combination has not been previously used to analyse decomposition VOCs, column suitability was assessed using a test mix adapted from the Philips mix [25]. The mixture contained a range of alkanes, alkylbenzenes, aromatic hydrocarbons, heterocyclic aromatics, chlorinated hydrocarbons, ketones, aldehydes, organosulphurs, phthalates, primary alcohols, secondary alcohols, fatty acid methyl esters, phthalates and Grob test mix compounds (see supplementary information, Table S1). A 100 ppm test mix in carbon disulphide (anhydrous, Sigma Aldrich) was analysed (n=5) by injecting 1 µL of the test mix onto a sorbent tube and using the specified method (section 2.4). Use of chromatographic space and separation of analytes of interest were assessed based on the 84 chemical standards provided by this text mix.

2.5 Data processing and analysis

ChromaTOF® (version 4.50.8.0, LECO, Australia) was used for data processing. Baseline tracking was performed with an 80% offset and automatic baseline smoothing. A 30 second peak width in 1D and 0.15 second peak width in 2D was used. A 150 signal-to-noise ratio (S/N) was used with a minimum similarity match > 800 to the NIST (2011) mass spectral library database. Peak identification was also confirmed by retention indices using the test mix described in 2.3. Peak areas were calculated using unique mass to allow peak alignment using the Statistical Compare software in ChromaTOF®. Samples from each day were input into Statistical Compare and separated into three classes (experimental, control, or field blank). Analytes were aligned and normalised using the internal standard peak. A 50% filter was applied to the data and peaks >20 S/N were included for analytes not identified by initial peak finding.

Output from Statistical Compare was exported into Microsoft Excel. All raw data was first investigated to see whether discrimination between control and experimental samples existed without data filtration. In order to further investigate trends based on specific compounds of interest, raw data for experimental and control replicates were logarithmically transformed and compared using an independent student’s t-test. An analyte was retained when exclusive to experimental samples or significantly higher than in control sites (using the student’s t-test, p < 0.05). The raw data was mean-centred and analysed using principal component analysis (PCA) in The Unscrambler® X (version 10.3, CAMO Software, Norway). This allowed for reduced data dimensionality, increasing the ability to visualise the data on PCA plots of scores and loadings. In this manner, relationships between experimental and control samples could be recognised based on compounds with the highest discriminatory power. The data filtration strategy could be assessed based on whether experimental and control soils could be distinguished at each time point.

3 Results and Discussion

3.1 Column suitability

Due to the fact that there are limited studies available in the literature for reference, this column combination (Rxi®-624Sil MS × Stabilwax®) was chosen based on the range of compounds reported in decomposition odour as well as the volatile nature of the analytes. The peak apex plot for the column suitability test mixture can be seen in Figure 1 and demonstrates the column performance. Many of the compound classes represented typical compounds expected in the decomposition soil analysis or those that may interfere with target analytes. For example, although phthalates were not considered to be compounds of interest, adequately separating phthalates that may have resulted from contamination was deemed important for accurately analysing compounds of interest. Overall, the separation of the test mixture provided good use of the chromatographic space while still providing a reasonable run time (51 minutes). In comparison to peak apex plots published previously in this field [13–15], superior use of chromatographic space occurred with this column set, especially for polar compounds. In the past, researchers have opted for a non-polar 1D column in combination with a mid-polar 2D column. The mid-polar 1D column (Rxi®-624Sil MS) used in this study provided improved 1D separation due to the exploitation of dipole-dipole interactions typical of compounds containing nitrogen, oxygen and sulphur that are common components of the decomposition VOC profile. The polar 2D column (Stabilwax®) provided improved separation of polar alcohols and aromatics.

3.2 GC×GC-TOFMS Analysis

While it has been mentioned in previous reports that GC×GC-TOFMS provides many benefits for decomposition odour analysis [13–15,18,19], direct comparison of samples analysed by GC-MS and GC×GC-TOFMS has not been demonstrated in the literature. Figure 2 represents a GC×GC surface plot for a carrion soil sample collected at t0. A trace showing the sum of the first dimension is represented in the back of the surface plot, depicting a theoretical one-dimensional representation of this sample. Although the one-dimensional trace looks relatively simple with few components, the surface plot clearly shows that many trace compounds are present in the sample. This demonstrates the potential for compound masking to occur in traditional GC-MS or the potential for trace compounds to appear hidden in the baseline.

Another benefit of GC×GC-TOFMS was the ability to separate early eluting VOCs from the solvent peak (which results from the internal standard injection). In GC-MS, separation of the many early-eluting compounds is challenging and may require cryo-cooling of the GC oven. Dimethyl sulphide (DMS) is identified in Figure 2 which has been previously noted as one of the predominant organosulphur compounds in decomposition odour [3,9]. This compound has a particularly low boiling point (37°C) meaning that it will elute very early in the analysis thereby coeluting with other light volatiles such as ethanol or methanol. Without the capabilities of GC×GC-TOFMS, peak identification of DMS would have been challenging. Even if deconvolution allowed for identification of DMS, accurate quantification would be challenging using traditional GC-MS due to the high amount of co-elution.

Representative contour plots obtained for each sample type (i.e. experimental, control and field blank) are shown in Figure 3. Many components were identified in the control and field blank samples (Figure 3b and 3c), demonstrating the increased complexity in the detection and identification of the background VOC profile. Many trace level components in Figure 3a can be attributed to background soil VOCs. Hence, it was important to develop a data filtration strategy to appropriately distinguish between the background VOC profile and analytes of interest.

3.3 Establishing a background VOC profile

In order to pre-filter data, a t-testing approach was carried out. While other studies have investigated the use of a fisher ratio (FR) threshold [19], the availability of replicate experimental and control data made it possible to perform a student’s t-test in this study. This is ideal because a threshold does not need to be chosen and the decision to retain or remove a compound is based on the replicate measurements distribution. Field and internal standard blanks were used as references after filtration to remove remaining VOCs that were not representative of decomposition following t-testing. Performing this step after t-testing resulted in fewer manual comparisons. Compounds that resulted from the internal standard solvent and from thermal desorption artefacts were removed. Manual comparisons can only be useful if the procedures are well described and can be replicated in future studies. The compounds removed manually were: acetonitrile, methyl isocyanide, diisobutyl phthalate, methylene chloride, 1,3,5-trifluorobenzene, carbon tetrachloride, and 1,1,2-trichloro-1,2,2-trifluoroethane. Polyfluorinated and polychlorinated compounds have been noted as artefacts of thermal desorption from Tenax TA [26,27]. These compounds were therefore excluded because their identification as decomposition by-products could not be made conclusively. Previous studies have identified some of these compounds (i.e. trifluorobenzene, carbon tetrachloride and 1,2,2-trichloro-1,2,2-trifluoroethane) as biomarkers of decomposition [1,4,12], yet a plausible mechanism for their release from the body as decomposition by-products has not yet been elucidated. It is therefore more likely that these compounds resulted from degradation of system components such as polytetrafluoroethylene (PTFE) o-rings in the thermal desorption unit. The high sensitivity of the GC×GC-TOFMS system means these artefacts are more likely to be detected and therefore caution must be used when classifying them as decomposition VOCs.

High variation in experimental soils meant that t-testing attempts on raw data excluded compounds that have been previously linked to decomposition and appeared to be elevated. Data transformation is commonly applied prior to statistical analysis to improve normality and reduce violations in assumptions of symmetry. Log-transformed data is used in chemistry and other life sciences for the purpose of stabilising variance in concentration measurements [28,29]. The student’s t-test requires homogeneity of variance between groups, and this transformation improved determination of data significance by reducing the large variance in experimental samples.

Once transformed data was t-tested, data visualisation by PCA allowed determination of whether experimental and control soils could be distinguished. Figure 4 demonstrates the data grouping without any data pre-treatment at t1, as well as after data treatment was undertaken. Because the carcasses had been artificially scavenged 3 months prior to sample collection, this time point represented how well the filtration performed once the influx of decomposition by-products into the soil had ceased. Prior to data treatment, distinct grouping of the samples did not exist, showing limited distinction between sample types. However, once insignificant compounds were removed, the discrimination between samples improved and a difference between the experimental and control samples existed, especially for two of the experimental soil samples that are distinctly separate from the controls along the first principal component axis (PC-1). Comparable results were also obtained at t0 and t2 (Figure S1 and Figure S2 available in supporting information).

The use of too many variables can cause overfitting of the PCA model [30–32]. The presence of “noise” variables reduces the ability to characterise the variables that underlie the true relationship. This study exhibits a statistically low number of replicates (i.e. 4 pigs) and many outcome variables that are measured (i.e. VOCs detected). This represents a situation which complicates traditional statistical approaches [29]. The reduction of background VOCs that fluctuate based on unrelated factors was useful in determining relevant compounds in the decomposition odour profile. Filtration of control compounds in this manner can be easily applied to prevent misidentification of compounds in control samples as being decomposition-specific.

3.4 VOC profile of soils

The described instrumental analysis and data handling procedure were used to determine key VOCs at each time point (Table 1). Before compound filtering, a total of 73, 54, and 136 components were identified at t0, t1, and t2, respectively. Following filtering, the number of VOCs was reduced to 41, 24, and 14 at each of the time points, respectively. The number of compounds identified after filtration does indeed indicate that the decomposition odour decreased following artificial scavenging of the remains. Nearly all compounds reported in this investigation have been previously noted in decomposition odour research (Table 1), providing further support for the success of the data handling approach.

An important consideration lies in the experimental setup, whereby multiple control sites were used to evaluate natural fluctuations in soil VOC profiles. Without analysis of replicate control sites, statistical approaches for removing background VOCs are not possible. Many previous studies have overlooked the importance of measuring multiple controls and make comparisons to only one or two controls. Considerable variation in control soils was seen (Figure 4a) prior to background VOC subtraction indicating that one or two controls are insufficient to characterise the magnitude of VOC fluctuations in control soils. Whilst the number of replicates used in this study would be considered statistically low (n=4), this is the highest number of replicates reported of any longitudinal decomposition VOC profiling study. This is largely due to feasibility of time associated with sample collection and analysis. In addition, the time required to collect a statistically higher number of replicates would span a long period (i.e. multiple days). Therefore, carrion replicates would not be true replicates because the decomposition process can change considerably over a short period of time. Obtaining a large number of carcasses for replication is not always possible, and would also require a large amount of space for carcass deposition. These are recognised limitations of decomposition odour profiling, and as such, appropriate data handling is even more essential in order to prevent results from being overstated.

4 Concluding remarks

Variation in methods for establishing an accurate and reproducible chemical profile for mammalian decomposition VOCs has undoubtedly led to differences in reported results. The use of GC×GC-TOFMS to characterise this matrix will lead to a better understanding of the evolution of VOCs during the decomposition process. Yet, increased sensitivity tests our previous notions of appropriate control measures and data handling. A new column combination for decomposition odour profiling was successfully used in this study. The importance of controls and establishing background VOCs in decomposition odour profiling has been overlooked for many years and this study presents a means to effectively exclude background from the decomposition odour profile. Data transformation, hypothesis testing, and appropriate control measures highly influenced the results obtained and provided a clearer picture of the decomposition VOC profile. Appropriate use of controls and background subtraction can also be applied to other areas of VOC profiling of complex mixtures, such as food and fragrance analysis in addition to environmental VOC monitoring in soils. At the very least, this study calls to attention the requirement for better documentation of data handling in published studies to allow advancement in this area of research. With these considerations in mind, our understanding of decomposition odour will improve and therefore will allow for confidence in subsequent studies examining how taphonomic variables affect the production of VOCs.

Acknowledgements

The authors would like to thank Dr. David Bishop, LECO Australia and SGE Analytical Science for support and equipment for this study. Mention is given to Dr. Jean-Marie Dimandja for providing chemical reference standards. This project was funded in part by the Australian Research Council (ARC) and the University of Technology, Sydney (UTS).

All authors declare that there are no financial or commercial conflicts of interest.

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Figure Captions

Figure 1. Peak apex plot showing separation of test mixture compounds using a primary 30 m Rxi®-624Sil MS column and secondary 2 m Stabilwax® column. Average primary and secondary retention times (1tr and 2tr) are shown in seconds for 5 replicate injections.

Figure 2. Surface plot of soil gas sample from beneath pig 1 at t0. A sum of the first dimension is projected on the back of the plot which is representative of a one-dimensional trace.

Figure 3. Contour plots for (a) decomposition soil beneath pig 3 at t0 (b) control soil at t0 and (c) field blank at t0. The Z-axis is logarithmically scaled and equal for each plot.

Figure 4. Principal component analysis (PCA) plots from 3 months (t1) a) unfiltered results scores plot with T2 Hotelling ellipse b) correlations loadings plot using unfiltered results c) scores plot with T2 Hotelling ellipse for filtered compounds of interest d) correlations loadings using filtered compounds of interest.