TAPHONOMY OF HUMAN REMAINS

Forensic Analysis of the Dead and the Depositional Environment

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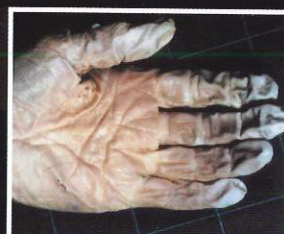

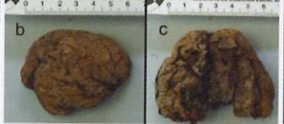

phonomy of Human Remains: Forensic Analysis of the Dead and the Depositional Environment a comprehensive interdisciplinary overview of taphonomy written by an international group of entists and forensic practitioners. A cadaver and its depositional environment are a complex l dynamic system. Without understanding the depositional environment, it is not possible to onstruct the original sequence of events leading to the deposition and discovery of the human nains. Moreover, the interpretation of suspicious circumstances surrounding death can only be de with due regard to the natural decay processes.

s volume focuses on the chemical and biological processes of soft tissue decomposition of man remains, the degradation of associated artefacts, and specific modifications to the body h as thermal alterations or the application of chemicals to human tissue.

pported by case studies and divided into five sections, chapter contributions cover:

- Essential knowledge of gross and microscopic post-mortem processes
- Analysis and interpretation of the depositional environment
- Anti-, peri- and post-mortem modifications to the body
- Case studies
- Past and future considerations for taphonomy

s book is primarily written by and for forensic scientists and practitioners working within erent jurisdictions and fields of expertise, including pathology, anthropology, archaeology, omology, botany, chemistry, microbiology and soil science. Additionally, this volume provides nvaluable resource for academics and students in a number of other areas of archaeology or hropology, and an ideal reference manual for professionals such as police officers, crime scene nagers, death investigators, coroners, forensic coordinators.

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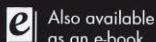

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3

Profiling Volatile Organic Compounds of Decomposition

Pierre-Hugues Stefanuto, Elien Rosier*, Jan Tytgat, Jean-François Focant and Eva Cuypers*

3.1 Introduction

During the decomposition of human or animal remains, a wide spectrum of volatile organic compounds (VOCs) are emitted into the environment, resulting in what is commonly known as 'the smell of death'. Due to the VOC's specific chemical profile and a canine's excellent olfactory capacity, human remains detection (HRD) canines (more commonly called 'cadaver dogs') can be trained to locate (buried) corpses. As these dogs can differentiate between odours produced by human and animal remains, it is believed that the decomposition of human remains generates a unique scent (Vass *et al.* 2004). HRD canines can even make a distinction between the odour of living persons and recently deceased individuals (DeGreeff *et al.* 2011). Training and cultivating such a selective sense of detection in HRD canines consequently requires a high level of understanding of cadaveric decomposition chemistry. Therefore, the physicochemical identification of specific VOCs generated by the complex and dynamic human decomposition process could contribute to increase the efficiency of HRD canine training. Additionally, HRD canines have a short working life and variable performances (depending on the dog) (Statheropoulos *et al.* 2007). The development of a portable device that could efficiently detect specific markers would be complementary to the work of HRD canines and enhance the power of forensic research in the discovery of buried bodies. However, for now, these detectors are not as sensitive as the HRD dogs.

In forensic entomology, insects that inhabit decomposing remains are used to aid forensic investigations. Although it can take days, weeks or longer to find a body, insects are typically attracted to the odour of decomposed bodies within minutes. Depending on the decomposition stage and the released VOCs, different insects will be attracted. The distribution, biology and behaviour of insects found on a decomposing body can provide information in an investigation (Amendt *et al.* 2006, 2011; Farinha *et al.* 2014). Since different insect activities are associated with each stage in the decomposition process, the most important application of forensic entomology is the estimation of the minimum post-mortem interval (PMI). Despite the fact that the exact mechanisms that attract insects are still not completely understood, it is believed that the nature of released VOCs plays a major role (Statheropoulos *et al.* 2011). The field of entomology would thus also benefit from the physicochemical identification of stage-specific VOCs to provide better insights in forensic investigations.

* Equal contribution

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To date, it is known that a wide variety of VOCs are formed during the decomposition process arising from degraded proteins, carbohydrates and fat in the human body. Several research groups have studied these post-mortem dynamic VOC mixtures and different chemical classes such as alkanes, alcohols, acids, esters, ketones, aldehydes, cyclic hydrocarbons, aromatic compounds, sulphur- and nitrogen-containing compounds have already been identified. Nevertheless, it is important to further study the VOC signature of the 'smell of death' and to determine whether the identified compounds are specific to human decomposition (Brasseur *et al.* 2012; Cablk *et al.* 2012; DeGreeff and Furton 2011; Dekeirsschieter *et al.* 2009, 2012; Forbes and Perrault 2014; Hoffman *et al.* 2009; Perrault *et al.* 2014; Rosier *et al.* 2014; Stadler *et al.* 2013; Statheropoulos *et al.* 2005, 2007; Stefanuto *et al.* 2014; Vass 2012; Vass *et al.* 2004, 2008).

3.2 Matrices and Sampling Methods

3.2.1 Matrices

The materials on which studies are carried out are defined as matrices. These can be divided into primary and secondary matrices. The decomposing body or tissue that is used in the study is considered a primary matrix. During decomposition investigations, different materials such as soil, water or air can also be used to determine the VOC signature. These matrices are considered to be secondary matrices.

3.2.1.1 Primary Matrices

The preferred matrix to use in human decomposition studies is human cadavers. Nevertheless, due to ethical restrictions and limited availability this is not always possible (Statheropoulos *et al.* 2011). Therefore, organs or tissues from humans such as muscle, fat tissue, bones, liver, intestines and blood can be used in studies. Domestic pigs (*Sus scrofa domesticus* L.) are also considered to be acceptable analogues for human decomposition studies due to the similarities including comparable internal organs/anatomy and fat distribution across the torso, similar hair coverage and comparable gut fauna. Pigs are therefore widely used in studies of decomposition (Brasseur *et al.* 2012; Dekeirsschieter *et al.* 2009, 2012; Forbes and Perraut 2014; Perrault *et al.* 2014; Stadler *et al.* 2013; Statheropoulos *et al.* 2011).

3.2.1.2 Secondary Matrices

When human or animal remains are decomposing in a specific environment, secondary matrices can also be analysed. Compounds produced during the decomposition process are released and migrate into different sub-media. Depending on their physicochemical properties (molecular weight, vapour pressure, boiling point, etc.), these compounds can be found predominantly in specific secondary matrices such as air (above the corpse or the grave), soil (above and below for buried corpses or below for corpses deposited on the surface), and water (moisture or immersion) (Brasseur *et al.* 2012; Forbes and Perrault 2014).

3.2.2 Sampling Methods

Since the composition of the air (i.e. headspace) above the body seems the most relevant in finding decomposition VOC signatures, most research groups focus on the secondary matrix. To study this matrix, air-sampling methods are required. Although the odour of decomposition might be very strong, the concentration of VOCs in the air is in most cases relatively low. A concentration step is therefore required in order to be able to detect trace VOCs. Concentrated air sampling can be carried out using two distinct methods.

3.2.2.1 Headspace Solid-Phase Microextraction

In this solvent-free sample preparation technique, a fused silica fibre is immersed for a certain time in the headspace/air collected above the remains. During this time, the VOCs that are released can be adsorbed in a *passive* way on the fibre coating. The fibre coating can be made of a single or multiple adsorbents. Selecting the more suitable coating will allow different types of molecules to be adsorbed. Trapped VOCs are then thermally desorbed from the fibre into the injection port of the gas chromatograph (GC) (see below). The advantage of headspace solid-phase microextraction (HS-SPME) is that no solvent is used, allowing solvent-independent VOC transfer (no solvent peak and no solubility issues), and preventing VOC dilution. Major disadvantages are:

1) thermally unstable compounds can be degraded when the fibre is heated;
2) this method can only be used in a passive way that requires the equilibrium stage to be reached; and
3) the trapping efficiency is dependent on the fibre coating (Ramírez *et al.* 2010; Ras *et al.* 2009; Woolfenden 2010).

3.2.2.2 Sorbent Tube

Sorbent tubes can collect the VOCs in a *dynamic way*, by drawing air through the tube, or in a *passive way*, by placing the tubes in the headspace so that the VOCs are passively adsorbed onto the sorbent. Sorbent tubes can be packed with one or multiple adsorbents. The adsorbent bed will influence the trapping efficiency. The collected and concentrated VOCs on the sorbent tube must be desorbed before chromatographic analysis.

Compared to HS-SPME, sorbent tubes have a higher loading capacity due to the larger amount of sorbent present. Another advantage of using sorbent tubes is the possibility of dynamic sampling, providing a higher volume of airflow through the tube. As a consequence, a higher number and abundance of VOCs can be collected and thus the sensitivity is greater compared to passive sampling. Two desorption techniques can be used with sorbent tubes: 1) solvent extraction (SE) and 2) thermal desorption (TD).

3.2.2.2.1 Solvent Extraction The VOCs that are adsorbed on the tubes can be eluted using *organic solvents* (e.g. carbon disulphide, ether). This is a simple method where VOCs with high molecular masses or thermally unstable compounds can be collected and analysed. However, there are also several drawbacks:

1) After concentration on the tubes, there is a dilution of the VOCs in the organic solvent.
2) When the VOCs are collected passively, the sampling period is longer.
3) More polar and reactive compounds often have poor desorption efficiencies.
4) The chemical nature of compounds collected depends on the type of organic solvent used.
5) Long-term storing is highly dependent on the solvent volatility and stability (Ramírez *et al.* 2010; Ras *et al.* 2009).

3.2.2.2.2 Thermal Desorption Thermal desorption (TD) is a *solvent-free* sampling technique in which sorbent tubes are used. Desorption is carried out by heating the tube to high temperatures. In TD, another concentration step can be introduced as there is a possibility of placing a cold trap between the sorbent tubes and the GC column. Using this cold trap, a focusing effect takes place to compress signals and increase the sensitivity.

Enhanced sensitivity of TD compared to SE is introduced due to the focusing step in the cold trap. However, thermally unstable compounds and compounds with high boiling points (>300°C) are not adequately analysed using TD because of their poor desorption efficiency (Ramírez *et al.* 2010; Ras *et al.* 2009; Woolfenden 2010).

3.2.3 Analytical Methods

Decrypting the composition of the volatile mixtures released by decaying mammalian bodies represents an analytical challenge. During the decomposition, complex reactions are taking place, leading to the chemical degradation of the body's constituents (Swann *et al.* 2010). The resulting by-products require a high-resolution separation for a complete analysis. Decomposition VOC mixtures are characterised by a high variability in chemical composition and a large dynamic range (Dekeirsschieter *et al.* 2012; Stadler *et al.* 2013). Nowadays, several analytical methods are commonly used for analyses of volatile matrices.

3.2.3.1 Gas Chromatography

The most common methods of VOC analysis are based on gas chromatography (GC) techniques. Chromatographic methods separate different components of a complex mixture based on particular affinities of the compounds between a stationary phase and a mobile phase. Compounds with a higher affinity for the mobile phase will elute faster than those more strongly retained on the stationary phase. Following separation, a detector is able to detect and identify the successive compounds eluting from the chromatographic system (Dekeirsschieter *et al.* 2009; Forbes and Perrault 2014; Hoffman *et al.* 2009; Perrault *et al.* 2014; Rosier *et al.* 2014; Statheropoulos *et al.* 2005, 2007, 2011; Vass 2012; Vass *et al.* 2004, 2008).

3.2.3.2 Comprehensive Two-Dimensional Gas Chromatography

The matrix complexity of decomposition odour has led to challenges in separation using classical GC (1DGC) techniques. To overpass these limitations, a higher peak capacity is required. To increase the separation power, the use of comprehensive two-dimensional gas chromatography (GC×GC) has been proposed (Brasseur *et al.* 2012; Dekeirsschieter *et al.* 2012; Stadler *et al.* 2013). GC×GC uses two separation dimensions (two GC columns) connected together. The stationary phase of each dimension is different to offer different separation mechanisms. These columns are serially connected and a special device called a modulator is placed between the two dimensions. The modulator allows the transfer of compounds eluting from the first dimension (^{1}D) to the second dimension (^{2}D), while maintaining the separation achieved in ^{1}D. Moreover, the two stationary phases should be different to ensure a maximum separation. The chemistry of separation taking place in ^{1}D (e.g. volatility) has to be different in the ^{2}D (e.g. polarity). This requirement is called the orthogonality of the two dimensions.

The modulator of choice for decomposition headspace analysis is a liquid nitrogen-based cryogenic system that allows efficient trapping-releasing of volatile analytes. It ensures a high sampling rate and a quantitative transfer between the two dimensions. In practice, the modulator continuously samples the eluent of ^{1}D into several slices that correspond to fast repeated injections of ^{1}D material into ^{2}D for further separation. This occurs based on a specific interval called the modulation period (P_M). Each P_M corresponds to a fast secondary separation. The modulator is the heart of the GC×GC system and allows both the conservation and the orthogonality rules to be fulfilled. Based on this 'sliced chromatogram', specific software can be used to create a multidimensional plot used to visualise the entire separation (Figure 3.1). Such an image can be used and considered as the fingerprint of the VOC profile. By fine tuning of the GC phase combinations in ^{1}D and ^{2}D, compounds potentially still co-eluting after ^{1}D elution can be resolved based on different separation mechanisms in ^{2}D. When properly selected, column combinations can also generate structured chromatograms that allow chemical similarities to be used during the identification process. The enhanced separation power is additionally completed by a global sensitivity enhancement as the modulation process

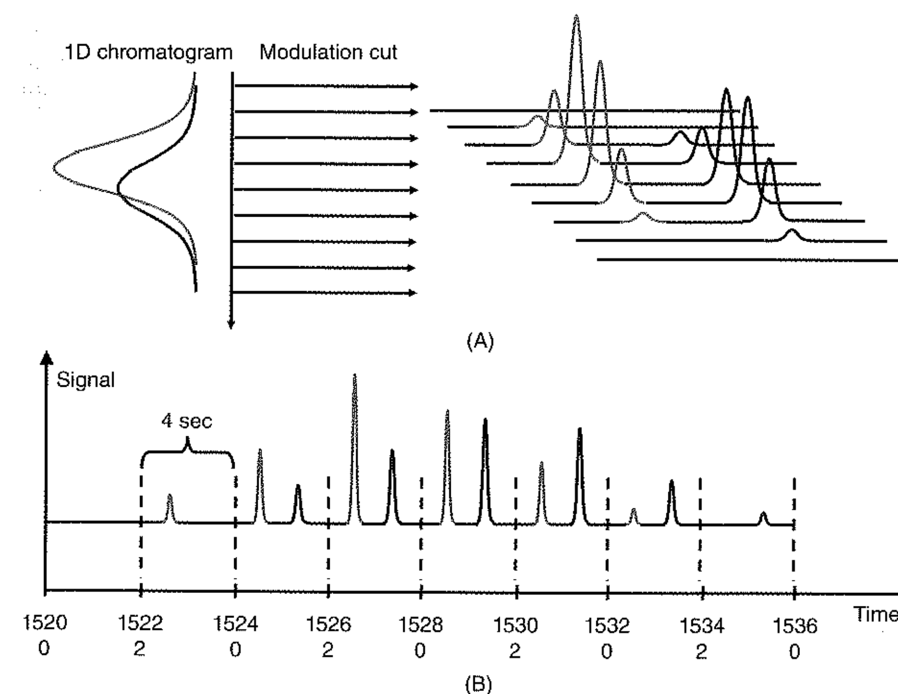

Figure 3.1 (A) In classical GC the two peaks are co-eluting. The modulator cuts this peak to generate a 2D (GC × GC) plot where the two peaks are resolved. (B) The 1D traces of a GC × GC analysis is a succession of fast GC separations that are software transformed to obtain the 2D plot.

also results in zone compression of the chromatographic signals while mass conservation is present (Patterson *et al.* 2011).

The decision of the use of either a 1DGC or a GC×GC system should be based on the application of interest. For the targeting of the main compounds produced in the decomposition headspace, the 1DGC approach provides adequate separation resolution. However, for a complete screening of the complex volatile mixture above decomposed remains, GC×GC is required.

3.2.3.3 Chromatography Detectors

For both chromatographic methods, mass spectrometry (MS) has been used to study the decomposition headspace. MS is used to ionise GC eluents and separate produced ions based on their respective masses. Resulting mass spectra are specific to each analyte by combining both fragment and parent ion information. They are compared to MS libraries for identification purposes. For classical 1DGC-MS, simple scanning quadrupole systems are commonly used as they offer good sensitivity and good dynamic range (Cablk *et al.* 2012; DeGreeff and Furton 2011; Dekeirsschieter *et al.* 2009; Forbes and Perrailt 2014; Hoffman *et al.* 2009; Perrault *et al.* 2014; Rosier *et al.* 2014; Statheropoulos *et al.* 2005, 2007, 2011; Vass 2012; Vass *et al.* 2004, 2008). For GC×GC, time-of-flight mass spectrometers (TOFMS) are typically used as they offer the fast acquisition rate required to characterise very narrow (<200 ms wide) ^{2}D peaks. Moreover, because TOFMS are non-scanning instruments, mass spectra are free from mass skewing, and signals of possibly co-eluting analytes can be deconvoluted to extract analyte-specific information and identification. Regardless of the type of MS used, electron

impact (EI) ionisation sources are used to ensure appropriate production of reproducible mass spectra, representing the fingerprint of chemical compounds, which can be compared with databases (e.g. NIST and Wiley). Retention indices can also be used to improve the identification for both 1DGC and GC×GC, at least in ^{1}D.

1DGC and GC×GC methods are powerful for describing the content of a complex mixture. However, the disadvantage to these techniques is the complexity of the data set generated. For each peak detected, several identification points are available: a retention time, intensity and a mass spectral identification. This becomes more complex when GC×GC is used as a ^{2}D retention time value and a chromatographic pattern description is obtained. Despite the added value of collecting more information, the decrypting of this information requires powerful statistical tools to be used. Principal component analysis (PCA) is the most commonly used multivariate method in decomposition studies (Brasseur *et al.* 2012; Dekeirsschieter *et al.* 2009, 2012; Forbes and Perrault 2014; Perrault *et al.* 2014; Stadler *et al.* 2013). PCA allows the combination of multiple variables to obtain a usable visualisation of the information present in a massive data set. More recently, Stefanuto *et al.* (2014) developed supervised filtration approaches to improve the PCA visualisation power. The establishment of a specific decomposition biomarkers database will only happen via specific statistical method development, in order to sort out compounds of interest from the hundreds of signals that are typically recorded during such analyses. The difficulty in replicating putrefaction experiments, and the potentially large biological variability, further make data treatment an analytical challenge.

3.2.3.4 Data Treatment and Validation

When such complex data are considered, the quality of the data treatment is as important as the quality of the analytical method itself. Indeed, the ethical and logistical limitations linked to decomposition studies require a powerful tool to obtain the maximum information from a limited number of replicates. Thus, the data handling requires the application of robust statistics. Statheropoulos *et al.* (2006) were the first to use strong univariant and multivariant statistics in the decomposition field. They applied various clustering methods to compare VOC mixtures from different origins. Dekeirsschieter *et al.* (2009) used PCA to study the link between differences in decomposition stage profiles and the environment. Based on these papers, the subsequent studies in decomposition also contained strong data handling methods.

For GC×GC-TOFMS, the larger amount of data increases the need for robust data handling methods. Indeed, behind each chromatographic peak, there are two retention times, full mass spectra and intensity information. Stadler *et al.* (2013) used a strong data alignment technique to monitor the decomposition headspace of pig decomposition samples. This method was based on peak table alignment to obtain an accurate day-to-day comparison. However, dealing with large tables of data is not user-friendly and requires grouping in chemical family prior to the statistical treatment. Based on the same approach, but shifting from family profiling to individual biomarker identification, Stefanuto *et al.* (2014) developed an image analysis approach to decrypt the decomposition volatile signature. This approach gives good preliminary results but more work is needed to produce a comprehensive decomposition VOC biomarkers list. All these studies used multivariate statistic methods to reduce the data dimensionality. The data filtration process in these kinds of mathematical methods allows conversion from chemical family comparison to individual compound comparison. This represents a major step in the methods of biomarker validation (Figure 3.2) (Stefanuto *et al.* 2014).

The combination of comprehensive hyphenated methods and statistics provides a powerful tool for decomposition investigation. However, this approach still requires a validation process for the

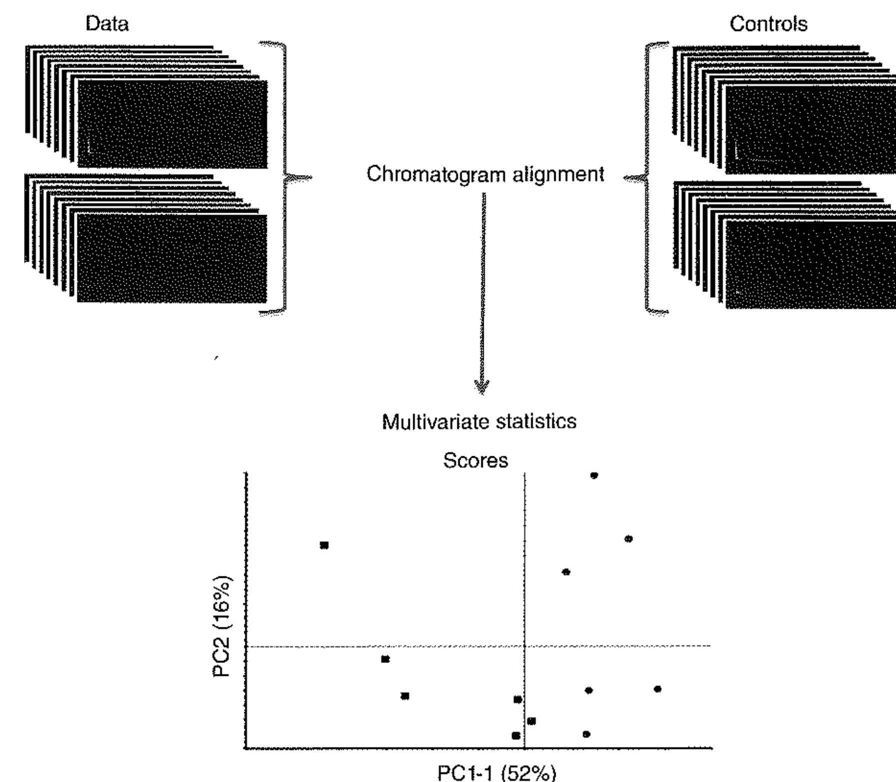

Figure 3.2 Illustration of the data processing method to analyse data from decomposition headspace compared to control samples.

chemical identification confirmation. Moreover, the quantification aspects need to be investigated and validated to obtain a full description of the decaying body headspaces. High-resolution mass spectrometry could provide such information. Accurate mass data would aid in confirming compound identities by reducing the range of possible attributions from a more accurate molecular formula. Tandem MS data could also give access to supplementary structural information to help in biomarker identification.

For all methods described, the validation process is a major step in decomposition headspace profiling. Indeed, several studies have already demonstrated the meaningful information that can be obtained from decomposition odour (DeGreeff and Furton *et al.* 2011; Stadler *et al.* 2012). However, to date there is no validated method for decomposition VOC biomarker identification that can be used to produce a list of specific compounds. The major reason for this lack of validation is directly linked to the limitation of decomposition studies. Indeed, it is almost impossible to obtain a true biological replicate. In classical biological study, this limitation is overcome by increasing the number of replicates. Nevertheless, for decomposition, access to bodies is limited for ethical and logistical reasons. Rosier *et al.* (2014) were the first group to focus on the validation of some decomposition markers and to establish a validation process. They developed a repeatable TD-GC-MS method. For the future, some effort will have to be directed towards the validation of decomposition monitoring methods.

Table 3.1 VOCs and their possible origin (Dekeirsschieter *et al.* 2012; Paczkowski and Schütz 2011; Stadler *et al.* 2013; Statheropoulos *et al.* 2011).

VOCs	Possible origin
Oxygenated compounds (acids, alcohols, ketones, aldehydes, esters and ethers)	Lipids (triglycerides) Sugars (carbohydrates)
Sulphur-containing compounds	Sulphur-containing amino acids (cysteine and methionine)
Nitrogen-containing compounds	Nucleic acids Amino acids
Aromatic compounds (phenol, indole, skatole, benzonitrile, benzaldehyde)	Amino acids (tyrosine, tryptophan, phenylalanine)

3.3 Results and Discussion

3.3.1 Identified Decomposition VOCs

Decomposition is initiated by endogenous enzymes, but during the active and the advanced stages of decay, microorganisms originating endogenously from for example the gut and mucous membrane of the respiratory system, but also from the environment, play the most important role. Lipids, proteins and carbohydrates will break down to amino acids, fatty acids and glucose (Paczkowski and Schütz 2011). An overview of VOCs and their possible origins can be found in Table 3.1. A wide variety of compounds that are released during the decomposition process have already been identified. VOCs from different chemical classes include alkanes, alkenes, aromatic and cyclic compounds, acids, esters, ketones, aldehydes, alcohols, ethers, halogen compounds, sulphur- and nitrogen-containing compounds (Brasseur *et al.* 2012; Cablk *et al.* 2012; DeGreeff and Furton 2011; Dekeirsschieter *et al.* 2009, 2012; Forbes and Perrault 2014; Hoffman *et al.* 2009; Perrault *et al.* 2014; Rosier *et al.* 2014; Stadler *et al.* 2012; Statheropoulos *et al.* 2005, 2007, 2011; Vass 2012; Vass *et al.* 2004, 2008).

The first investigation of decomposition headspace was conducted by Vass *et al.* (2004). The authors used a TD-GC-MS method to study the VOC emission from buried human bodies. The aim of this study was to create a 'decompositional odour analysis database'. This research reports hundreds of compounds that were grouped by chemical family for the data interpretation. Grouping compounds by chemical family or decomposition stage allows for the identification of the main contributors of decomposition odour. Dimethyl disulphide (DMDS) and other sulphides are typically the compounds with the highest concentration. They are produced by the degradation of amino acids (cysteine and methionine) that are the building block sulphide bridges in protein. A large number of amine and hydrocarbon compounds are also usually detected. However, these compounds do not seem to be specific to one species. They are typically detected in the headspace of animal as well as human decomposition. These compounds probably come from amino acid degradation and are the by-products of deamination and decarboxylation. The volatile fatty acids (e.g. butyric acid) and their derivatives (ester) are mainly produced via the degradation of sugars. Tracking the origin of VOCs is not straightforward. The chemicals produced from biomolecule degradation generally undergo secondary reactions (e.g. oxidation, reduction, esterification). Until now, there is no specific validated list for human decomposition VOCs. Nevertheless, almost all the articles published contain a compound list from the decomposition headspace. The aim of decomposition odour profiling is to advance the biomarkers list to improve HRD dog-training methods or to develop target detectors.

Remarkably, during all described decomposition studies, cadaverine and putrescine have never been reported. They are both produced from the decarboxylation of specific amino acids. Cadaverine is produced from lysine and putrescine from ornithine (resulting from arginine) (Statheropoulos *et al.* 2005). These two diamine compounds were previously associated with human decomposition and were assumed to be essential for HRD canines to find human remains. Hence, these two compounds were used in training formulations for dogs (Stadler *et al.* 2012; Tipple *et al.* 2014; Vass *et al.* 2004). Several hypotheses to explain the lack of cadaverine and putrescine detection have been described, including their fast metabolism by bacteria, or that they are thermally labile and therefore destroyed in GC and not detected, hence liquid chromatography is therefore more suitable to detect cadaverine and putrescine. Dekeirsschieter *et al.* (2009, 2012) detected piperidin-2-one and reported that this could be a metabolite of cadaverine. Another hypothesis is that cadaverine and putrescine are not produced in partial-pressure oxygen atmospheres (Dekeirsschieter *et al.* 2009, 2012; Statheropoulos *et al.* 2005; Vass *et al.* 2004).

3.3.2 Differences in Decomposition VOCs Between Primary Matrices

Since cadaver dogs can differentiate between odours emitted by human and animal remains, differences between species are likely (Vass *et al.* 2004). Several research groups have therefore tried to identify differences between human and animal specific markers from the headspace of the remains (Cablk *et al.* 2012; DeGreeff and Furton 2011; Rosier *et al.* 2014).

DeGreeff *et al.* (2011) used the STU-100 to collect samples and analysed the samples with SPME-GC-MS. The air was sampled above human remains in a morgue and above animal remains in a laboratory (canine, tuna, chicken, beef, hamburger, lamb and pork chops). They found many similarities between the profiles, but styrene and benzoic acid methyl ester were only detected in the human remains samples (DeGreeff and Furton 2011). However, styrene was also detected in pig remains by Statheropoulos *et al.* (2011) and Forbes and Perrault (2014).

Cablk *et al.* (2012) sampled the air above bone, fat, muscle and skin samples of cow, pig and chicken and compared their VOC profile with a study by Hoffman *et al.* (2009) who sampled the air above human remains. They found 11 'human specific' compounds (Cablk *et al.* 2012), but of these 11 compounds only 4 compounds were not detected in pig remains in other decomposition studies: (1) hexanoic acid, hexyl ester, (2) 2-octene-3-ol, (3) 2-hexenal and (4) tetrachloroethylene. Notably, these compounds were also never detected in other human sample studies.

Rosier *et al.* (2015) compared the released VOCs of 6 decomposing human and 26 decomposing animal remains in a lab-controlled environment using a validated TD-GC-MS-method. They found a combination of eight compounds that separated human and pig remains from the other animal remains using PCA-analyses: ethyl propionate, propyl propionate, propyl butyrate, ethyl pentanoate, pyridine, diethyl disulphide, methyl(methylthio)ethyl disulphide and 3-methylthio-1-propanol. Further research in the field using full bodies has to corroborate these results.

Stefanuto *et al.* (2015) analysed the VOCs emitted by human bodies and pig carcasses during the first days of decomposition. This study was conducted to demonstrate the differences between species, since pigs are commonly used as a human analogue. Different factors were compared in order to demonstrate their impacts on the decomposition process of both species. Two trials were conducted (summer and winter) on two humans and one pig. One human was protected from insect colonisation. This study was conducted in an open environment.

3.3.3 Differences in Decomposition VOCs Between Secondary Matrices

The secondary matrix will also impact the decomposition VOCs detected. In 2014, Forbes and Perrault (2014) placed domestic pig carcasses on the soil surface and compared the VOC profile obtained

from the air above the pig remains with the profile obtained from the soil below the remains. In order to accumulate the VOCs in the headspace, they placed a stainless steel hood over the carcasses prior to sampling. To facilitate the sampling in soil, they used VOC-Mole™ Soil Probes. Both samples were analysed with TD-GC-MS. When they compared the results they found 207 compounds in soil compared to 100 compounds in air samples. Only 23% of all described VOCs were found in both air and soil. This demonstrates that sampling in soil and air are complementary techniques. An explanation for the increased number in soil samples could be:

1) the microorganisms in the soil, hence more VOCs can be produced and detected;
2) adsorption of VOCs on soil particle causing a longer retention; and
3) rapid dispersion of VOCs in air due to wind and evaporation.

They also noted that the majority of the compounds identified in the air samples were detected during bloat, active decay, advanced decay stages and skeletonisation, while in soil samples the majority of VOCs were detected starting from the active decay stage. Since the liquefaction of the soft tissue starts during active decay, and there is a release and leaching of the compounds into the surrounding soil, this could explain why VOCs were not detected earlier. When VOC profiles of the air and the soil samples were compared, a comparable profile was reported (Forbes and Perrault 2014).

The majority of other published decomposition studies focused on air samples only. In the studies where soil samples were collected, VOCs were dominated by ketones, alcohols and esters. This was comparable with the results of the Forbes and Perrault (2014) study, where the soil samples mainly consisted of aromatics, esters, ketones, alcohols and hydrocarbons. In air samples, Forbes and Perrault (2014) found mainly sulphides, nitrogen-containing compounds, aromatics and alcohols. In other studies, air samples were dominated by sulphur and nitrogen-containing compounds, ketones, alcohols, acids and aromatics. Acids were mainly found in air samples, also described by Forbes and Perrault (2014). The corresponding esters were also detected in soil (Forbes and Perrault 2014), but this result was not reported in other decomposition studies.

3.3.4 Differences in Decomposition VOCs Based on Sampling Methods

TD is the most commonly used method for sampling VOCs from various decomposition matrices. However, other sampling techniques are available for VOC profiling, for example HS-SPME and SE. Based on non-forensic VOC studies, it may be presumed that TD is the most suitable method for sample collection and introduction into the GC (Agelopoulos and Pickett 1998; Brokl *et al.* 2013). Indeed, the HS-SPME technique is a static equilibrium procedure. The time required to reach this equilibrium state is different for every compound based on their physicochemical properties.

In 2014, Perrault *et al.* compared the efficiency of TD and HS-SPME to monitor the VOCs in soil beneath decomposing pig remains. They illustrated the differences between TD and HS-SPME sampling for decomposition matrices. Depending on the chemical property of the compound, differences in trapping efficiency were reported between different methods. All polar compounds (sulphur, nitrogen) were more efficiently trapped on the sorbent tubes, while carbonyl compounds (aldehydes, ketones, carboxylic acids) were more efficiently trapped using HS-SPME. The authors also demonstrated that the VOC profile obtained by TD is more discriminatory for the analysis of the decomposition process. HS-SPME appears to be more suitable when specific target compounds are monitored. In the latter case, a good choice of the fibre coating is required in order to obtain good results.

The SE approach has not been widely used for decomposition studies. Indeed, several disadvantages are linked to SE application (see Section 3.2.2 above). However, this method was applied with GC×GC-TOFMS producing interesting results (Brasseur *et al.* 2012; Dekeirsschieter *et al.* 2009, 2012). Dekeirsschieter *et al.* (2009, 2012) demonstrated the time evolution in the headspace

above pig remains. The consistency of these results was confirmed by Stadler *et al.* (2013), who also studied pig carcasses on the soil surface using TD-GC×GC-TOFMS. In both studies, the same main chemical families were detected: alcohols, carboxylic acids, aromatics, and sulphides and along with ketones and aldehydes, were the major contributors to the overall VOC profile. Moreover, the main compounds of each family were the same. Although these two studies were conducted in different geographical areas (i.e. Belgium and Canada) with different meteorological parameters, the general profile was consistent.

3.3.5 Differences in Decomposition VOCs Based on Analytical Methods

Most research groups studying decomposition have used GC-MS methods. It was only very recently that GC×GC-TOFMS was implemented into the study of decomposition VOCs (Dekeirsschieter *et al.* 2012). An overview of research groups and their developed methods can be found in the supplementary information.

Dekeirsschieter *et al.* (2012) conducted the first study using GC×GC that monitored the headspace of pig remains in 2012. The authors applied the same data treatment previously used with classical 1DGC, but demonstrated an enhancement for the number of compounds detected due to the separation capacity and the deconvolution of the TOFMS. This research presented the main compounds detected through the first 40 days of decomposition. Around 830 compounds were detected. This number represented a vast increase from the first database previously reported (Vass *et al.* 2004). Studying the occurrence of these compounds, the 1H-indole, DMDS, dimethyl trisulphide (DMTS) and other well-known compounds of the decomposition signature were identified as the most detected compounds. The time evolution of the number of compounds also displayed the parallel that can be drawn between the decomposition activities and the number of VOCs detected in the corpse headspace. Moreover, the evolution of the occurrence of different chemical classes in the headspace was monitored and appeared to be dependent on the decomposition stage. For example, the carbonyl families (aldehydes, carboxylic acid) were the major compounds detected during active decay. Stadler *et al.* (2013) confirmed this time evolution during another study on pig carcasses.

3.3.6 Factors that Influence the Detection of Decomposition VOCs

Differences in detected compounds can be explained by the use of different soil (sample) types. Indeed, differences in physical, chemical and microbial properties of the soil can influence the speed and/or type of compounds formed and released. When the soil is dry, water will compete with the VOCs on the soil surface, but when the soil is wet, water can act as a solvent for VOCs. Temperature can also influence the detection of VOCs, the number of detected compounds can increase and different types can be identified when other microorganisms are involved in the decomposition process (Forbes and Perrault 2014). Therefore, the comparison of results obtained from decomposition profiling studies represents a challenging task. Indeed, each group applies its own analytical approach limiting the comparison possibilities.

3.4 Conclusion and Future Research

The study of 'the smell of death' has increased considerably since robust analytical methods appeared in the field, for the results to date have assisted in better understanding of canine olfaction processes and entomological colonisation. Although several research groups are exploring this field and hundreds of compounds have already been described, it remains challenging to compare these studies in order to find 'specific human markers'. This is because different matrices are typically studied, different analysis techniques are often used and even different data analysis tools are introduced.

Fortunately, increasing attention is being given to the validation of methods and data analysis. This standardisation of methods will certainly lead to a better comparison of analysis results and ultimately an increased understanding of animal and human decomposition odour.

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4

Blood Degradation and Bloodstain Age Estimation

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4.1 Introduction: Forensic relevance of bloodstains

Blood, the major biological fluid of the human body, is one of the most common traces encountered at a crime scene. Bloodstains can be of great value in forensic investigations, as they provide information at the source level, useful for the identification of the donor, and at the activity level, to enable the reconstruction of events at the crime scene (Cook *et al.* 1998). Historically, blood typing was used to gain information about the bloodstain donor, a classification technique limited by the low variation of blood groups. Following the advances in forensic genetics, DNA analysis nowadays enables the identification of victims, perpetrators or witnesses with high accuracy and reliability. Current developments in 'DNA intelligence' are expected to help police investigations concentrate towards finding unknown individuals, by predicting the donor's physical characteristics, such as eye and hair colour, skin pigmentation, bio-ancestry and facial features (Walsh *et al.* 2011).

Apart from source level information, bloodstain pattern analysis (BPA) may also provide information on the activity level. The analysis of the size, shape and distribution of bloodstains enables forensic investigators to reconstruct a crime, to verify statements, or to identify bloodstains which are likely to be donated by an offender (Peschel *et al.* 2008). By calculating the area of origin of a bloodstain pattern, information can be provided about the position of the victim (e.g. standing, sitting, lying) at the moment of the bloodshed. Sometimes the sequence of events can also be determined using BPA, for example a bloodied shoeprint overlaid by an impact pattern indicates that the impact happened after the shoeprint was created (Laber *et al.* 2014). Likewise, altered bloodstain patterns, for example a bloodstain wiped prior to drying, provides evidence of movement after creation of the stain. This information can be used to generate a relative timeline of events.

To gain even more information, several techniques have been explored for the age estimation of bloodstains. Knowledge of the absolute time of bleeding can be highly relevant in forensic investigations. The age of bloodstains of a victim may give an indication of the moment the person was injured. When bloodstains of a suspect are found, estimation of the age can indicate their presence at the scene in a certain timeframe. Some bloodstains may not be related to the crime, but may be caused during an earlier or later incident. This is relevant information that crime scene investigators can use in their selection of traces for further analysis.

Driven by the forensic relevance, scientists have explored techniques to determine the age of bloodstains for more than a century. An overview of these techniques, mainly developed and tested under laboratory conditions, is given below. During the last decade the main focus was on optical, non-destructive techniques. Current developments in optical methods are described in

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