Analysis of the Volatile Organic Compounds Produced by the Decomposition of Pig Carcasses and Human Remains

by

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Abstract

Complex processes of decomposition produce a variety of chemicals as soft tissues and their component parts are degraded. Among others, these decomposition by-products include volatile organic compounds (VOCs) responsible for the odour of decomposition. Human remains detection (HRD) canines utilize this odour signature to locate human remains during police investigations and recovery missions in the event of a mass disaster. Currently, it is unknown which compounds or combinations of compounds are recognized by the HRD canines. In this study decomposition VOCs were collected from the decomposition headspace of pig carcasses and were analyzed using thermal desorption gas chromatography mass spectrometry (TD-GC-MS). The difficulties associated with the non-target analysis of complex samples led to the further analysis of decomposition odour using a novel application of thermal desorption coupled to comprehensive two-dimensional gas chromatography time-of-flight mass spectrometry (TD-GC×GC-TOFMS). The additional peak capacity and spectral deconvolution of the GC×GC-TOFMS system generated a characteristic profile of decomposition VOCs across the various stages of soft tissue decomposition. The profile was comprised of numerous chemical families, particularly alcohols, carboxylic acids, aromatics and sulfides. Characteristic compounds identified in this study included 1-butanol, 1-octen-3-ol, 2-and 3-methyl butanoic acid, hexanoic acid, octanal, indole, phenol, benzaldehyde, dimethyl disulfide and trisulfide, which represent potential target compounds of decomposition odour.

Currently there is a demand for improved canine training aids and pig carcasses have been proposed as an alternative due to their acceptance as human body analogues. This work investigated the similarities in the decomposition odour profile of pig carcasses and human remains through surface decomposition trials and comparisons to the published literature. It was determined that pig carcasses cannot be eliminated as potential human decomposition odour mimics. Additionally, following the examination of commercially available synthetic training aids, pig carcasses demonstrated a more suitable profile for the training of cadaver dogs. Further investigation into the chemical composition of decomposition odour utilizing TD-GC×GC-TOFMS will aid in determining the signature

of human decomposition odour and facilitate the comparisons of these profiles between environments, individuals and species.

Keywords: Forensic chemistry, Decomposition, Remains detection, VOCs, Human analogue, Cadaver dog, Thermal desorption, Comprehensive two-dimensional gas chromatography – time-of-flight mass spectrometry

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"No man is an island entire of itself; every man is a piece of the continent, a part of the main"

-Meditation XVII, John Donne

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List of Abbreviations

^{1}D	First Dimension
$^{1}t_R$	First Dimension Retention Time
^{2}D	Second Dimension
2D	Two dimensional
$^{2}t_R$	Second Dimension Retention Time
3D	Three dimensional
ADD	Accumulated Degree Days
ATP	Adenosine Triphosphate
BADD	Burial Accumulated Degree Days
$C_{\#}$ (e.g.: C_8)	Number of Carbon atoms (e.g.: eight carbons)
CDI	Cadaver Decomposition Island
CFCs	Chlorofluorocarbons
CH_3SH	Methyl Mercaptan
CIS	Cooled Injection System
CO_2	Carbon Dioxide
COD	Cause of Death
DBU	Pyrimido[1,2-a]azepine, 2,3,4,6,7,8,9,10-octahydro-
DEUSA	Department of Experimental Surgery at the University of Athens Medical School
DHS	Dynamic Headspace
DMDS	Dimethyl Disulfide
DMQS	Dimethyl Tetrasulfide
DMTS	Dimethyl Trisulfide
DOD	Date of Death
EPA	Environmental Protection Agency
FARF	Forensic Anthropology Research Facility
GABA	4-aminobutanoic acid
GC	Gas Chromatography
GC×GC	Comprehensive Two Dimensional Gas Chromatography
GC-GC	Two Dimensional Gas Chromatography or Heart-Cut Gas Chromatography

GRF	Geoforensic Research Facility
H_2O	Water
H_2S	Hydrogen Sulfide
HC	Hydrocarbon
HCFCs	Hydrochlorofluorocarbons
HFCs	Hydrofluorocarbons
HRD	Human Remains Detection
HRTOFMS	High Resolution Time-of-flight Mass Spectrometry
MeSH	Methane Thiol
MS	Mass Spectroscopy
NIST	National Institute of Standards and Technology
n_M	Modulation Number
ORNs	Olfactory Receptor Neurons
PCA	Principal Component Analysis
PIG	Pig In Ground
P_M	Modulation Period
PMI	Post Mortem Interval
RIC	Reconstructed Ion Count
RT	Retention Time
S/N	Signal to Noise Ratio
SPME	Solid Phase Micro-Extraction
TD	Thermal Desorption
TDU	Thermal Desorption Unit
TOFMS	Time-of-flight Mass Spectrometry
TSU	Texas State University
UAGA	Universal Anatomical Gift Act
UDDA	Uniform Determination of Death Act
UOIT	University of Ontario Institute of Technology
VFAs	Volatile Fatty Acids
VOCs	Volatile Organic Compounds
VSCs	Volatile Sulfur Compounds

WHC	Water Holding Capacity

Chapter 1: *Introduction*

Forensic taphonomy is a multidisciplinary field within forensic science that investigates the chemical processes that occur after death and their impact on the surrounding environment. As soft tissue decomposes a variety of compounds are produced including volatile organic compounds (VOCs). These decomposition VOCs are responsible for the odour of decomposition and are utilised by cadaver dogs to locate human remains during police investigations and recovery missions in the event of a mass disaster. In order to be effective, cadaver dogs require ongoing training in the recognition of decomposition odour. Due to difficulties associated with accessing and storing current training aids, pig carcasses have been proposed as an alternative. Pig carcasses are commonly utilized in decomposition chemistry as human body analogues however it is unclear if they can function as decomposition odour analogues. Currently it is unknown what compounds or combinations of compounds are recognized by cadaver dogs and there is little consensus within the literature on the odour signature of human decomposition. This research investigates the chemical profile of decomposition odour from both pig carcasses and human remains and compares it to synthetic training aids commercially available for canine training. The characterization of the VOCs present within the headspace of decomposition will produce a better understanding of the target odour of cadaver dogs and therefore lead to the production of improved training aids.

1.1 *Forensic Taphonomy*

Taphonomy has its origins within paleontology where it is defined as the transition of animal remains to the fossil record (Efremov 1940; Dirkmaat et al., 2008). Taphonomy has influenced disciplines such as archaeology and anthropology. These fields began to incorporate taphonomic theories and methods into their excavation, analysis and interpretation of animal and human remains, particularly by including the depositional context in their examination (Haglund and Sorg 1997; Dirkmaat et al., 2008). This multi-disciplinary field can be summarized as the study of the post-mortem processes and their interaction with the surrounding environment. The principles from paleontology, archaeology and anthropology can be applied to the recovery of remains and their analysis during forensic investigations. Forensic taphonomy focuses on the detection and recovery of human remains, the identification of taphonomic factors affecting

decomposition, the determination of the post-mortem interval and the reconstruction of peri-mortem events (Haglund and Sorg 1997; Dirkmaat et al., 2008).

1.2 *Detection of Remains*

As criminals may attempt to conceal their victims, locating the remains can pose a problem for forensic investigators. France and associates (1992 and 1997) conducted a multidisciplinary examination of a wide range of current methods for the detection of buried remains. Their research is the result of two collaborations, Project PIG and NecroSearch International Inc. Project PIG (Pigs In Ground) is an ongoing research endeavour to evaluate the methods and technologies used in the detection and recovery of clandestine graves (France et al., 1992). This later developed into NecroSearch International, Inc. which is a volunteer organization that provides research, training and assistance to law enforcement agencies in locating human remains and associated evidence (France et al., 1997; NecroSearch International 2007). The collaborators explored methods such as aerial photography, thermal imagery, geology, botany, magnetometry, ground penetrating radar, decomposition dogs and archaeology. All of these methods work on the principle that the burial of remains will have an effect on the surrounding environment by changing its visible appearance, vegetation, soil characteristics, gases and odours (France et al., 1997). The changes to the environment are detected and the most likely locations for a clandestine grave are indicated. The authors found that each method has its advantages and disadvantages and put forward a multidisciplinary approach to the search for remains at crime scenes. They also recommend that the various techniques should be tested in different environments as their efficacy could be altered.

The recovery of remains is important not only during forensic investigations, but also in the event of a mass disaster. Numerous methods that are utilized for human remains detection within forensic investigations are utilized for the recovery of disaster victims including thermal imagery, video devices and scent-detection canines (Statheropoulos et al., 2011). However, the unique environment of collapsed buildings and entrapped victims requires specific methods that can accurately and rapidly locate victim remains (Statheropoulos et al., 2006[b]; Statheropoulos et al., 2007; Statheropoulos et al., 2011).

One potential method is the use of the chemical signal, specifically the volatile organic compounds (VOCs) produced by decomposition (Statheropoulos et al., 2011).

1.3 *Cadaver Dogs*

Cadaver dogs (*Canis lupis var. familiaris*) (Linnaeus 1758) are a group of specially trained air scent dogs that recognize and locate human decomposition scent (Rebmann et al., 2000). Air scent and cadaver dogs work on the scent cone theory which is based on the dispersal of scent molecules in air (Rebmann et al., 2000). The scent source has the highest concentration of odour-causing molecules which become more diffuse further from the source, creating a "scent cone". Cadaver dogs follow the increasing scent to the apex of the cone thereby locating the source. The dispersal of scent within an environment is highly influenced by environmental factors; dynamic features such as the wind and temperature as well as static features like terrain, trees and water (Rebmann et al., 2000). All these factors need to be considered by the handler in order to perform a productive search. The optimal environmental conditions for a cadaver dog search include temperatures between 4 and 16°C, 20% humidity or higher, and wind speeds of 8+km/h (France et al., 1997), although some research has shown that cadaver dogs are effective at temperatures below zero (Komar 1999). At high temperatures, dogs cool themselves by panting and may experience some discomfort. Panting reduces a dog's ability to smell thereby making their searches less effective (Lasseter et al., 2003).

The process of decomposition produces a variety of gasses and compounds that are responsible for various odours. The scent produced is not uniform but rather a range of scents that changes over time as the body progresses from the fresh stage through to skeletonization. The cadaver dog should be trained on a variety of decomposition material including fresh, putrefied and skeletal material, in order to be able to recognize the complete range of scents (Cablk et al., 2012; DeGreeff et al., 2012). Since these dogs are trained to detect decomposition scent, not necessarily cadavers, some have reclassified their dogs as "decomposition dogs" or "human remains detection" (HRD) dogs rather than "cadaver dogs" (France et al., 1997). Due to the difficulties associated with the access, storage and ethical restraints on the utilization of complete human remains, canine handlers utilize a variety of training aids (Cablk et al., 2012).

1.3.1 *Cadaver Dog Training Aids*

Natural scent sources are commonly used and include items such as human tissue, blood, bone, decomposition fluid or grave soil (Sachs 1996; Komar 1999; Rebmann et al., 2000; Lorenzo et al., 2003; DeGreeff et al., 2012). However these natural scent sources are a potential biohazard and are difficult to obtain therefore artificial scents have been presented as an alternative (Sachs 1996; Rebmann et al., 2000). Some artificial scents are thought to contain putrescine and cadaverine (select chemical structures can be found in Appendix A). These two compounds are products of amino acid breakdown and were previously thought to be the main contributors to decomposition odour (Gill-King 1997). It was also believed that these volatile compounds are a target for cadaver dogs (Gill-King 1997; Rebmann et al., 2000). However, research into the VOCs produced by pig and human decomposition was unable to identify these two diamines (Vass et al., 2004; Statheropoulos et al., 2005[a;] Statheropoulos et al., 2007; Vass et al., 2008; Dekeirsschieter et al., 2009; Hoffman et al., 2009; Statheropoulos et al., 2011; DeGreeff and Furton 2011[b;] Brasseur et al., 2012; Dekeirsschieter et al., 2012). This casts doubt on the importance of putrescine and cadaverine as key components in decomposition odour (Oesterhelweg et al., 2008; DeGreeff et al., 2012).

The chemical composition of commercially available training aids and pseudo-scents is largely unknown. The chemical composition of one such product from Sigma-Aldrich® was recently investigated (Stadler et al., 2012). Although these products are specifically marketed as canine training aids it was found that their chemical composition was overly simplistic and contained none of the compounds reported within the headspace of decomposition (Stadler et al., 2012).

The scent produced by decomposition is complex and is made up of a range of scents produced over time as the organism decomposes (Rebmann et al., 2000). Each scent produced is comprised of many odorous compounds, VOCs, whose composition varies depending on the conditions (Rebmann et al., 2000; Vass et al., 2004; Dekeirsschieter et al., 2009; Hoffman et al., 2009; Dekeirsschieter et al., 2012). The large number of olfactory receptors within a dog's nose responds to the compounds present within a complex scent creating a distinct scent picture. This response allows for the scent-

specific work of trained canines (Rebmann et al., 2000). However, it is unknown what chemicals or combination of chemicals are responsible for the canines recognition of target odours (Cablk et al., 2012; DeGreeff et al., 2012). Therefore it is unlikely that an artificial scent that has reduced the complex volatile profile of decomposition to a few compounds will accurately represent the entire odour profile required for effective training of HRD canines (Oesterhelweg et al., 2008; Cablk et al., 2012; DeGreeff et al., 2012; Stadler et al., 2012). This is further supported by a study that worked to develop and validate a synthetic swine odour (Zahn et al., 2001). Using a human panel and chromatographic methods they compared the odour of an effluent air sample, liquid sample and the synthetic mixture. The synthetic mixture contained 19 VOCs, and it was found that although some individual components did produce a manure odour, only the combination of VOCs produced the same olfactory response as the swine effluent (Zahn et al., 2001).

A novel HRD training aid that utilizes VOCs from natural scent sources was recently developed by DeGreeff et al. (2011[a], 2011[b], 2012). They optimized a method for the collection of VOCs onto gauze pads (DeGreeff et al., 2011[a]), analyzed the compounds collected (DeGreeff and Furton 2011[b]) and evaluated its effectiveness as a canine training aid (DeGreeff et al., 2012). The collection system involves the dynamic sampling of headspace from a target odour through a gauze pad which traps the VOCs (DeGreeff et al., 2011[a]; DeGreeff and Furton 2011[b]). The gauze pad can then be safely stored and transported to the canine handler at which point it can be presented to the canine to initiate a search or in the form of a 'hide' for training exercises (DeGreeff et al., 2012). The analysis of the VOCs trapped on the gauze found only a portion of the overall profile reported from human remains (DeGreeff and Furton 2011[b]; DeGreeff et al., 2012). The training aid was however effective in canine testing and therefore may serve to elucidate the chemical signature utilized by HRD canines (DeGreeff et al., 2012). Conversely, in order to achieve holistic training the canine must be exposed to the entire range of target odours (whole or scattered remains, fresh, putrefied and skeletonised) and the fabrication of this training aid still requires access to human remains, which can be difficult for many canine handlers.

It has been proposed that pig carcasses could be used as an alternative training aid for cadaver dogs as the canines could be exposed to the entire decomposition process and therefore the whole spectrum of decomposition odours. Additionally, pig carcasses are commonly used as human analogues in decomposition chemistry, taphonomy and entomology studies. Due to the similarities between pigs and humans it would be expected that the decomposition products, including VOCs, are also similar (Rebmann et al., 2000; Lorenzo et al., 2003; Statheropoulos et al., 2011) and it is hypothesized that if the canines are trained on pig carcasses they can extrapolate the scent profile to locate human remains (France et al., 1997).

However, one unpublished study showed that the dogs could differentiate between pig and human remains (see (Lorenzo et al., 2003)) and animal remains are commonly used in HRD testing as distractor odours (DeGreeff et al., 2012). Additionally the chemical foundation of using pig carcasses as human decomposition odour analogues has been questioned. Studies that utilized skeletal material and individual tissue types of human and various animal species have argued that the human VOC profile is unique and can be used for species differentiation (Vass et al., 2008; Cablk et al., 2012). However no study has conducted a comparison of the VOCs from complete remains of pig and human decomposition within the same decomposition environment or with the same methodology.

Research into the similarities and differences of the VOCs produced by current training aids, pig carcasses and human remains is necessary to improve cadaver dog training and increase the effectiveness of their searches (Lorenzo et al., 2003). The chemical composition of decomposition odour has received increasing attention however an understanding of decomposition VOCs and their relationship to canine scent recognition is still elusive. This knowledge could be applied to the development of canine training aids that accurately represent the complex nature of decomposition odour throughout the decomposition process therefore allowing for a more holistic training process and more effective searches.

1.4 *Volatile Organic Compounds*

1.4.1 *Volatility*

Volatile Organic Compounds (VOCs) are compounds from a variety of chemical classes including alkanes, alkenes, alcohols, aldehydes, ketones, acids, esters, aromatics, amides, sulfides and compounds containing halogens. VOCs are imprecisely defined based on either their environmental effects or their physical properties. Environment Canada defines a VOC as any compound that undergoes atmospheric photochemical reactions, excluding methane, ethane and a range of chlorine and fluorine containing compounds such as chlorofluorocarbons (CFCs), hydrochlorofluorocarbons (HCFCs) and hydrofluorocarbons (HFCs) (Environment Canada). Environment Canada is interested in the compounds encompassed by this definition because they are the precursors for the formation of ground level ozone and particulate matter. The formation of ozone and particulate matter produces air pollution known as smog (Environment Canada).

An alternative definition that focuses on the chemical properties of the compounds is used by Health Canada. Health Canada defines a VOC as an organic compound that has a boiling point of 50-250 °C (Health Canada 2007). The boiling point of a compound corresponds to the temperature at which the vapour pressure is equal to the atmospheric pressure (Fox and Whitesell 2004). Within a closed system, vapour pressure is the pressure of a gas that is in equilibrium with its solid or liquid phase (Petrucci et al., 2002). Vapour pressure indicates the tendency of a compound to enter the gaseous phase. As the vapour pressure increases so does the volatility of the compound thus making it more gaseous (Petrucci et al., 2002). Compounds with a lower boiling point have a higher volatility because they require a lower temperature at which their vapour pressure will equal atmospheric pressure, favouring the phase change. The boiling point of a compound is influenced by the molecular interactions it is involved with. The molecular interactions of hydrocarbons include Van der Waals attractions. Functionalized hydrocarbons can participate in hydrogen bonding as well as dipole-dipole interactions. The amount and strength of these interactions dictate changes in boiling point and thus volatility (Fox and Whitesell 2004).

1.4.2 *Human Odour Analysis*

The study of biological VOCs, their correlation to human odour and their applications to health sciences, policing and forensic science has increased over the last several years. The analysis of odours from humans has centered on establishing a VOC database for healthy individuals. The establishment of the VOCs normally present within human odour is the first step in many research projects. Once the range of human variation is known it can be used to develop cosmetic products, search and rescue techniques, diagnostics and forensic applications (Ostrovskaya et al., 2001; Curran et al., 2005[a]; Curran et al., 2005[b]; Statheropoulos et al., 2005[b]; Statheropoulos et al., 2006[b]; Curran et al., 2007; Gallagher et al., 2008; Kusano et al., 2012). The "individual odour hypothesis" states that each individual has a unique scent that is persistent over time and can be differentiated from another individual (Kusano et al., 2012). Much of the published literature has identified a wide range of VOCs produced from human odour and have found quantitative similarities and differences between individuals (Curran et al., 2005[a]; Curran et al., 2005[b]; Curran et al., 2007; Gallagher et al., 2008). Although there is support for the conclusion that the relative ratios of compounds present within human odour is different among individuals, there is debate as to whether human odour is unique enough for the purposes of individualization (Curran et al., 2005[b]; Preti et al., 2006).

Work on comparing the VOC profile from healthy individuals and stressed individuals has been carried out by Statheropoulos et al. in 2005, 2006[a], 2006[b] and Gallagher et al. in 2008. Their work focused on the differentiation between healthy individuals, fasting individuals, household waste, human decomposition and urban air (Statheropoulos et al., 2005[b]; Statheropoulos et al., 2006[a]; Statheropoulos et al., 2006[b]). The research aimed to improve search and rescue techniques for people trapped in disasters such as earthquakes (Statheropoulos et al., 2005[b]; Statheropoulos et al., 2006[b]).

In the field of health science, researchers are developing a VOC database for healthy individuals in order to identify potential VOC biomarkers for various diseases including lung, breast and skin cancer (Curran et al., 2007; Gallagher et al., 2008; Boots et al., 2012). The forensic applications of VOC research have focused primarily on tracking/trailing canines and scent identification line-ups (Curran et al., 2005[a]; Kusano et

al., 2012). Preliminary research was also conducted by Gallagher et al. in 2008 to generate a VOC profile from latent fingerprints. The goal of these forensic studies are to investigate the potential of human odour to establish links between individuals, objects and biological evidence found at crime scenes (Kusano et al., 2012). However, questions concerning the uniqueness and permanence of human odour need to be answered prior to the acceptance of this evidence in court (Ostrovskaya et al., 2001; Kusano et al., 2012).

1.5 *Chemistry of Decomposition*

In Canada there is no national legal criterion for the determination of death however the United States Uniform Determination of Death Act (UDDA) states that death can be established by the irreversible loss of brain function or the irreversible cessation of cardiorespiratory function (American Medical Association 1981; Shemie et al., 2006). As circulatory stasis and anoxia occurs, decomposition changes begin within the body.

When the heart and respiration cease, the body's natural buffer system fails and cells revert to a fermentative metabolic pathway, resulting in a decrease in blood and tissue pH (Gill-King 1997). Without the presence of oxygen, oxidative phosphorylation and the formation of adenosine triphosphate (ATP) slows and eventually ceases (Gill-King 1997). Without ATP to fuel active transport or the biosynthesis of cell membrane structures, the integrity of the cell membrane decreases. The lack of membrane selectivity causes the cell and organelles to swell and rupture (Gill-King 1997). The rupture of lysosomes releases self-digestive enzymes which are activated by the decreasing pH (Gill-King 1997). The digestive enzymes cause the release of intracellular components; mainly proteins, carbohydrates and lipids. This process of self-digestion is known as autolysis (Gill-King 1997). Autolysis occurs in the most metabolically active cells first. Generally the order is as follows: digestive and circulatory system – lungs and air ways – kidneys and bladder – brain and nervous tissue – skeletal muscles – connective tissue (Gill-King 1997). As autolysis reaches completion an anaerobic environment is created within the body allowing enteric bacteria to thrive (Gill-King 1997).

The anaerobic environment at the end of autolysis supports the activity of enteric bacteria and bacteria from the surrounding environment. Collectively the bacteria degrade the cellular components released during autolysis into their constituent building blocks in a process called putrefaction (Gill-King 1997). Putrefaction produces several of the visible features of decomposition, most notably colour changes, odours and bloating. The various gases produced begin to accumulate within the bowels and soft tissues creating an overall distended appearance known as bloating (Clark et al., 1997; Gill-King 1997). As the pressure of these gases increases it may force fluids to purge from wounds and the natural orifices of the body. The release of fluids and gases may cause an increase in odour associated with the remains. The odours are likely the result of the decomposition by-products such as VOCs and volatile fatty acids (VFAs) being released from the remains (Vass et al., 2004; Statheropoulos et al., 2005[a;] Rosenfeld et al., 2007; Statheropoulos et al., 2007; Vass et al., 2008; Dekeirsschieter et al., 2009; Hoffman et al., 2009).

1.5.1 *Putrefactive Breakdown of Macromolecules*

The human body consists of approximately 20% protein, 10% fat and 1% carbohydrates (Janaway et al., 2009). During soft tissue decomposition these macromolecules are broken down by enzymatic and microbiological activity into their respective constituents (Vass et al., 2002).

The enzymatic breakdown of proteins is called proteolysis and forms proteoses, peptones, polypeptides and amino acids (Dent et al., 2004). Further breakdown of amino acids occurs through deamination, decarboxylation or desulfhydralation for sulfur-containing amino acids (Gill-King 1997; Dent et al., 2004). Some mirco-organisms present during decomposition have been shown to utilize amino acids as a carbon source for fermentative metabolism (Vass et al., 2002; Boumba et al., 2008; Janaway et al., 2009; Paczkowski and Schutz 2011). This and other bacterial metabolic processes could produce a variety of decomposition VOCs including aldehydes, alcohols, aromatics, acids and sulfides (Boumba et al., 2008; Paczkowski and Schutz 2011).

During deamination the amino terminus of the amino acid is cleaved off and the nitrogen is released. In the case of the amino acid L-phenylalanine deamination produces phenylpyruvic acid and the nitrogen is released as ammonia (NH_3) (Gill-King 1997). The ammonia can be released as a gas or converted into ammonium ions which can be utilized by surrounding plants and microbes (Gill-King 1997; Dent et al., 2004).

The removal of the carboxyl group from amino acids during decarboxylation releases carbon dioxide gas (CO_2) (Gill-King 1997). The decarboxylation of ornithine and lysine produces putrescine and cadaverine, respectively. These two compounds are thought to produce the characteristic odours of decomposition and be target odours for cadaver dogs (Gill-King 1997). Recent research into the VOCs produced by decomposition has not been able to identify these compounds within the decomposition odour calling this precept into question (Vass et al., 2004; Statheropoulos et al., 2005[a;] Statheropoulos et al., 2007; Vass et al., 2008; Dekeirsschieter et al., 2009; Hoffman et al., 2009; Statheropoulos et al., 2011; DeGreeff and Furton 2011[b;] Brasseur et al., 2012; Dekeirsschieter et al., 2012). Along with putrescine and cadaverine two additional compounds resulting from protein breakdown are skatole and indole (Vass et al., 2002; Cooke et al., 2003; Dent et al., 2004). These four compounds are thought to be significant decomposition products (Vass et al., 2002). However current research may not support this (Stadler et al., 2013).

The desulfhydralation of sulfur containing amino acids produce hydrogen sulfide gas (H_2S), various sulfides and thiols (Gill-King 1997). Specifically, cysteine degradation produces hydrogen sulfide (H_2S) and methionine breaks down to produce methyl mercaptan (CH_3SH) (Higgins et al., 2006). This breakdown of sulfur containing amino acids is known to be carried out by a variety of bacteria in both aerobic and anaerobic environments (Persson et al., 1990; del Castillo-Lozano et al., 2008; Paczkowski and Schutz 2011). The oxidation of methyl mercaptan produces dimethyl disulfide (DMDS) and the oxidation of methyl mercaptan along with hydrogen sulfide produces dimethyl trisulfide (DMTS) (Chin and Lindsay 1994[a;] Chin and Lindsay 1994[b;] Zahn et al., 2001) and dimethyl tetrasulfide (DMQS) (del Castillo-Lozano et al., 2008).

Dimethyl disulfide and dimethyl trisulfide are predominant compounds found in association with decomposing remains (Vass et al., 2004; Statheropoulos et al., 2005[a;] Statheropoulos et al., 2007; Vass et al., 2008; Dekeirsschieter et al., 2009; Hoffman et al., 2009; Statheropoulos et al., 2011; DeGreeff and Furton 2011[b;] Brasseur et al., 2012; Dekeirsschieter et al., 2012). The exact mechanism for the oxidation reaction producing DMDS and DMTS is unknown (Chin and Lindsay 1994[b]) however under anaerobic conditions the oxidation could be facilitated by inner transition metals such as copper and iron (Chin and Lindsay 1994[a]). These compounds have a low sensory threshold (del Castillo-Lozano et al., 2008) and therefore could be key compounds in decomposition odour.

Carbohydrates are broken down into their sugar building blocks, such as glucose, by various micro-organisms. The sugar monomers are further broken down and depending on the amount of oxygen available produce carbon dioxide (CO_2), water (H_2O), a variety of organic acids, alcohols and fermentative gases (Dent et al., 2004; Paczkowski and Schutz 2011). In aerobic conditions the sugars are completely broken down through a series of steps (pyruvic acid to lactic acid to acetaldehyde) to form carbon dioxide and water (Dent et al., 2004; Forbes 2008). The oxidation of sugars can also produce gases such as methane, hydrogen and hydrogen sulfide (Dent et al., 2004). Under anaerobic conditions the breakdown and fermentation of sugars can produce lactic acid, butyric acid, acetic acid, alcohols including ethanol, butanol, butyl alcohol, ethyl alcohol as well as acetate, acetone and butyrate (Dent et al., 2004; Boumba et al., 2008; Forbes 2008; Paczkowski and Schutz 2011).

The decomposition of lipids begins with the breakdown of triglyceride molecules. Triglycerides are comprised of one glycerol molecule and three fatty acid chains. Hydrolysis of the triglyceride molecule produces a mixture of saturated and unsaturated free fatty acids, which can subsequently undergo hydrogenation or oxidation (Dent et al., 2004). Hydrogenation of unsaturated fatty acids will yield saturated fatty acids. If there is sufficient water and lipase enzymes available this process can convert all triglycerides to free fatty acids (Dent et al., 2004). The conversion of triglycerides to free fatty acids will occur in anaerobic environments and is enhanced by bacterial action (Forbes 2008).

This mix of saturated and unsaturated fatty acids can produce a waxy substance called adipocere, under the right conditions (Dent et al., 2004). If exposed to oxygen and light, the oxidation of unsaturated fatty acids can yield aldehydes and ketones (Dent et al., 2004). This process of oxidation can be facilitated by micro-organisms such as bacteria and fungi (Paczkowski and Schutz 2011). The breakdown of lipids by micro-organisms can also produce progressively shorter fatty acid chains and volatile fatty acids (VFAs) (Vass et al., 2002) eventually resulting in the complete degradation of fatty acids yielding carbon dioxide and water (Dent et al., 2004).

1.5.2 *Process of Decomposition*

Commonly, the complex processes of decomposition are described by a series of stages. Decomposition stages identify the gross morphological changes that result from the underlying chemical processes. Payne (1965) described six stages of decomposition; Fresh, Bloated, Active decay, Advanced decay, Dry and Remains. However the visible distinctions between the dry and the remains stage were problematic (Payne 1965). Therefore in 1996, Anderson and VanLaerhoven adapted Payne's stages by combining the dry and remains stages into one. They described five stages; Fresh, Bloat, Active decay, Advanced decay and Dry remains (Anderson and VanLaerhoven 1996). The fresh stage begins at death and continues until bloat is evident. During this stage no visible changes occur and no decomposition odour is associated with the remains. Bloat is characterized by the distension of the torso due to the accumulation of gases. The pressure from the gases may cause fluid to purge from the orifices. During this stage, colour changes occur and a strong decomposition odour may be present. Active decay begins once the carcass has deflated. During active decay there is a strong putrid odour, a decrease in the amount of soft tissue and the carcass will have an overall wet appearance, due to the liquefaction of tissue. In advanced decay most of the soft tissue will be absent and the remaining tissue may be mummified, there will also be a decrease in odour. Dry remains are characterized by minimal soft tissue with the majority of skeletal elements exposed and a lack of odour (Payne 1965; Anderson and VanLaerhoven 1996).

These stages are said to occur within certain time intervals and may be used in the determination of post-mortem interval (PMI). The fresh stage occurs 0-1 day after death, the bloat stage occurs on days 2-10 after death, active decay begins around day 11 and continues until 16 days after death, advanced decay occurs on days 17-42 and dry remains occurs 43+ days after death (Anderson and VanLaerhoven 1996) according to studies conducted in British Columbia, Canada. These time frames are variable and affected by several intrinsic and extrinsic factors, making PMI estimates difficult.

Temperature has a large effect on decomposition; generally an increase in temperature will relate to an increase in the rate of decomposition (Mann et al., 1990; Gill-King 1997; Megyesi et al., 2005). Due to the close relationship of temperature and rate of decomposition, accumulated degree days (ADD) have been used for PMI estimates. Accumulated degree days (ADD) sum the average daily temperatures over the time period of decomposition (Vass et al., 1992; Megyesi et al., 2005). For example, one body may require four days with an average temperature of 20 °C/day to reach an ADD score of 80, whereas another may require 16 days with an average temperature of 5 °C/day to reach the same ADD score of 80. Both cases have reached the same ADD and are in the same stage of decomposition even though the amount of time is different (Vass et al., 1992). The presence of a decomposition biomarker is often associated with a particular ADD (Vass et al., 1992; Vass et al., 2002; Megyesi et al., 2005). Therefore by determining the number of days required to reach the ADD needed for the production of a decomposition biomarker, PMI can be estimated. As more research is carried out to link biomarkers with ADD it can be used along with weather data to determine PMI.

The amount of humidity in the air influences the rate of decomposition. In arid environments of both temperature extremes, mummification can occur due to the loss of fluids via evaporation (Clark et al., 1997). Mummification slows down the decomposition process as it preserves soft tissue (Mann et al., 1990). Conversely, a humid environment will support interactions with plants, bacteria and insects thereby increasing the rate of decomposition (Mann et al., 1990; Gill-King 1997).

Maggot activity is another driving force in the decomposition process. The voracious feeding of fly larvae facilitates the removal of soft tissue from the remains thereby

accelerating the rate of decomposition (Mann et al., 1990; Carter et al., 2007; Simmons et al., 2010[a]) and in their absence, the rate of decomposition has been shown to be significantly slower (Simmons et al., 2010[b]). In addition to their feeding, a maggot mass generates heat and the resultant increase in the temperature of the remains above ambient may also be an influencing factor (Simmons et al., 2010[b]).

Microorganisms such as bacteria and fungi are also key factors in the decomposition process. Initially, the anaerobic microorganisms residing in the respiratory system and gastro-intestinal tracts proliferate and spread throughout the body (Gill-King 1997; Carter et al., 2007; Janaway et al., 2009). This activity is supported by the predominately anaerobic environment present within the remains, although aerobic bacteria likely remain active on the outer surface of remains (Carter et al., 2007; Janaway et al., 2009). In the later stages of decay, there is an exchange between the remains and the surrounding environment. As the putrefactive processes continue, the liquefied tissues are purged from the remains creating a nutrient rich influx into the surrounding environment (Carter et al., 2007). As a result of the remains the associated area may be further colonized by both aerobic and anaerobic soil microorganisms (Janaway et al., 2009). Currently, little is known about this faunal community and its role in soft tissue decomposition, however it is clear that cadaveric material is a nutrient source utilized by both the intrinsic and extrinsic microorganism (Carter et al., 2007; Janaway et al., 2009; Paczkowski and Schutz 2011). The macromolecules and the metabolic pathways utilized by the bacteria and fungi present will result in numerous breakdown products, including volatile organic compounds (Paczkowski and Schutz 2011).

1.6 *Current Research on Decomposition VOCs*

A limited number of publications are available that investigate the VOCs produced from decomposition. These studies have focused on identifying the chemical compounds produced by decomposition in an effort to understand what elicits a response in cadaver dogs and attracts carrion insects. This information can also be used to develop portable instrumentation for the detection of remains as well as provide insight into the chemical process of decomposition.

Initial studies were conducted by Vass et al. in 2004 and 2008. In 2004, research focused on establishing a Decomposition Odor Analysis Database (DOA Database). The database identified the volatile and semi-volatile compounds produced by buried human remains. To achieve this, pipes were buried above and below four separate human cadavers to allow sampling of VOCs at the level of the body (Vass et al., 2004; Vass et al., 2008). A hood was placed on the soil surface above the remains to sample the VOCs that migrated to the surface (Vass et al., 2004; Vass et al., 2008). The surface hood was heated to prevent water condensation. The VOCs from the three areas (below and above the body and at the soil surface) were sampled using triple sorbent traps and analysed using thermal desorption - gas chromatography - mass spectrometry (TD-GC-MS) (Vass et al., 2004; Vass et al., 2008). In this study, a total of 424 volatile compounds were identified, however only the 36 most prevalent compounds were published (Vass et al., 2004). The identified compounds were grouped into eight chemical classes; cyclic hydrocarbons (HCs), non-cyclic HCs, nitrogen, sulfur, oxygen and halogen containing compounds, acids/esters and others. Vass et al. (2004) note that the burial environment may have altered the concentrations or structure of the chemical compounds as they migrated to the surface due to soil permeability or bacterial action.

In 2008, Vass et al. continued investigating the VOCs that reach the surface from buried human remains. The second study identified a total of 478 VOCs, publishing the 30 compounds they identified as the key markers of decomposition (Vass et al., 2008). The key compounds were chosen because they were identified at the body as well as at the surface, unmodified by the burial environment (Vass et al., 2008). Interestingly, the authors state that these key markers of human decomposition are not unique and may be found in a variety of locations (Vass et al., 2008). Although the 2004 study noted that the VOC profile changed over time, the 2008 study identified the compounds within three time intervals; those found throughout the decomposition process, compounds found only early in the decomposition process and compounds that persist until the absence of soft tissue (Vass et al., 2008). The periods of ‘early decomposition’ and ‘until the absence of soft tissue’ were also defined by burial accumulated degree days (BADDs) of approximately 7,300 and 18,000 respectively.

Also included in the 2008 study was a smaller investigation into the VOCs from the bones of various species, including pigs and humans. Bones taken from a University collection were placed in Tedlar® bags and the headspace was sampled using triple sorbent traps and analysed by TD-GC-MS (Vass et al., 2008). A total of 72 VOCs were identified from the bone samples, 12 of which were also found within the key markers for human decomposition (Vass et al., 2008). The compounds were grouped into the chemical classes and the ratios of aldehydes, amides, alcohols and ketones were compared between the species. The authors state that the ratio of chemical classes was unique enough to be used for identification purposes (Vass et al., 2008). These studies have demonstrated that identifying the chemical signature of human decomposition is attainable but more research needs to be carried out in order to improve the instrumentation for the detection of the VOCs.

Studies conducted by Statheropoulos et al. in 2005 and 2007 used a similar approach to the analysis of VOCs, using triple sorbent traps and analysis with TD-GC-MS (Statheropoulos et al., 2005[a;] Statheropoulos et al., 2007). However the human cadavers were analysed within a different environment. The human remains were placed in plastic body bags and the accumulated headspace was sampled. In 2005, two cadavers were used and a total of 86 VOCs were identified. In concurrence with the previous studies a wide range of chemical classes were represented. In 2007, one cadaver was sampled four times over 24 hours. A total of 32 compounds were identified from a range of chemical classes although the majority were benzene derivatives (Statheropoulos et al., 2007). Eleven of the 32 identified compounds were detected in all four sampling cycles (Statheropoulos et al., 2007).

The authors also note that the majority of compounds were only detected during the last sampling cycle, which equates to a PMI of approximately four days. This indicates that further research needs to be conducted over longer periods of time that would allow for the qualitative and quantitative analysis of VOCs produced over the decomposition process (Statheropoulos et al., 2005[a;] Statheropoulos et al., 2007). The authors propose that this could be achieved through the use of animal models. They also remark on the assumption that cadaver dogs have been thought to alert to putrescine and cadaverine,

despite these compounds not being detected in their studies and that further investigation into this discrepancy is required (Statheropoulos et al., 2007).

In 2011, Statheropoulos et al. conducted a study investigating post-mortem volatiles utilizing 20kg pig carcasses as human analogues. This study investigated the post-mortem chemistry within a simulated void of a collapsed building in order to improve victim remains recovery in the event of mass disasters. In total the study found over 150 VOCs using TD-GC- time-of-flight mass spectrometry (TOFMS) and reported the 30 most abundant compounds (Statheropoulos et al., 2011). The resulting list of compounds was representative of the variety of chemical classes commonly observed and select compounds including DMDS, DMTS, trimethylamine, acetone and 2-butanone were discussed in depth (Statheropoulos et al., 2011).

The authors noted that there were differences in the profile of volatile sulfur compounds (VSCs) and CO_2 levels between pig carcasses obtained from a pig farm and those from the department of experimental surgery at the University of Athens Medical School (DESUA). This difference was also seen in the observations of soft tissue decomposition and was attributed to the differences in microbial load of the pig skin as well as an increased organic load of the farm pigs (Statheropoulos et al., 2011). However, it should be noted that the DESUA pigs had some internal organs removed which may have altered the VOCs profile. This study also demonstrated the usefulness of hyphenated analytical techniques for the analysis of decomposition odour.

Hoffman et al. (2009) conducted a study aimed at identifying the VOC profiles generated by various canine training aids. Various human tissues that had previously been used by canine handlers as training aids were sampled (Hoffman et al., 2009). The tissues included blood clots, blood, muscle tissue, skin, adipose tissue, adipocere and bone. The sampling of VOCs was carried out using solid phase micro-extraction (SPME) fibers and analysis was completed by GC-MS (Hoffman et al., 2009). The 14 tissue samples produced a total of 33 VOCs from a range of chemical classes and although there were some similarities between the samples, the results of this paper indicated that no single compound was found in all tissue profiles (Hoffman et al., 2009). The majority of compounds detected were alcohols and aldehydes (Hoffman et al., 2009).

This study was successful in identifying indole in the headspace of four human tissue samples. Cadaver dog training is based on the assumption that the scent of decomposition can be generalized. From this assumption it follows that a dog trained on one type of human tissue will be able to detect all human decomposition materials (Hoffman et al., 2009). However, this study found that a dog trained only on one tissue type may not have been provided with the appropriate range of odour targets required to locate a variety of specimens (Hoffman et al., 2009). Therefore a more holistic approach to the selection of training aids may be beneficial. The authors also noted that in comparison with previous studies (Vass et al. 2004), the VOCs they identified were a subset of the total VOC profile and that a more effective extraction technique along with additional analytical methods should be explored (Hoffman et al., 2009).

In 2012 the Hoffmann et al. (2009) study was replicated by Cablk and colleagues (Cablk et al., 2012). The same sampling and analysis methodologies was utilized to analyze the VOCs from the isolated tissues of cow, chicken and pig (Cablk et al., 2012). Bone, fat, muscle and skin tissue were collected from each animal. The results were then compared with those generated from human tissues by Hoffmann et al. (2009). As was previously demonstrated (Hoffman et al., 2009) the different tissue types produced different combinations of VOCs. Additionally it was observed that the VOC profile changed as decomposition progressed resulting in a dynamic profile with different compounds being detected across tissue types and decomposition status (Cablk et al., 2012).

The comparison of VOC profiles between animal and human tissues led the authors to report that the profiles were sufficiently different to support the hypothesis that humans and animals could be differentiated by canine olfaction. The main focus of the study was to improve human remains detection by developing a unique human VOC profile for the development of canine training aids and portable instrumentation. The study noted that the halogenated compounds, poly chlorinated and fluorinated hydrocarbons, could be key compounds in the unique human odour signature (Cablk et al., 2012). They noted that if these compounds are unique to humans and ubiquitous across populations then they could be used to develop instrumentation that would be human specific. Alternatively, if the goal is to identify the compounds responsible for a canine's target odour signature,

then other factors need to be considered when evaluating a VOC profile (Cablk et al., 2012).

Several studies have utilized pig carcasses to investigate decomposition VOCs (Dekeirsschieter et al., 2009; Brasseur et al., 2012; Dekeirsschieter et al., 2012). The 2009 study was conducted to better understand the role that VOCs play in the attraction of carrion insects and used 25 kg pigs (*Sus scrofa*) placed in three different environments; forest, agricultural and urban (Dekeirsschieter et al., 2009). The VOCs were passively sampled using adsorbent tubes and analysed with TD-GC-MS. In total, 104 VOCs were identified and a common core of 35 compounds was found which spanned the various chemical classes (Dekeirsschieter et al., 2009). The decomposition product indole was identified however it was only found within one sample and is therefore not included as a core compound.

Observations of the decomposition stages were conducted during the study and it was found that no VOCs were detected during the fresh stage; however the bloat, active and advanced decay stages all produced VOCs. A principle component analysis of the data showed that the environment and the decomposition stage had an influence on the VOCs produced (Dekeirsschieter et al., 2009). This study found that the VOC profile produced by pig decomposition is similar to that produced by human decomposition and the authors continued their research using pigs as human models (Dekeirsschieter et al., 2009).

In 2012, Dekeirsschieter et al. were the first to utilize comprehensive two dimensional gas chromatography – time-of-flight mass spectrometry for the analysis of decomposition headspace. The samples were collected from a 25 kg pig carcass using sorbent cartridges and were analysed following solvent desorption. The additional peak capacity of this technique was evidenced by the more than 4,000 peaks identified in the samples (Dekeirsschieter et al., 2012). Following the removal of compounds present in reference soils and instrumental background, 830 decomposition VOCs were identified, however only 225 compounds were reported more than once (Dekeirsschieter et al., 2012).

The compounds represented a variety of chemical classes and were present throughout all stages of soft tissue decomposition. The authors noted that as decomposition progressed there was a transition in the dominant compounds. The fresh stage had higher levels of alcohols (1-butanol) which persisted into the bloat stage along with aldehydes (hexanal, 3-methyl butanal), aromatics (1H-indole) and sulfur (DMTS) compounds. Active decay was characterized by sulfur and nitrogen (trimethylamine) along with carboxylic acids (butanoic acid, butanoic acid-2-methyl & 3-methyl, propanoic acid-2-methyl) and aromatics (phenol-4-methyl). The profile during the advanced decay stage was dominated by ketones (2-hexanone, 3-hexanone, cyclohexanone) and alkanes (1,3-diethylcyclopentane). Dekeirsschieter et al. (2009) noted both similarities and differences between the volatile compounds identified from the headspace of pig carcasses and human remains. However the amount of variability in the compounds reported in the literature may be due to the range of analytical methods being utilized (Dekeirsschieter et al., 2012).

A complimentary study was conducted by Brasseur et al. (2012), in which the headspace of grave soil from the burial of pig carcasses was analyzed using solvent desorption – two dimensional gas chromatography – time-of-flight mass spectrometry. Soil samples were collected above and below the carcass as well as through the soil column above the grave (Brasseur et al., 2012). This study identified two main fractions of compounds, one set of 20 compounds was specific to the soil from below the carcass, the other group of 34 compounds was identified in gravesoil samples from all depths.

The VOCs from the soil below the corpse seemed to represent a sub-set of the larger decomposition VOC profile with some but not all of the major chemical classes being identified (Brasseur et al., 2012). The depth gravesoil samples were characterized by the presence of methyl-branched alkanes, additionally a depth trend was observed which illustrated the migration of the methyl-branched alkanes from the carcass through the soil column to the surface (Brasseur et al., 2012). This study was able to further demonstrate the power of GC×GC-TOFMS and its data handling strategies for applications to forensic geophysics and taphonomy.

1.7 Thermal Desorption

The method of sample collection is important for ensuring the complete VOC profile is trapped and analyzed. The most common sample collection techniques for the analysis of decomposition VOCs have been solid-phase microextraction (SPME) (Hoffman et al., 2009; DeGreeff and Furton 2011[b;] Cablk et al., 2012), thermal desorption (TD) (Vass et al., 2004; Statheropoulos et al., 2005[a;] Statheropoulos et al., 2007; Vass et al., 2008; Statheropoulos et al., 2011) and solvent desorption (Dekeirsschieter et al., 2009; Brasseur et al., 2012; Dekeirsschieter et al., 2012). Although solvent desorption provides a liquid sample that can be stored, the resulting large solvent peak can mask the more volatile compounds (Agelopoulos and Pickett 1998; Brasseur et al., 2012; Dekeirsschieter et al., 2012).

Few studies have combined thermal desorption (TD) and GC×GC-TOFMS (Xu et al., 2003; Sanchez and Sacks 2006), and to date no published studies have utilized this methodology for decomposition headspace analysis. However air sampling using TD tubes provides several advantages compared to other approaches. TD tubes are available in single or multiple sorbents formats, they are made of robust stainless material, they can easily be loaded using simple pumping devices, they present high field portability, analysis with TD devices allows splitting of the trapped VOCs between the injector and a secondary tube that can be used for duplicate analysis or archiving (McClenny 1999; Ribes et al., 2007). In contrast, SPME offers less possibilities in terms of sorbent phase combinations, and generally requires an equilibrium status to be reached between the sample matrix, the headspace and the fiber, which is not always feasible in a field setting (Agelopoulos and Pickett 1998; Augusto et al., 2001). Both methods can be used for qualitative and quantitative analysis, however the introduction of an internal standard is more challenging with SPME analyses.

A two-step desorption sequence for sample introduction of TD samples also provides advantages in the analysis of VOCs. Included in the two-step desorption process is a dry purge to remove moisture and a cold trap focusing step. During primary desorption the volatiles are condensed onto a sorbent bed and are then rapidly desorbed and transferred onto the head of the capillary column in a discreet plug. This focusing step functions to

improve peak shape and compound resolution (McClenny 1999; Sanchez and Sacks 2006).

1.8 *Two Dimensional Gas Chromatography – Time-of-flight Mass Spectrometry*

Two dimensional gas chromatography (GC×GC) is an emerging technique that provides the additional peak capacity required for the analysis of complex samples, however its wide range of potential applications are not well represented in the literature (Dalluge et al., 2003). Recent studies into decomposition odour have shown the applicability of this analytical technique to this unique sample type (Brasseur et al., 2012; Dekeirsschieter et al., 2012).

GC×GC has been developed for the trace analysis or in-depth investigations of complex samples and matrices. In these situations, the peak capacity of conventional GC systems might not be adequate to achieve efficient separation of sample components. Sample preparation techniques such as column chromatography or liquid chromatography could be applied to samples prior to analysis, however these methods can be time consuming (Dalluge et al., 2003) and are not applicable to volatile samples. GC×GC-TOFMS provides multi-dimensional information about the entire sample in approximately the same amount of time as conventional one GC dimensional systems and can be combined with a variety of sample types and injection systems (Semard et al., 2009).

Multidimensional GC techniques started as heart-cut GC (GC-GC) where individual fractions of the column effluent were subjected to a secondary separation. The additional peak capacity gained from this analysis can be represented as the sum of the two columns (^{1}D peak capacity + ^{2}D peak capacity) (Dalluge et al., 2003). This is in contrast to *comprehensive* two dimensional GC (GC×GC) where all the column effluent from the first dimension (^{1}D) undergoes separation on a second GC column in the second dimension (^{2}D) (Dalluge et al., 2003; Shoenmakers et al., 2003). In this case, the overall peak capacity can be represented as the product of the two columns (^{1}D peak capacity x ^{2}D peak capacity) (Giddings 1987; Venkatramani et al., 1996; Dalluge et al., 2003). Comprehensive GC×GC provides a distinct advantage because the entire sample

undergoes thorough analysis and therefore the maximum amount of information is gained.

The first and second dimension columns of GC×GC are connected in series (Figure 1.1) and are selected to ensure the orthogonal separation of components. In analytical chemistry the term *orthogonal* refers to two different separation mechanisms. Orthogonal separation occurs when a compound's elution times in the two dimensions are statistically independent of each other (Venkatramani et al., 1996; Shoenmakers et al., 2003). Typically this is achieved with a longer non-polar column in the ^{1}D followed by a shorter more polar column in the ^{2}D (Dalluge et al., 2003). This allows the compounds to be separated by two different properties; boiling point and their activity co-efficient respectively (Semard et al., 2009). As two compounds are not likely to have the same boiling point and the same 2nd dimension column interaction, they can be efficiently resolved.

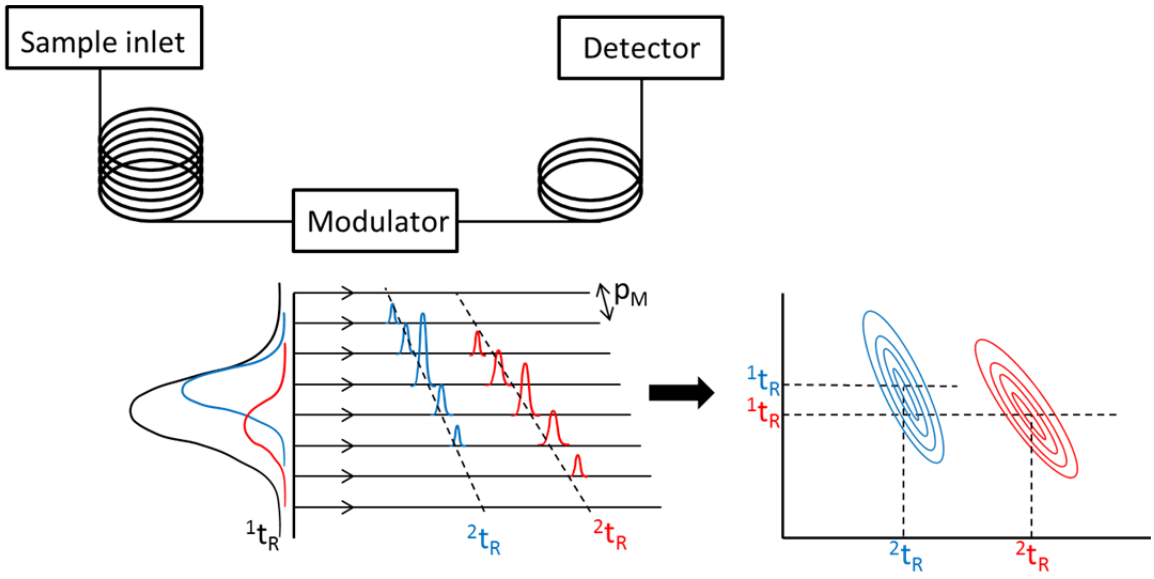


Figure 1.1: Schematic of GC×GC system. Sample inlet can be liquid injector or thermal desorption apparatus, detector is typically a TOFMS. $^{1}t_R$: first dimension retention time, $^{2}t_R$: second dimension retention time, p_M: modulation period. Figure adapted from Semard et al., 2009.

Along with phase type, the dimensions of the two GC columns also differ. Generally, the ^{1}D column is a typical column utilized in one-dimensional GC analysis with a length of 15-30 m and an internal diameter of 0.25 mm. The ^{2}D column is a short column of approximately 0.5-2 m with a narrow internal diameter of 0.1mm which allows for fast ^{2}D separation and analysis (Dalluge et al., 2003). The ^{2}D column can be housed within a separate oven, the temperature of which can be independently controlled allowing for optimization of retention times in the second dimension. If located in a secondary oven, the difference between the temperature programing of the two ovens is known as the *column offset*. Although the secondary oven utilizes the same temperature ramps, the temperatures will be offset by +5-15 °C.

The key feature of the GC×GC instrumentation is the modulator. The modulator is situated between the two columns and serves several functions; to continuously trap and re-focus fractions of effluent from the first dimension column, and to re-inject these fractions onto the second dimension column (Dalluge et al., 2003). The trapping and subsequent release of fractions is achieved by the alternate cooling and heating of the GC column, known as a *modulation*. The time required for one cooling and heating cycle is known as the *modulation period* (P_M) and is optimized so that each modulation peak has eluted from the second dimension column prior to the injection of the next (Dalluge et al., 2003). This avoids a phenomenon known as *wraparound* which can result in the overlap or co-elution of peaks from different modulation cycles. The P_M includes the time required for both the focusing step (*cool time*) and the injection step (*hot pulse time*) of modulation. Similar to the column offset, the *modulator offset* describes the difference in temperature between the secondary oven and the modulator. The modulator functions as a sample inlet for the secondary column, and just as injectors are kept at temperatures higher than that of the column, the modulator must also be set at a temperature higher than that of the secondary oven.

As a peak elutes from the ^{1}D column the modulator collects small fractions of it at a time, producing multiple slices across the peak (Figure 1.1). In order to maintain the separation achieved in the first dimension, the number of slices per peak or a *modulation number* (n_M) of at least four is desirable (Dalluge et al., 2003). The peaks resulting from

the two dimensional separation and modulation are quite narrow (100 – 600 milliseconds) and require fast detectors in order to reconstruct the two dimensional chromatograms. Time of flight mass spectrometry (TOFMS) fulfils this requirement with its acquisition rates of >50 Hz (Dalluge et al., 2003). In contrast to other mass spectrometers such as quadrupoles or ion traps, TOFMS is a non-scanning instrument and collects full mass spectra during each acquisition (Cochran 2002; Semard et al., 2009). These nonskewed spectra ensure that consistent ion ratios are maintained as a peak elutes into the detector (Erickson et al., 1990). These nonskewed spectra allow for software algorithms to perform peak finding and spectral deconvolution of overlapping peaks (Cochran 2002). The combination of the chromatographic resolution provided by GC×GC along with the analytical or mass spectral resolution provides an extremely powerful tool for the analysis of complex samples as each sample component will be described by its first dimension retention time (^1t_R), second dimension retention time(^2t_R) and mass spectra.

The initial data processing required to generate an output for GC×GC-TOFMS analysis requires extensive computing power and integrated software tools (Dalluge et al., 2002; Dalluge et al., 2003). As a part of the automated data processing, the software calculates the baseline, performs peak finding and deconvolution, library searching and peak integration and generates two dimensional contour plots. The aim of the automated peak find is to locate all peaks larger than a user defined signal-to-noise threshold. The pure mass spectra of each peak are then generated with deconvolution algorithms and these pure spectra are searched against mass spectral libraries for identification (Dalluge et al., 2002).

The raw output of the TOFMS is a linear trace of the modulated peaks or slices. Each slice has four pieces of information associated with it; the first dimension RT (^1t_R), the second dimension RT (^2t_R), a mass spectrum and signal intensity. The first RT identifies which modulation the peak occurred in and the second RT identifies the peaks' RT within the modulation (LECO 2009). A comparison of the ^2t_R and pure or deconvoluted mass spectra allows for the individual slices to be combined. This reconstructed peak matrix is then plotted by their first and second retention times. In a 3D surface plot the

intensity of a peak is shown on the z-axis. A contour plot is a 2D plot of the data and is essentially a birds-eye view of the 3D surface plot (Figure 1.1). In both, the colour scale indicates the intensity of a peak. Once processed, the data may also be presented as an apex plot. In this case there is no scaling to indicate the intensity of the peak and the two retention times of the peak apex are plotted as one point. The contour plot can be overlaid with the apex plot for further clarity (Dalluge et al., 2002).

1.9 Study Objectives

The purpose of this study is to investigate the use of pig carcasses as an alternative canine training aid. The first objective is to generate a VOC profile for pig carcass decomposition by conducting a preliminary microcosm study and full scale outdoor decomposition trials utilizing human analogues. The preliminary microcosm will allow for the identification of decomposition VOCs in the absence of environmental VOCs and the outdoor trials will elucidate the VOC profile across soft tissue decomposition. The second objective is to compare the decomposition odour profiles of pig carcasses to that of human remains. This will be accomplished by conducting decomposition trials in which both pig carcasses and human remains are placed within the same environment and comparing the resulting VOC profiles. The final objective is to investigate the composition of a commercially available synthetic canine training aid in order to evaluate current training aids and pig carcasses as human decomposition odour mimics.

Chapter 2: *Materials & Methods*

2.1 *Introduction*

This study involves four distinct projects; an indoor microcosm trial, outdoor field trials utilising human analogues, outdoor field trials utilising human remains, and an examination of artificial canine training aids. The microcosm was a preliminary investigation to determine the types of compounds produced by decomposition in the absence of environmental VOCs. This project utilized pig carcasses and was analysed with thermal desorption – gas chromatography – mass spectrometry (TD-GC-MS).

The outdoor field trials that utilised human analogues took place at the UOIT Geoforensic Research Facility (GRF) and monitored the surface decomposition of pig carcasses to determine the VOC profile of soft tissue decomposition. This trial was replicated over three years, each occurring during the spring-summer months and commencing in June; Trial 1 (2010), Trial 2 (2011) and Trial 4 (2012). Trial 3 (2011) was also a surface decomposition trial using pig carcasses at the UOIT-GRF but it commenced in July and was conducted to collect samples for further characterization of decomposition VOCs using a more advanced technique, thermal desorption – two dimensional gas chromatography – time-of-flight mass spectrometry (TD-GC×GC-TOFMS).

In order to compare the VOC profile of pig carcasses and human remains within the same decomposition environment, research trials were conducted at the Forensic Anthropology Research Facility (FARF) at Texas State University in collaboration with the recipients of a US Department of Justice grant (No. 2010-DN-BX-K243) (Hamilton et al., 2010). One of the aims of this grant was to investigate the effect of insect colonization on the microbial community of decomposing remains. In order to achieve this objective, two experimental treatment groups were established; insect inclusion and insect exclusion. The insect inclusion treatment was allowed to be colonized by insects, in contrast to the insect exclusion treatment, where arthropods were prevented access to the remains. Within this larger project, the current study monitored the surface decomposition of human remains and pig carcasses in the same environment and collected VOC samples for analyses using TD-GC×GC-TOFMS. These trials were conducted three times, fall 2011 (November), spring 2012 (May) and fall 2012 (November). The trials will be

herein referred to as human remains trial 1, human remains trial 2, and human remains trial 3, respectively.

To investigate the chemical composition of commercially available canine training aids and evaluate their accuracy as decomposition odour mimics, Sigma Pseudo™ Corpse Scents were analysed with GC×GC-TOFMS.

2.2 *Experimental Supplies*

2.2.1 *Chemicals and Consumables*

The microcosms were housed in five 30 gallon steel drums purchased from U-LINE®. The interior and underside of the lid of each barrel was wiped down with a soft sponge and reverse osmosis water to remove any dirt from the interior of the barrels. A decomposition surface of sand was selected and 24 kg of loose sand (Hard-Co, Whitby Ontario, Canada) was added to each barrel.

Weather stations equipped with temperature loggers were present at all field trials. For the microcosm study the probe of a HOBO® data logger was placed within the microcosm. This data logger was then installed on a weather station located within the UOIT-GRF for the outdoor trials conducted in Oshawa, Ontario, Canada. All carcass deposition sites were located within 20m of the weather station. The Forensic Anthropology Research Facility at Texas State University was equipped with NexSens DS1923 micro-T temperatures loggers (Fondriest Environmental, Inc., Alpha, OH, USA) which were placed within 1 m of each set of remains.

To prevent avian and mammalian scavenging at the UOIT-GRF, cages (1.1 m x 0.8 m x 0.4 m) constructed of rebar and 1 cm^2 wire mesh were placed over the carcasses and secured to the ground between sampling periods.

To prevent scavenging at the Forensic Anthropology Research Facility at Texas State University the human remains were placed under modified 5 m x 5 m x 3 m (l x w x h) vulture exclusion cages (Quanset, Growers Solutions, Cookeville, TN, USA) as shown in Figure 2.1. The frame was covered with Tenax 15 m x 1.3 m (l x h) plastic hardware cloth (TENAX Corporation, Baltimore, Maryland, USA).

Figure 2.1: Modified vulture exclusion cage used to prevent scavenging of remains at the Forensic Anthropology Research Facility for human trials 1, 2 and 3.

The bottom 0.5 m of the cage was additionally covered with 2.5 cm 20-gauge galvanized wire poultry netting to prevent scavenging. The top portion (roof) of the cage extending down from the top center approximately 2 m was covered with Easy Gardner sun screen fabric with 70 % shade (Easy Gardener Products, Inc., Waco, TX, USA) in order to reduce exposure to sunlight. For each trial one set of remains was allocated to the insect inclusion and insect exclusion treatment group. The insect inclusion remains were placed under the vulture exclusion cage without any additional screen to exclude arthropods. Alternatively, the insect exclusion treatment group were placed under a 3.7 m x 1.8 m x 1.8 m (l x w x h) Lumite® screen (18 x 14 mesh size) (BioQuip Products, Rancho Dominguez, CA, USA) inside the vulture exclusion cage in order to inhibit arthropod access to the remains (Figure 2.2). At the Forensic Anthropology Research Facility, the pig carcasses were placed under 0.9 m x 0.6 m x 0.6 m (l x w x h) anti-scavenging cages constructed of a metal grating covered with wire poultry netting.

Figure 2.2: Insect exclusion netting within the vulture exclusion cage for the insect exclusion treatment of the human remains trials 1, 2 and 3. The additional screening prevents access to remains by avian and terrestrial arthropods.

To accumulate a headspace during outdoor field trials a sampling hood was fabricated of stainless steel with the dimensions 100 cm x 70 cm x 40 cm (l x w x h) producing a total headspace volume of 280 L (280000 cm^3 or 0.28 m^3). Stainless steel was chosen for the hood in order to ensure no loss of VOCs through adsorption (Restek 2008). A stainless steel Swagelok® bulk-head connector formed a continuous sample path from under the hood to the sampling tube. The design of the volatile collection hood was carried out in accordance with previous studies on the collection and analysis of decomposition VOCs (Vass et al., 2004; LeBlanc 2008; Vass et al., 2008; LeBlanc and Logan 2010).

The volatile collection hood for the human remains was transported in two pieces each with the dimensions 120 cm x 76 cm x 76 cm (l x w x h). At the time of collection, the hood is assembled by aligning the two pieces to overlap at the midpoint. Similar to the Canadian trials, a Swagelok® bulk-head connector was used as a sampling port for connection of the sample tubes to the hood.

All volatile samples were collected using multi-sorbent thermal desorption tubes comprising of Tenax® GR and Carbopack™ B, purchased from Markes International Ltd (Llantrisant, United Kindom). This combination of sorbents provides the greatest range in volatility (C_6-C_{20}) (McClenny 1999), thus allowing for the collection of a wide range of VOCs. A LaMotte model BD (Chestertown, Maryland, USA) constant flow air sampling pump was used to draw the volatile sample onto the thermal desorption tubes.

Puriss (≥99.5 %) bromobenzene was purchased from Sigma Aldrich® (Oakville, Ontario, Canada) and utilised as the internal standard (IS). This compound was chosen as it is not expected to be produced from soft tissue decomposition, is not present in the atmosphere and will not co-elute with compounds of interest. Additionally, bromobezene was selected as the internal standard in accordance with previous decomposition VOC literature (Vass et al., 2004; Vass et al., 2008). The bromobenzene standard was made up to a concentration of 20 ng/µL using methanol (HPLC grade; Sigma Aldrich®, Oakville, Ontario, Canada). The standard was spiked onto the sampling tubes resulting in a mass loading of 160 ng of bromobenzene.

For the investigation of current canine training aids, the Sigma Pseudo™ Corpse Scent kit was purchased from Sigma Aldrich® (Oakville, Ontario, Canada) and comprised of Sigma Pseudo™ Corpse Scent Formulation I- for early detection and Sigma Pseudo™ Corpse Scent Formulation II- for post putrefactive decay.

Chemicals and standards used in the GC×GC-TOFMS analysis were purchased from Sigma Aldrich® in Schnelldorf, Germany. This included the internal standard bromobenzene, a GC grade alkane standard (C_8-C_{20}), and standards for compound identification; 4-aminobutanoic acid (GABA), 1,4-diaminobutane (putrescine), 1,5-diaminopentane (cadaverine), pyrimido[1,2-a]azepine, 2,3,4,6,7,8,9,10-octahydro- (DBU), butyrolactone and tridecylamine.

2.2.2 *Research Subjects*

The difficulty in obtaining human cadavers for research in Canada requires an alternative be used for research and training. Pig carcasses (*Sus scrofa domesticus*) (Linnaeus 1758) are commonly used as human analogues in decomposition and entomology studies. Pigs

are similar to humans in several respects; they have similar internal anatomy, fat distribution, lack of heavy fur and are omnivores therefore they are likely to share a similar gut flora as humans (Anderson and VanLaerhoven 1996; Schoenly et al., 2006). Earlier studies used a variety of animals including cats, dogs, rabbits, lizards and elephants however it was determined that the differences in carcass size or coat type had an effect on insect colonization and decomposition (Anderson and VanLaerhoven 1996). The extrapolation of these studies to forensic cases involving humans has been questioned; an appropriate human analogue should accurately replicate the pattern of human decomposition, be easy to obtain, inexpensive and not generate public scrutiny (Catts and Goff 1992). A 22 kg pig carcass approximates an adult male torso and fulfils all these criteria thereby facilitating comparisons to human forensic cases (Catts and Goff 1992; Anderson and VanLaerhoven 1996).

The microcosm utilised four pig carcasses of approximately 13.6 kg (30 lbs) and the spring trials (Trial 1, 2, 4) each utilised four pig carcasses of approximately 23 kg (50 lbs). The summer trial (Trial 3) utilised two carcasses of approximately 23 kg. All pigs utilized in the Canadian trials were euthanized by captive headbolt in accordance with the government of Ontario's Ministry of Agriculture, Food and Rural Affairs approximately an hour prior to deposition at the research site. A schematic of the deposition sites within the UOIT-GRF is provided in Figure 2.3.

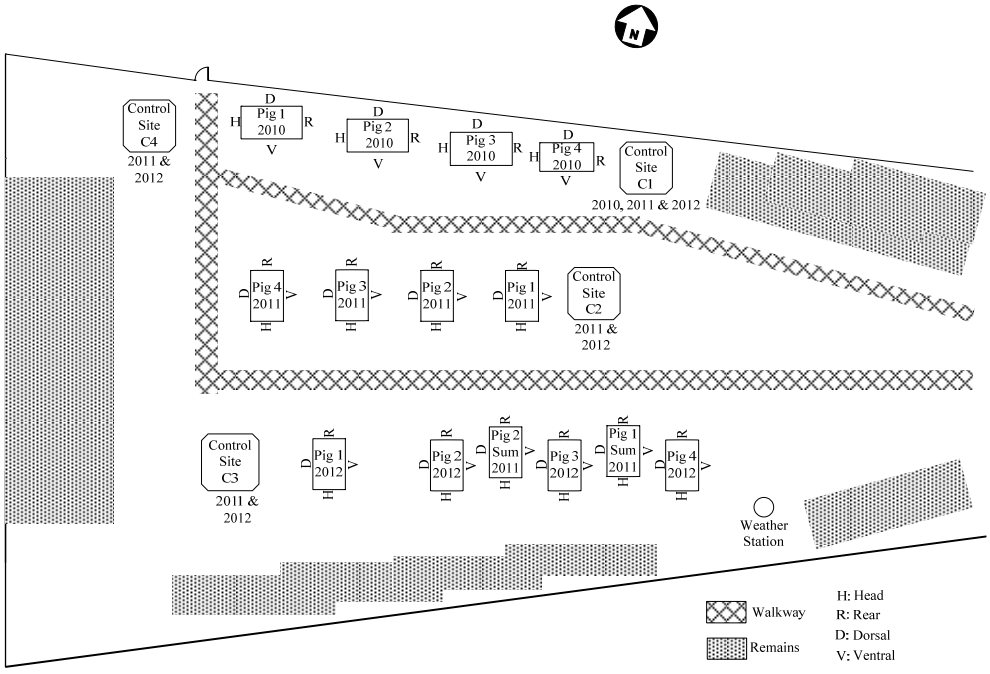


Figure 2.3: Schematic of the UOIT-GRF displaying the location of carcass deposition and control sites for outdoor Trials 1, 2, 3 and 4. Rectangles denote carcass deposition sites (Pig 1-4); the northern most sites are for Trial 1 (2010), sites for Trial 2 (2011) are located in the middle of the facility, and sites for Trial 3 (2011) and Trial 4 (2012) are the southernmost in the facility. Control sites (C1-C4) are indicated by the square shapes and were used throughout the outdoor studies.

The human remains utilized in human remains trial 1, 2 and 3 were donated to the Forensic Anthropology Centre at Texas State University in San Marcos, Texas under the United States Universal Anatomical Gift Act (UAGA). Three treatment groups were utilized in each of these research trials; insect inclusion where entomological colonization was not inhibited, insect exclusion where insects were inhibited from accessing the remains and a pig carcass control (also insect inclusion).

The first trial was conducted in November 2011. The donor for the insect inclusion treatment was an 84 year old black male (H1). The date of death (DOD) was October 25th 2011. The cause of death (COD) was prostate cancer, which was diagnosed approximately 2 months prior to death. The individual was 6' 2" (187.96 cm) in height

and weighed 47.6 kg (105 lbs) at death. Field placement occurred on November 2nd 2011, with a pre-deposition PMI of 8 days.

The donor for the insect exclusion treatment was a 75 year old Caucasian female (H2). The DOD was October 28th 2011 and the COD appeared to be due to natural causes. The individual was 5'7" (170.18 cm) and 61.2 kg (135 lbs). Field placement occurred on November 2nd 2011, with a pre-deposition PMI of 5 ½ days.

The pig carcass control (P1) for this trial was 19.1 kg (42.1 lbs) and was euthanized on October 30th 2011 and kept on ice at 4°C until field placement on November 2nd, a pre-deposition PMI of 3 days.

The second trial was conducted in May 2012. The donor for the insect inclusion treatment was a 59 year old Caucasian male (H3). The DOD was May 15th 2012 and the immediate COD was anoxic encephalopathy. The individual was 178 cm and weighed approximately 153 kg (337 lbs). Field placement occurred on May 22nd 2011, with a pre-deposition PMI of 7 days.

The donor for the insect exclusion treatment was a 90 year old Caucasian female (H4). The DOD was May 15th 2012 and the COD was determined to be respiratory failure. The individual was 168 cm and weighed 61 kg (134.5 lbs). Field placement occurred on May 22nd 2011, with a pre-deposition PMI of 7 days. While at the TSU anthropology facility the remains for the May 2012 trial were stored in a human remains cooler at approximately 4.5 °C.

The pig carcass control (P2) for this trial was approximately 77 kg (170 lbs) and was euthanized on May 22nd 2012 and transported on ice until field placement that day at 5:00 pm.

The third trial was conducted in November 2012. The donor for the insect inclusion treatment was a 67 year old Hispanic female (H5). The DOD was October 29th 2012 and the COD was metastatic breast cancer. The individual was 157 cm and weighed 54.4 kg (120 lbs). Field placement occurred on November 1st 2012, with a pre-deposition PMI of 3 days.

The donor for the insect exclusion treatment was a 57 year old Caucasian male (H6). The DOD was October 23rd 2012 and the immediate COD was pneumonia with an underlying COD of lung cancer. The individual was 190 cm and weighed approximately 59 kg (130 lbs). Field placement occurred on November 1st 2012, with a pre-deposition PMI of 9 days. While at the TSU anthropology facility the remains for the November 2012 trial were stored in a human remains cooler at 4.4 °C

The pig carcass control (P3) for this trial was approximately 68 kg (150 lbs) and was euthanized on the morning of November 1st 2012 and transported on ice until field placement that day at 6:24 pm.

2.3 Research Trials

2.3.1 *Microcosm*

Indoor microcosms were established to investigate the VOCs produced by decomposition within a controlled environment. The VOCs from both experimental and control microcosms were analysed. Each experimental microcosm consisted of a pig carcass placed on a sand substrate housed within a steel drum (Figure 2.4). The control microcosm consisted of only the sand substrate housed within the microcosm and was utilized to monitor background levels of VOCs from the indoor environment. A data logger recorded the temperatures within the microcosm at hourly intervals throughout the research trial.

Figure 2.4: Stainless steel barrel used for experimental set-up for the Microcosm trial. Inset: placement of carcass on sand substrate within the microcosm on experimental day 0.

Sand was selected as the surface substrate as it has little to no organic content which could produce its own volatile signature. The water holding capacity (WHC) of the sand was adjusted to 25 % to ensure moisture availability for soft tissue decomposition and to prevent tissue desiccation. The water holding capacity of the sand was determined following the method outlined in Tibbett et al. (2004). Briefly; fifty grams of sand was soaked in water for 24 hours. The saturated sand was then transferred to a Büchner funnel until water ceased to drip (Tibbett et al., 2004), approximately 2 hours. The sand was removed from the Büchner funnel and weighed to determine its mass at a water holding capacity (WHC) of 100 %. The mass of water retained within the sand when saturated was determined by placing it in an 80 °C oven overnight and subtracting the dry weight from the wet weight. Based on this calculation, 1 L of water was added to the 24 kg of sand in each barrel to establish a WHC of 25 %.

Following preparation of the microcosm, where the interior surfaces of the barrels were wiped with reverse osmosis water and addition of the sand substrate, the pig carcasses were placed on the decomposition surface and the lid was sealed. Two access holes were located in the lid, a larger hole for viewing and a smaller sampling port. When not in use, both access holes were sealed.

Throughout the research period the headspace within the microcosm was replaced with ambient air in conjunction with volatile sample collection. During the fresh stage of decomposition the headspace was replaced post-sampling but once the pig carcasses reached the bloat stage of decomposition the amount of gases and VOCs being produced had overwhelmed the GC-MS analysis. In order to reduce the amount of VOCs sampled an alternative venting-sampling regime was developed. The alternate procedure used is described herein; first, the headspace of each microcosm was replaced using an air pump and vented into the fume hood for a period of 30 minutes; second, the microcosms were sealed for another 30 minutes, allowing for the re-accumulation of VOCs prior to sampling. At the time of sampling, a sample tube was inserted into the microcosm and a rubber stopper formed a seal between the sampling tube and the access hole. The VOCs were sampled at a constant flow rate of 0.2 L/min for 5 minutes, producing a total volume of 1 L of air being sampled. These sampling parameters were selected in accordance with the Environmental Protection Agency (EPA) compendium method T0-17 (McClenny 1999). Immediately after sampling the tube was sealed using long-term storage caps and placed within an air tight container.

Samples, photographs and observations on the state of decomposition were collected daily for two weeks, then decreased to 2-3 times /week and decreased again to weekly intervals until the trial was terminated on experimental day 96. At this point the decomposition process appeared to have slowed and minimal changes were occurring.

2.3.2 *Human Analogue Field Research*

Outdoor research trials utilizing pig carcasses were conducted in Oshawa Ontario at the University Of Ontario Institute Of Technology's Geoforensic Research Facility (UOIT-GRF). UOIT is located in the City of Oshawa at the latitude/longitude of 43.947 °N,

78.898 °W (Natural Resources Canada[a]). It is located within the Mixedwood Plains forest ecozone of Canada (Natural Resources Canada[b]). This forest ecozone is composed of a variety of hardwood species, however it is also a densely populated area. UOIT is adjacent to both urban development and agricultural land. During the month of June, when field trials commenced, Oshawa has a daily average temperature of 17.2 °C (maximum 21.9 °C and minimum 12.4 °C) and receives 80.6 mm of rainfall (Environment Canada 2010).

The GRF is a quarter acre of open grassed land enclosed by a chain link fence. The vegetation within the GRF is mixed (Watson and Forbes 2008), mostly composed of grasses, dandelions, clovers and wild flowers of southern Ontario with some bare patches. Prior to the deposition of the carcasses the vegetation was trimmed using a string trimmer and the refuse was raked from the test areas. Control sites were delineated within the GRF and underwent the same preparation as experimental sites. The areas chosen for control sites included similar vegetation to those of the experimental sites and had not previously been used for decomposition trials. The deposition and control sites were approximately 3.0 m apart to prevent contamination of the control area by fluid purging and/or maggot migration. Separating the carcass from a mixed forest were two fences, a chain link and a small wooden fence.

The GRF also contained additional graduate experiments utilizing pig carcasses as well as the skeletal remains of various species: Domestic pig (*Sus scrofa domesticus)*(Linnaeus, 1758), Cattle *(Bos primigenius taurus)*(Linnaeus, 1758), White tailed deer *(Odocoileus virginianus)*(Zimmermann, 1780), Moose*(Alces Alces)*(Linnaeus, 1758) and Sable antelope (*Hippotragus niger)*(Harris, 1838). The deposition sites of these experiments and remains were located at least 3 m away from the current study.

At the start of each trial, the pig carcasses were placed directly on the soil surface, on top of light grassy vegetation at the experimental sites. Cages were secured over the remains between sampling periods in order to prevent access by avian and mammalian scavengers. Samples were collected from the experimental and control sites on alternate

days until the majority of soft tissue had been removed with the onset of the advanced decay stage. Samples were then collected 2-3 times a week for two weeks followed by once a week until no visible changes were occurring during the dry remains stage (approximately 55 days).

Throughout each trial the hourly temperature was recorded by the weather station and the accumulated degree days (ADD) for each sampling day was calculated by summing the average daily temperature (°C) recorded by the data logger. ADD is used as a temporal measurement and facilitates the comparison of studies across seasons and geographical locations (Adlam and Simmons 2007). Precipitation was measured with a field rain gauge (mm) and accumulations were recorded during field observations. Photographs and observations on the stage of decomposition were also taken on each experimental day.

Volatile sample collection was carried out by placing the sampling hood over the remains/control site for a period of 30 minutes prior to sampling in order to collect the VOCs and to prevent wind dispersal (Figure 2.5). By covering the remains for only brief intervals the impact on decomposition was limited. This also reduced the formation of condensation on the inside of the hood which can affect the presence of compounds within the vapour phase. At the time of sampling, an adsorbent tube was removed from storage, uncapped and connected to the sampling port on the hood. The other end of the sampling tube was connected to the sampling pump and 1 L of decomposition headspace was sampled at a constant flow rate of 0.2 L/min for 5 minutes (Figure 2.5 inset). Following sample collection, all tubes were wrapped in aluminum foil and placed within an air tight container until analysis was carried out.

Figure 2.5: Stainless steel sampling hood and outdoor VOCs sampling set-up. Inset: connection of TD sampling tube and pump.

2.3.3 *Human Remains Field Research*

The human remains trials took place at Texas State University in San Marcos Texas, United States of America. The research site was located at Freeman Ranch (N 29° 55' 54.98" S 98° 00' 00.62"). Freeman Ranch is an open grassy field surrounded by trees.

The human remains were placed a minimum of 10 m apart, nude and in an extended and supine position directly on the ground with the head pointed towards cardinal north. The pig carcasses were placed parallel to the human remains a minimum of 10 m away. The carcass was oriented similarly, with their heads towards cardinal north; however their dorsal side was oriented west. The temperature loggers were placed within 1 m of each set of remains and temperature recorded at 0.25 h intervals.

A control site that contained no decomposition material was delineated within the area of deposition sites at a distance of 10 m.

The same procedure as outlined for the UOIT field trials was utilized to collect VOCs from human remains. For all trials, volatile samples were collected daily from both human remains, pig carcass and control site (November 2011: four experimental days; May 2012: 6 experimental days; November 2012: 6 experimental days). The duration of the studies was previously outlined in the grant proposal and was governed by access to the Forensic Anthropology Research Facility. Additionally, at each sampling interval photographs of the remains were taken.

2.3.4 *Canine Training Aids*

To investigate the chemical composition of commercially available training aids one microliter of formulation I and II were manually injected in split mode (10:1 and 20:1 respectively) on the GC×GC-TOFMS instruments. Each formulation was analysed in triplicate. A mixed standard of compounds identified by spectral match was also analysed in triplicate. The GABA standard was additionally analysed by headspace and direct thermal desorption. For headspace analysis, crystalline GABA was placed in a 20 mL headspace vial and 25 mL of headspace was sampled onto a Tenax® tube. Direct thermal desorption was carried out by placing GABA crystals in a sample cup inside an empty thermal desorption tube. An alkane standard (C_8-C_{20}) was spiked into both the liquid and headspace samples for retention index calculations. The retention index of a compound is a standardized RT under a set of known conditions and can aid in compound identification. Analysis of both formulations with GC×GC- High resolution TOFMS (GC×GC- HRTOFMS) was used to obtain exact mass measurements for further confirmation of compound identity.

2.4 ***Sample Analysis***

Prior to desorption and analysis of volatile samples, the IS (160 ng of bromobenzene) was spiked onto sampling tubes using a standard GC injection syringe.

2.4.1 *Decomposition VOCs: Thermal Desorption*

The Markes International Ltd. Unity 2 series thermal desorber is a sample introduction system for samples collected on solid sorbents sampling tubes. The two step desorption

sequence combines sample desorption, focusing and injection. Primary desorption removes the analytes from the sample tube via heating under a flow of carrier gas and transfers them to the secondary cold trap. Secondary desorption rapidly heats the cold trap to remove the analytes which are then injected onto the GC column via a heated transfer line. The precise sequence of operation utilized is as follows: sample tubes are leak checked and undergo a dry-purge prior to being desorbed at 300 °C for 4 minutes under a reverse flow of helium carrier gas (primary desorption). The volatiles are re-condensed on a -10 °C Tenax® TA cold trap which is then rapidly heated to 300 °C for 3 minutes (secondary desorption). The volatile sample is injected on the GC column via a heated transfer line (120 °C). Following desorption each sample tube was conditioned at 330 °C for 15 minutes. Depending on the state of decomposition, samples were split following tube and/or trap desorption as required to avoid saturation of the detector. Following sample introduction via thermal desorption (TD), analysis was performed with either gas chromatography – mass spectroscopy (GC-MS) or two-dimensional gas chromatography – time-of-flight mass spectrometry (GC×GC-TOFMS).

2.4.2 *Gas Chromatography – Mass Spectrometry*

The Varian Inc. 450-GC gas chromatograph was equipped with a non-polar Varian VF-5ms phase (5% phenyl/95% dimethyl polysiloxane, 0.25µm phase thickness) 30 m x 0.25 mm I.D. x 0.39 mm O.D. capillary column. The helium carrier gas used was a constant flow of 1.0 ml/min. The GC oven parameters were as follows: an initial temperature of 35 °C was held for 4 minutes followed by the first temperature ramp of 3 °C/min to 80 °C, the second temperature ramp of 10 °C/min to 120 °C followed by the final temperature ramp of 40 °C/min to 240 °C which was held for 7 minutes. The total run time was 33 minutes. The 240-MS ion trap mass spectrometer employed electron ionization (EI) in full scan mode with a mass range of 35-450 m/z. The MS had a delay of 0.1 minutes.

2.4.3 *Two Dimensional Gas Chromatography – Time-of-Flight Mass Spectrometry*

An Agilent© 7890 gas chromatograph (Palo Alto, CA, USA) equipped with a secondary oven and a quad-jet dual stage modulator was coupled with a Pegasus® 4D GC×GC TOFMS from LECO Corporation (St Joseph, MI, USA). For the human analogue Trial 3, human remains trial 1 and analysis of the synthetic training aid, the first dimension column was a Rxi®-5Sil MS (Restek, Bellefonte, PA, USA) low polarity Crossbond® silarylene phase equivalent to a 5 % phenyl/95 % dimethyl polysiloxane Restek® (29.5 m x 0.25 mm id x125 0.25 μm df) and the second dimension column was a BPX-50 (SGE Analytical Science, Austin, TX, USA) 50% phenyl polysilphenylene-siloxane phase (1.0 m x 0.1 mm id x 0.10 μm df). For human remains trial 2 and 3, the first dimension was unchanged with a Rxi-5SilMS (28 m x 0.25 mm id x 0.25 um df) and the second dimension column was an Rxi-17SilMS (1.5 m x 0.15 mm id x 0.15 um) of the same polarity with a 50 % diphenyl-polysiloxane/50 % dimethyl polysiloxane phase.

For the analysis of decomposition VOCs the GC oven temperature was initially set at 35 °C and held for 5 minutes, before being increased at 5 °C /min to 240 °C where it was held for another 5 minutes. The temperature offset for the secondary oven was 5 °C. The modulation period was 4 sec, with a hot pulse time of 0.70 sec and a temperature offset of 10 °C. The transfer line was held at 250 °C. The TOFMS was operated in electron ionization mode at 70 eV, with a mass range of 29-450 amu, an acquisition rate of 100 Hz and a detector voltage of 1500 V.

The analysis of the canine trainings aid solutions (formulation I & II) was carried out with the same instrumentation as described above, however the sample introduction was performed with direct liquid injection. The liquid injection port was maintained at 300 °C. The GC oven temperature ramp was as follows: an initial temperature of 60 °C was held for 3 minutes, before being increased at a rate of 5 °C/min to 205 °C followed by the final temperature ramp of 30 °C/min to 250 °C which was held for 5 minutes. The temperature offset for the secondary oven was 5 °C. The modulation period was 4 and 6 s for formulation I and II respectively, with a hot pulse time of 0.70 s and a temperature offset of 15 °C. The transfer line was held at 250 °C. The low resolution (LR, unit mass

resolution) TOFMS was operated in electron ionization mode at 70 eV with a mass range of 29–550 amu, an acquisition rate of 100 Hz, and a detector voltage of 1500 V.

For headspace analyses, a GERSTEL multipurpose sampler (MPS) equipped with GERSTEL dynamic headspace (DHS), thermal desorption unit (TDU) and cooled injection system (CIS-4) was used. Various incubation parameters were tested; 30 °C and 40 °C for 1 min as well as 50 °C for 5 min. Thermal desorption tubes containing a bed of Tenax® TA were utilised to trap 25 mL of headspace. The tube was desorbed at 300 °C and refocused with cryogenic cooling at −20 °C. Direct thermal desorption of samples was also performed; desorption and injection was carried out at both 300 °C and 120 °C. Compounds that were identified in the two formulations using the LRTOFMS instrument were confirmed with exact mass measurements of HRTOFMS (Stadler et al., 2012).

2.5 ***Data Processing***

2.5.1 *Thermal Desorption – Gas Chromatography – Mass Spectrometry*

Peaks from the reconstructed ion chromatogram (RIC) were initially identified by searching the unknown spectra within the NIST Mass Spectral library using the MS Search Program v.2.0. Peaks with match factors greater than 900 were accepted, where a perfect spectral match has a match factor of 999 and spectra with no peaks in common receives a value of 0. The retention time and representative spectra of the accepted matches were input into a sample list. Using this sample list the Varian MS Workstation v. 6.9 conducted peak identification and integration of target compounds. Peak integrations were completed using the quantification ion of each compound.

Computer processing and peak identifications completed by the Workstation software were conducted on a mass spectral match factor threshold of 700. Identifications were verified throughout the study by performing a manual search of each peak's mass spectra; independent of the data processing performed by the workstation software, and ensuring that compounds were correctly identified. Manual verifications of peak integrations were also performed throughout the study.

Prior to the analysis of samples, system checks of the GC-MS were performed, including verification of the mass spectrometer's mass accuracy using a perfluorotributylamine (PFTBA) calibration gas solution. Auto tunes of the mass spectrometer, including mass calibration against the PFTBA calibration gas were performed as required. Further verification of mass spectral identifications were completed by the analysis of standards representative of the compounds within the sample list. Standards for dimethyl disulfide, phenol and indole were analysed using TD-GC-MS. Following a mass spectral search within the NIST Mass Spectral library the three standards were correctly identified with high match factors and probability scores (Table 2.1). The probability value indicates the uniqueness of the spectra as well as providing an indication that the library match is correct.

Table 2.1: Mass spectral identifications of standards representative of compounds in target sample list

Compound	Reverse Match / Forward Match	Probability
Dimethyl Disulfide	874 / 874	97.53
Phenol	912 / 912	52.87
Indole	924 / 924	61.11

2.5.2 *Thermal Desorption - Two Dimensional Gas Chromatography – Time-of-Flight Mass Spectrometry*

TOFMS data were processed with LECO's proprietary software, ChromaTOF® 4.33 and 4.42. This software was used to generate two dimensional contour plots, calculate baselines, peak finding and deconvolution, library searching and peak integration. For library identifications the Wiley (2008) and NIST (2008) databases were used. Following manual review, peaks of interest were selected to confirm the preliminary library identification. The following thresholds were used to process the data: S/N > 100, baseline offset of one and library match factors >700. The forward and reverse match factors along with the probability value generated by the NIST search algorithm were used to evaluate the potential library identifications. A forward match factor is a number

from 0 – 999 that describes how well the unknown spectra matches the library hit, with 999 being a perfect match. The reverse match factor (0-999) describes the opposite; i.e. how well the library hit matches the sample spectra. For both match factors, generally a score of 700+ is considered acceptable, 800+ is good and 900+ is excellent. The probability value has a maximum of 10,000 and indicates the likelihood that the library hit is the correct one. A probability of 9000+ indicates a strong match (Dalluge et al., 2002; Lu et al., 2003)

2.5.3 *Data Handling & Statistical Analysis*

The results of the above data processing were exported as ASCII files to Microsoft Excel. Within Excel, peak areas of identified compounds were normalized against the IS by calculating the peak area ratio. Where applicable principal components analysis (PCA) was conducted using PAST 2.14 statistical software. PCA is an Eigen analysis that projects multivariate data onto a new principle components axis which account for as much of the variance of the original data set as possible. Each principle component (PC) extracts a portion of the variance in the original data, with the first PC extracting the greatest amount of variance. The component loadings, in the form of coefficients, can be utilised to interpret the relationship between the new PC and original variables. This relationship can also be displayed on the PCA scatter plot as vectors.

Within the ChromaTOF® software v.4.42 a data handling tool called *Statistical Compare* is available. By comparing the peak tables generated from initial data processing within ChromaTOF® the unknown chemical variations between sample groups i.e. experimental and control, can be determined. Specifically this tool allows for the statistical comparison of the aligned peak tables from groups or classes of samples. A mass spectral match criterion of 60 % is utilized to align multiple chromatograms and integrate peak areas (Koh et al., 2010).

Chapter 3: *TD – GC – MS Analysis of Decomposition VOCs from Human Analogues*

Thermal Desorption – Gas Chromatography – Mass Spectrometry (TD-GC-MS) was utilized to analyse the decomposition VOCs collected from the microcosm trials as well as the three outdoor spring trials. These projects monitored the decomposition of pig carcasses and the headspace was collected for analysis.

3.1 *Climatic Conditions*

The temperatures within the microcosms were consistent across the duration of the study with an average temperature of 19.25 °C, a minimum of 17.84 °C and a maximum of 22.85 °C.

Trial 1, 2 and 4 all commenced the first week of June in 2010, 2011 and 2012, respectively and continued until the remains had reached skeletonization in late July. The temperature profiles of the three trials were similar with an average daily temperature of approximately 20 °C (Figure 3.1). During Trial 1 (2010), the average daily temperature was 19.97 °C with an absolute minimum of 6.36 °C and an absolute maximum of 33.73 °C. Trial 2 (2011) had an average daily temperature of 21.26 °C, an absolute minimum of 5.15 °C and an absolute maximum of 41.91 °C. Trial 4 (2012) had an average daily temperature of 20.68 °C, an absolute minimum of 7.97 °C and an absolute maximum of 36.34 °C. The precipitation for Trials 1 and 4 were similar with 224 mm and 219 mm of total rainfall, respectively. However Trial 2 received only 59.5 mm of total rainfall throughout the study.

Environment Canada reported an average daily temperature of 17.2 °C and 20.3 °C for June and July, respectively for Oshawa, Ontario recorded over the years 1971-2000 (Environment Canada). The Canadian Climate Normals (1971-2000) also reported an average precipitation of 80.6 mm and 67.3 mm for June and July, respectively (Environment Canada).

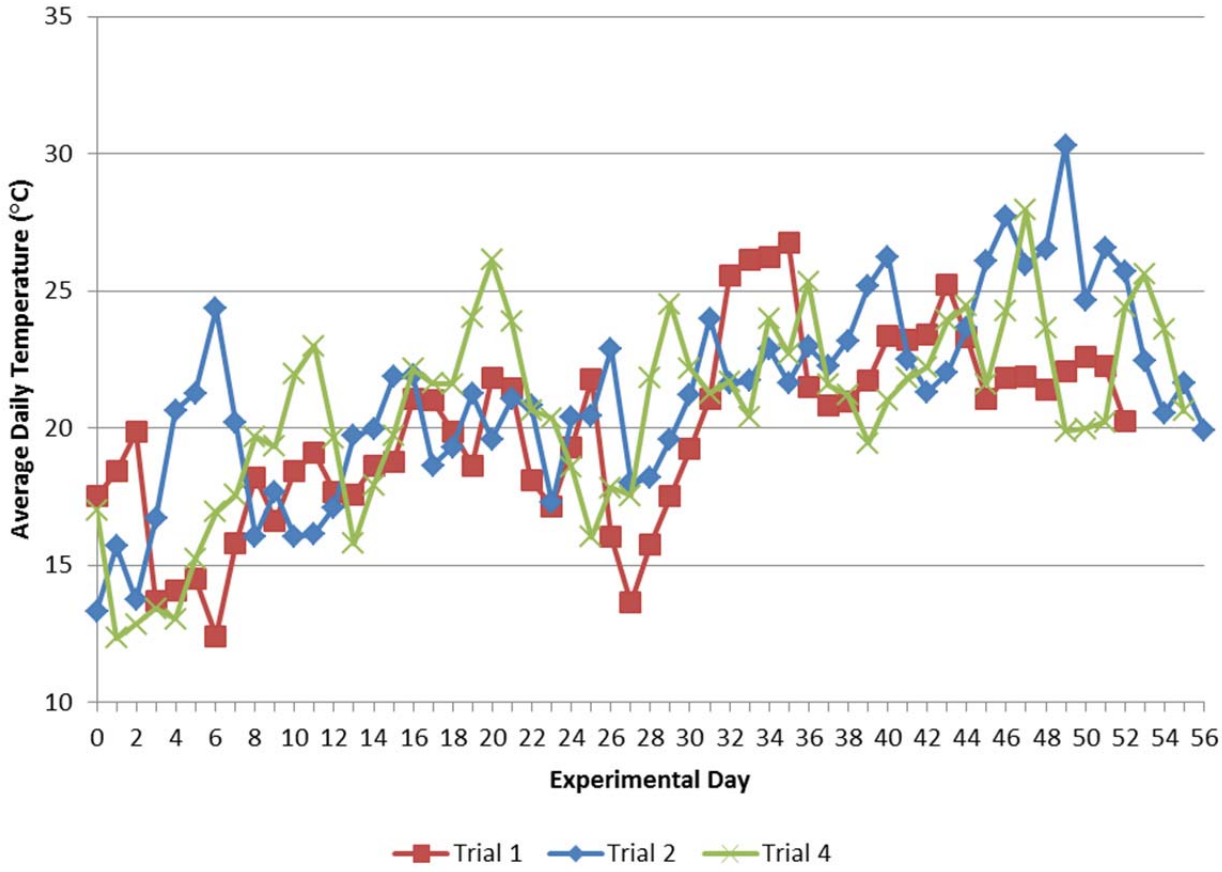


Figure 3.1: Average daily temperatures for the three spring/summer outdoor trials utilizing pig carcasses as human analogues. Trial 1 (2010) - average: 19.97 °C, minimum: 6.36 °C, maximum: 33.73 °C. Trial 2 (2011) - average: 21.26 °C, minimum: 5.15 °C, maximum: 41.91 °C. Trial 4 (2012) - average: 20.68 °C, minimum: 7.97 °C, maximum: 36.34 °C.

Table 3.1: Description of decomposition stages used in this research as adapted from Payne et al. 1965 and Anderson and VanLaerhoven 1996.

Decomposition Stage	Description & Visible Features
Fresh	No visible decompositional features are present during this stage. - No decomposition odour associated with the remains Ends when bloat is evident
Bloat	Characterized by distension of the carcass due to the accumulation of putrefactive gases. - Torso is firm to the touch - Splayed/lifted limbs - Potential purging of fluids - Protrusion of the intestines - Decomposition colour changes may be visible (green complex, black precipitate and marbling) - Skin/fluid blister and skin sloughing may occur - Decomposition odour may be present - Egg masses and small maggots may be visible
Active	Begins once the carcass has deflated. Marked decrease in the amount of soft tissue due to liquefaction. - Large maggot masses - Overall wet appearance and liquefaction of tissue - Formation of Cadaver Decomposition Island (CDI) - Strong putrid odour associated with the remains - Some skeletal elements may be exposed
Advanced	Majority of soft tissue will be absent and remaining tissue may be mummified. - Decrease in the decomposition odour - Skin may become dark in colour and have a leathery texture - Additional skeletal elements may become exposed - A distinct CDI will encircle the remains - Maggots have migrated away from the remains
Dry Remains	Minimal soft tissue remains aside from skin and the majority of skeletal elements are exposed. - Lack of odour

3.2 *Carcass Decomposition*

Observations on the stage of decomposition were completed on each sampling day. Field notes and photos were used to record observations. Due to the short time between euthanasia and carcass deposition all carcasses were classified as fresh at the time of deposition. No carcasses exhibited visual signs of soft tissue decomposition at this time. The stages used to characterise decomposition are those outlined previously, as adapted from Payne (1965) and Anderson and VanLaerhoven (1996). Decomposition is not a discrete process and carcasses did exhibit features of more than one stage at a time. Therefore carcasses were categorized based on the predominant features for a particular stage. Descriptions and features of the decomposition stages are provided in Table 3.1 and photographs of each stage are shown in Figure 3.2 for all trials. In all studies the carcasses followed the general progression of decomposition however there was some variability observed. Figure 3.3 depicts the onset and duration of the decomposition stages by accumulated degree days for the decomposition trials carried out (ADDs are provided in Appendix B). The early stages of decomposition were comparable, with bloat first observed on experimental day 2 (ADD 36, 29 and 29) for the outdoor trials and on experimental day 3 for the microcosm trial (ADD 59). However, as decomposition progressed the onset and duration of the stages became more variable. The types of variation in the process of decomposition observed included differential decomposition, liquefaction and insect colonization.

Observations of the decomposition stage within the microcosms were completed on each sampling day by viewing the carcass through the access hole in the lid. The bloat stage was identified by the first signs of carcass distension and gas production and began approximately on day 3 of experimentation (ADD 59.1). During the microcosm trial, the decomposition was slowed considerably as indicated by the elongated bloat and active decay stages (see Figure 3.3). Active decomposition was identified when the carcass showed areas of deflation and began on experimental day 26 (ADD 500.0). The carcasses did not progress past the active decay stage and therefore no data is available for the advanced decay and dry remains stages. Instead, following the collapse of the abdomen the liquefaction of soft tissue was evident. As a result of soft tissue

decomposition, fluids begin to accumulate within the remains (Clark et al., 1997; Dent et al., 2004). The decomposition fluid is released into the surrounding environment through the natural orifices or through skin ruptures produced during the bloat stage (Clark et al., 1997; Carter et al., 2007). In contrast to the confined microcosm, within a terrestrial environment the decomposition fluid is absorbed by soil and may not be observed (Carter et al., 2007). During this indoor study, little to no insect activity was noted within the decomposition containers and active decay was not characterized by the rapid loss of tissues. However, small unidentified flies were attracted to the microcosms during sampling and were seen within the system. Prior to the purging of decomposition fluid small larvae were observed on the carcasses and the sand substrate.

The carcasses in Trial 1 (2010) were characterized as fresh until bloat became apparent on experimental day 2 (ADD 35.9). Deflation of the carcasses and onset of the active decay stage occurred by experimental day 6 (ADD 98.1). It was during onset and progression of the active decay stage of decomposition that differential decomposition was generally observed. During differential decomposition the head would progress to a more advanced stage of decomposition than the rest of the remains. Consequently skeletal elements of the head and limbs would become visible when the torso was still observed to be in an active or advanced state of decay. Advanced decay became apparent due to the overall lack of soft tissue on experimental day 11 (ADD 179.5) and continued until the carcasses consisted of only skin and bones and were characterized as being in the dry remains stage on experimental day 22 (ADD 395.1).

In the spring trial of 2011 (Trial 2), the carcasses entered the bloat stage on experimental day 2 (ADD 29.0) and progressed to the active decay stage by experimental day 6 (ADD 101.3). During this stage an atypical migration event of blowfly larvae occurred. On experimental day 7 (ADD 126) a large number of maggots migrated away from the carcasses in an east-south east direction as one large mass. Prior to the migration event, the maggots appeared to have consumed the majority of soft tissue, effectively leading to the early onset of advanced decay (experimental day 8, ADD 145.8). This event occurred after a rain fall of approximately 2 mm. During the advanced decay stage the skin began to desiccate, however the underlying tissue retained its moisture and persisted

for an extended period of time. The carcasses were characterized as being in the dry remains stage on experimental day 34 (ADD 658.1) when no soft tissue remained and the carcasses only consisted of mummified skin and bones.

During Trial 4 (2012) the bloat stage was elongated compared to the previous studies. It developed slowly with early bloat being observed on experimental day 2 (ADD 29). In this stage the torsos were slightly distended and firm to the touch however the limbs were not splayed. The splayed limbs typically observed during the bloat stage is due to the accumulation of gasses within the tissues causing the stiffening and straightening of the appendages, sometimes referred to as putrefactive rigor (Gill and Landi 2010). By experimental day 4 (ADD 56) the carcasses had entered the bloat stage with the torso and neck being fully distended, this became more pronounced on experimental day 6 (ADD 84) where carcasses were observed to be in full bloat. By experimental day 8 (ADD 118), differential decomposition was observed due to the maggot activity at the heads of the carcasses. Although the head and neck portion of the remains could be classified as active decay, the rest of the torso still had an accumulation of gases and had not fully collapsed therefore the carcasses were categorized as being in a post-bloat stage. All carcasses were classified as being in the active decay stage on experimental day 10 (ADD 157.3) and had progressed to the advanced decay stage on experimental day 14 (ADD 237.7). By experimental day 17 (ADD 297.5), the carcasses were classified in the dry remains stage.

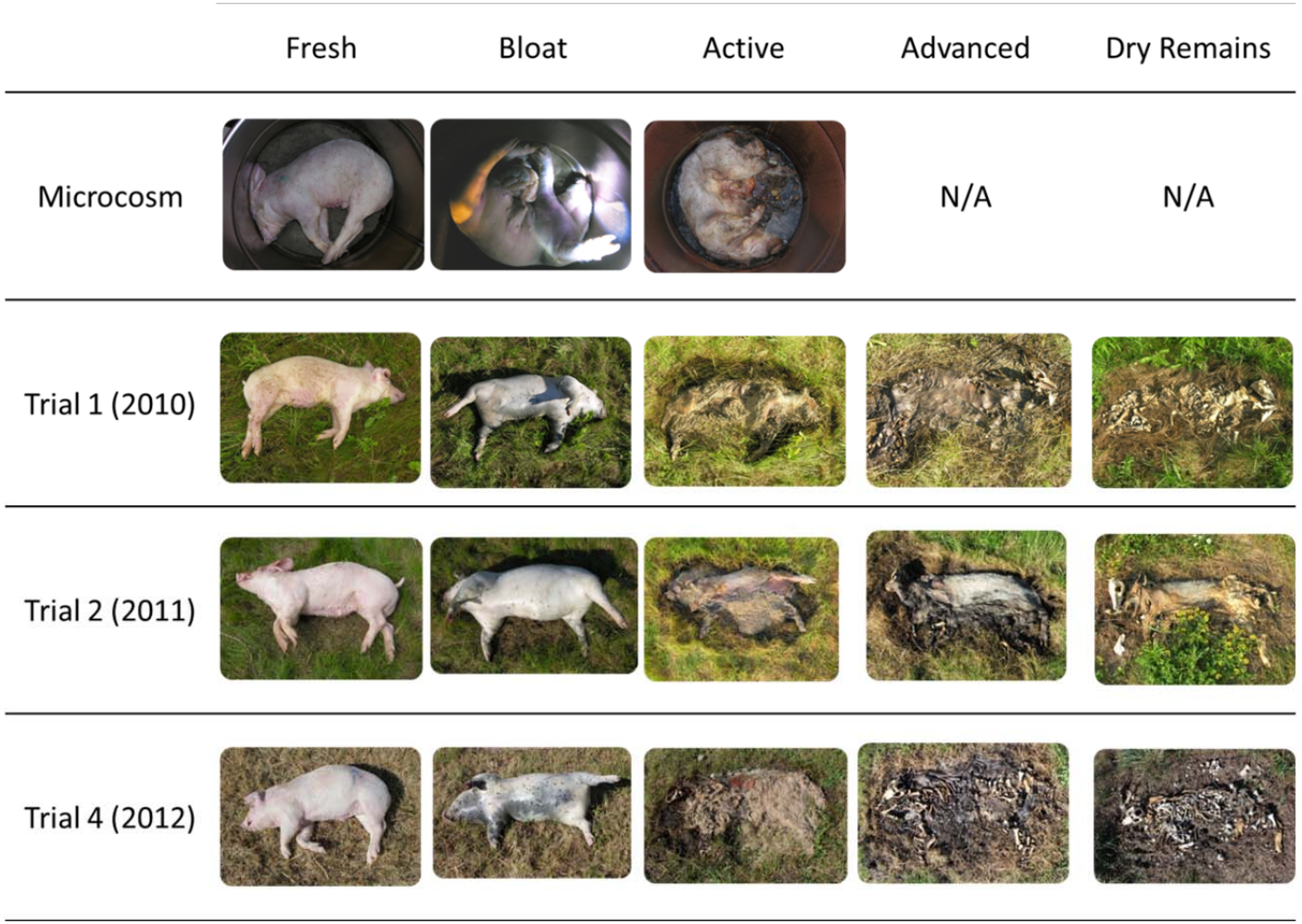


Figure 3.2: Photographs of each decomposition stage for the microcosm trial and Trials 1, 2 and 4.

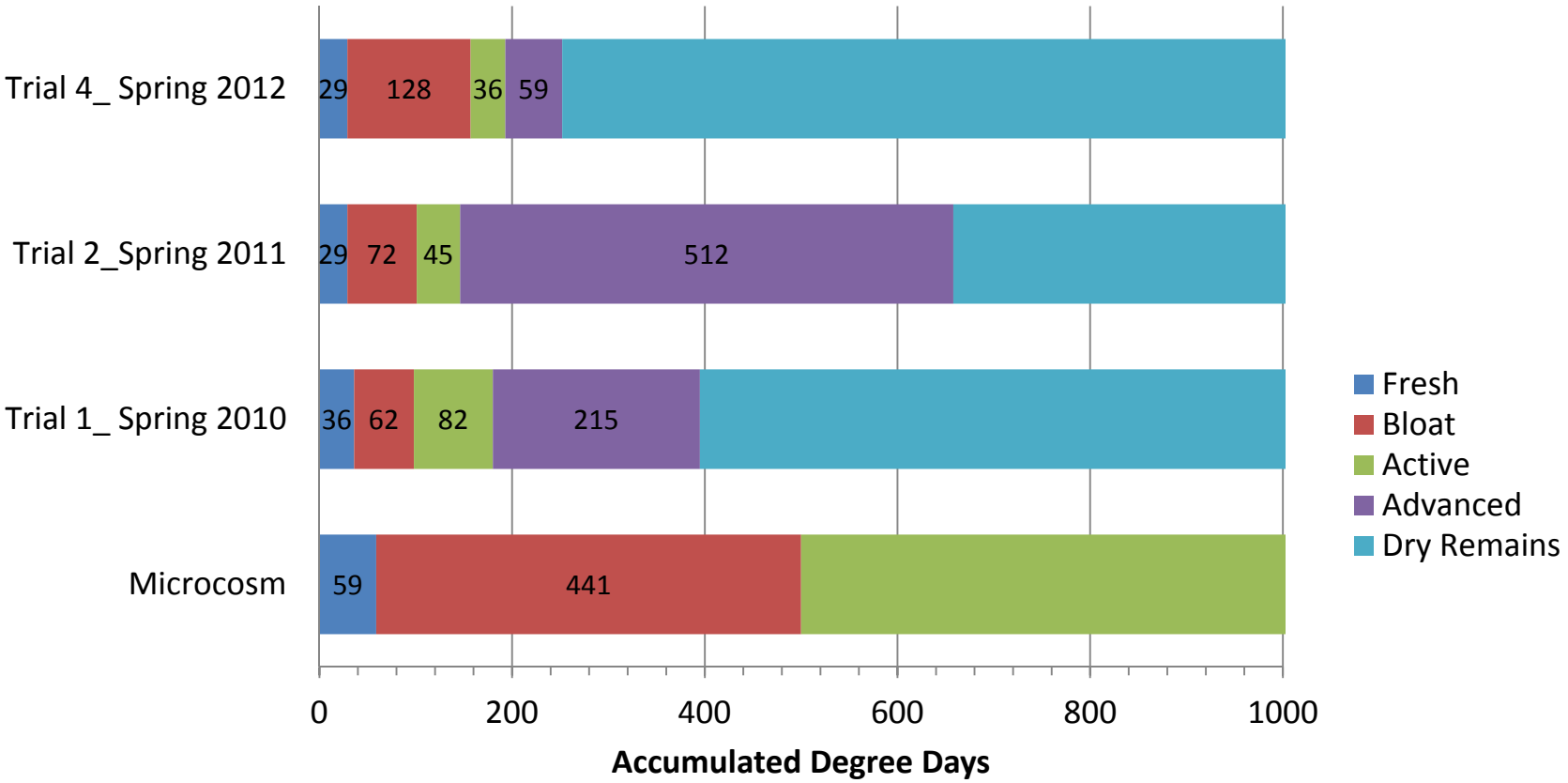


Figure 3.3: Onset and progression of the decomposition stages by accumulated degree days (ADD) for the microcosm trial and Trials 1, 2 and 4. Values indicate duration of the decomposition stage in ADD.

3.3 *Decomposition VOCs*

Only ten compounds were identified within the headspace of the microcosm; dimethyl disulfide (DMDS), dimethyl trisulfide (DMTS), dimethyl tetrasulfide (DMQS), methyl ethyl disulfide, 2,4-dithiapentane, phenol, phenol-4-methyl, indole, indole-3-methyl (skatole) and 2-pentanone. The VOC profiles for the outdoor trials were remarkably similar to the enclosed microcosm environment with only three additional compounds identified; hexanal, heptanal, and octanal. Table 3.2 shows the retention times and reference spectra for the total number of compounds identified. These compounds were selected for inclusion in the VOC profile because the compounds were consistently detected within all carcass replicates and displayed distinctly different trends within the experimental treatment compared to the control treatment which contained no carcass. The levels of the compounds and the overall composition of the profile changed over time in the decomposition samples whereas the levels remained relatively consistent in the control samples. Furthermore, these compounds were selected due to the confidence in the identifications via spectral match (>900) to the NIST mass spectral library. The accuracy of the mass spectra and resultant identifications were confirmed with representative standards (see Table 2.1). The peak areas for all identified compounds were normalized against the area of the internal standard by calculating the peak area ratio. The peak area ratios for the experimental and control treatments for each experimental day were averaged and a background subtraction was performed for those compounds detected within the corresponding control samples.

The compounds identified within the microcosm were consistently detected throughout the trial. However due to the slowed decomposition process, full trends were not observed within the microcosms. Overall, the polysulfide compounds were at the highest levels during the bloat stages and the aromatic compounds, i.e. phenol and indole, were increasing during the active decay stage.

Table 3.2: The retention time (min) and mass spectrum (m/z) of compounds identified within the decomposition headspace of pig carcasses with a match threshold of 900+. The quantitation ion within the reference spectra is shown in bold.

Compound	Retention Time (min)	Reference Spectrum (m/z)
2-Pentanone	2.5	**43.0**, 41.1, 85.9
Dimethyl Disulfide	3.6	**93.9**, 79.0, 45.0
Hexanal	5.1	**41.2**, 56.1, 67.1
Methyl Ethyl Disulfide	6.7	**107.9**, 80.0, 64.0
2,4-Dithiapentane	9.0	**61.0**, 107.7, 45.0
Heptanal	9.4	**41.1**, 55.2, 70.1
Dimethyl Trisulfide	12.9	**125.9**, 79.0, 45.0
Phenol	13.8	**93.9**, 66.0, 50.0
Octanal	14.6	**41.1**, 67.0, 55.1
Phenol-4-methyl	18.9	**107.1**, 108, 77.2
Dimethyl Tetrasulfide	23.2	**79.0**, 157.8, 64.0
Indole	24.3	**117.0**, 90.0, 89.0
1-H-Indole, 3-methyl (Skatole)	25.0	**130.1**, 131.0, 77.1

More detailed temporal trends were observed during the three outdoor trials. The predominant class of compounds within the VOC profile for all trials were the polysulfide compounds (DMDS, DMTS, DMQS, methyl ethyl disulfide, 2,4-dithiapentane). DMDS was the most prevalent of the polysulfides and was first seen on experimental day 2 of the outdoor trials (ADDs of 35.9, 29.0 and 29.4 for trials 1, 2 and 4, respectively). This is representative of the other polysulfide compounds. The levels of polysulfides reach their apex during the bloat stage (Trials 1 and 2: experimental day 2 – 6, Trial 4: experimental day 2 – 10) (Figure 3.4a-d) and represent over 90% of the VOC profile during this stage (Data tables for Trials 1, 2 and 4 can be found in Appendix D). Following the bloat stage, 2,4-dithiapentane and methyl ethyl disulfide decrease and are no longer detected whereas DMQS persists through to the advanced decay stage (experimental day 11, 8 and 14 for Trial 1, 2 and 4, respectively). DMTS remains part of

the profile through advanced decay and into the dry remains stage (experimental day 22, 34 and 17 for Trial 1, 2 and 4, respectively) and DMDS remained detectable at low levels throughout the remainder of the trials.

Indolic (indole and 1H-indole,3-methyl) and phenolic (phenol and phenol-4-methyl) compounds were the only aromatics included in the VOC profile in this study. The general trends for the aromatics varied over time, however as a class these compounds were predominant during the active and advanced decay stages (experimental days 6 – 22, 6 – 34 and 10 – 17 for Trial 1, 2 and 4, respectively) (Figure 3.5a). Indole was present within the VOC profile during early decomposition, peaked at the end of the bloat stage and remained at elevated levels through to the end of the active decay stage (experimental days 6 - 11, 6 – 8 and 10 – 14 for Trial 1, 2 and 4, respectively) (Figure 3.5b). On average indole represented over 55 % of the aromatics during the bloat stage. However, the levels of indole observed during Trial 4 were distinctly higher than those observed in the other trials and during the bloat stage it represented over 90 % of the aromatics. In comparison to the higher levels of indole, 1H-indole,3-methyl also known as skatole, was present in trace amounts but was not observed during soft tissue decomposition in Trial 2. Phenol was the most abundant of the aromatics and appears to have a two peak cyclic trend. The first peak occurred during active decay at the transition between the active and advanced decay stages (Trials 1 and 2: experimental day 8, ADD 126.3 and 145.8, respectively, Trial 4: experimental day 10, ADD 157.4) (Figure 3.5c). The second maxima for phenol occurred with the onset of the dry remains stage (Trial 1: experimental day 22, ADD 395.1, Trial 2: experimental day 34, ADD 523.8) (Figure 3.5c). However, this second peak was not seen in Trial 4. Although not present in the same amounts as phenol, phenol-4-methyl also displayed maximums during the bloat and active decay stages for all three trials (Figure 3.5d).

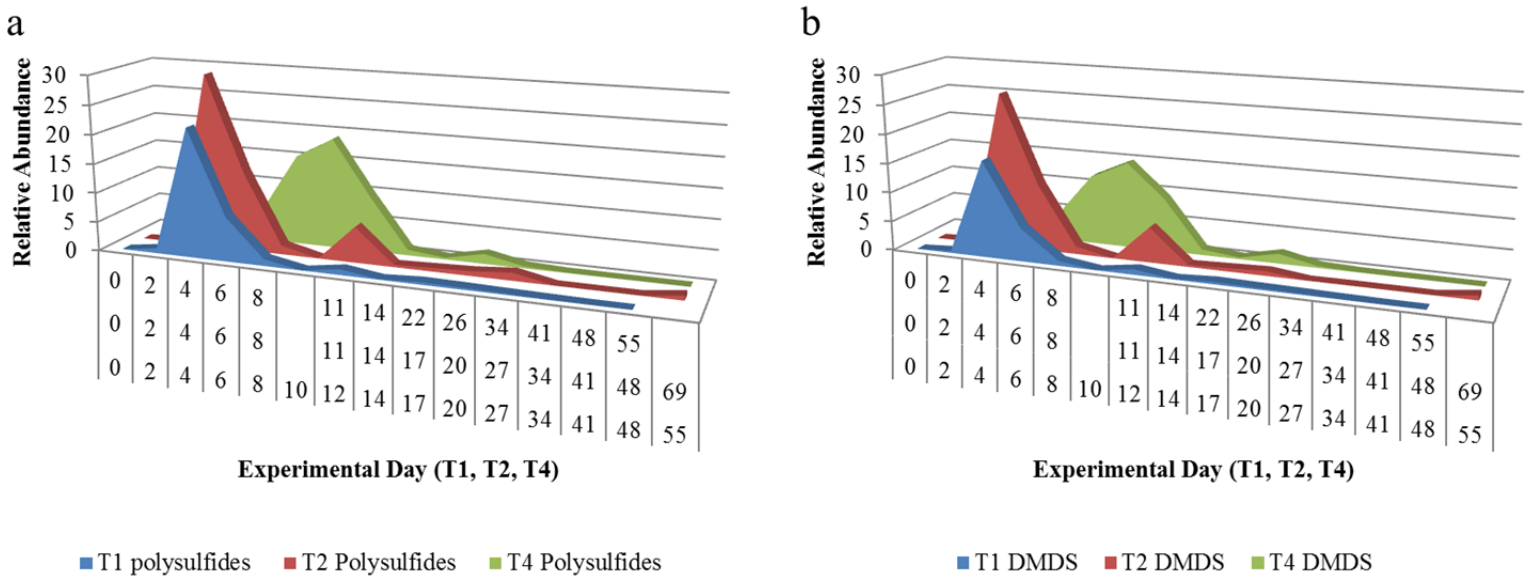


Figure 3.4: The relative abundances for the sulfide compounds across decomposition for Trials 1, 2 and 4. The x-axis displays the experimental days for Trials 1, 2 and 4 respectively. a) Polysulfide compounds: DMDS, DMTS, DMQS, 2,4-dithiapentane and methyl ethyl disulfide. b) DMDS

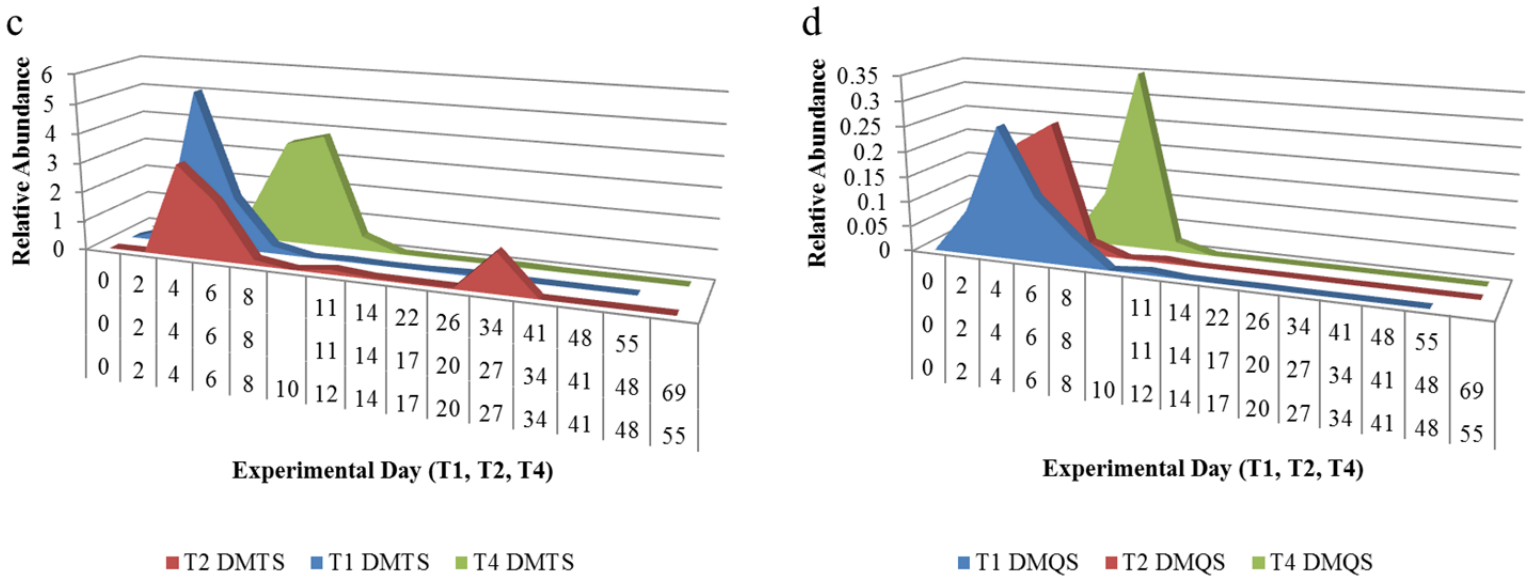


Figure 3.4: The relative abundances for the sulfide compounds across decomposition for Trials 1, 2 and 4. The x-axis displays the experimental days for Trials 1, 2 and 4 respectively c) DMTS d) DMQS

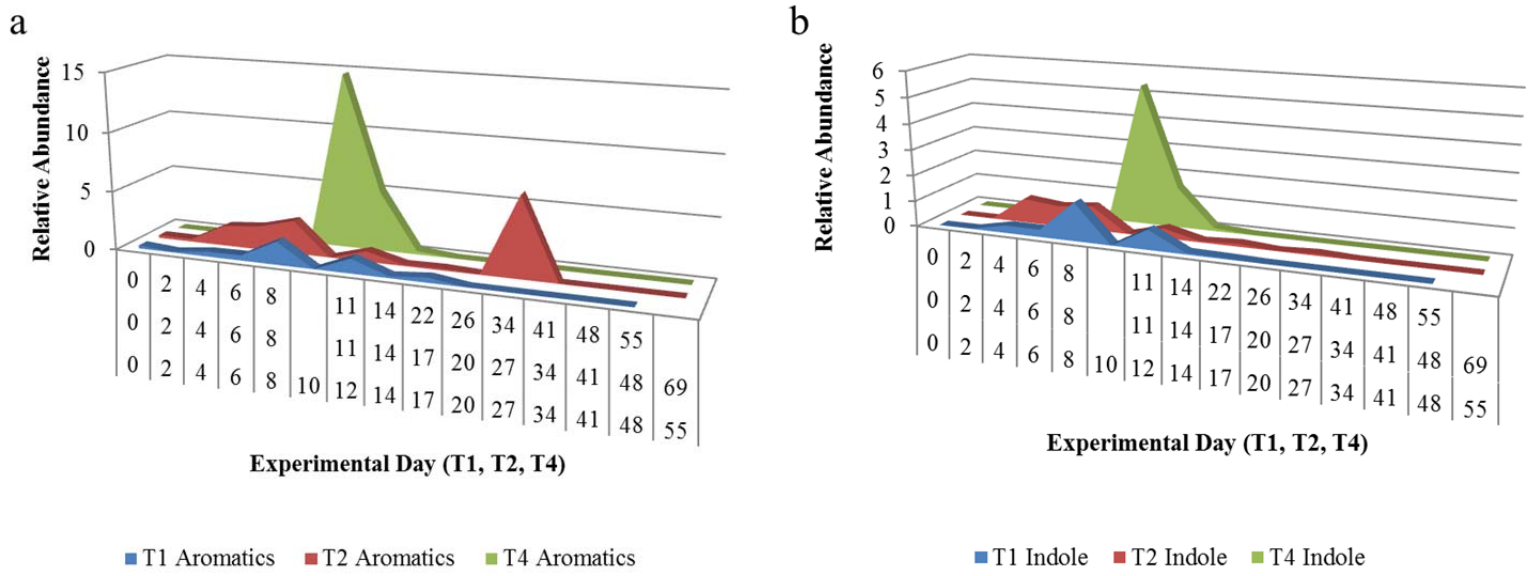


Figure 3.5: The relative abundances for the aromatics across decomposition for Trials 1, 2 and 4. The x-axis displays the experimental days for Trials 1, 2 and 4 respectively. a) Aromatic compounds: indole, skatole, phenol, phenol-4-methyl b) indole

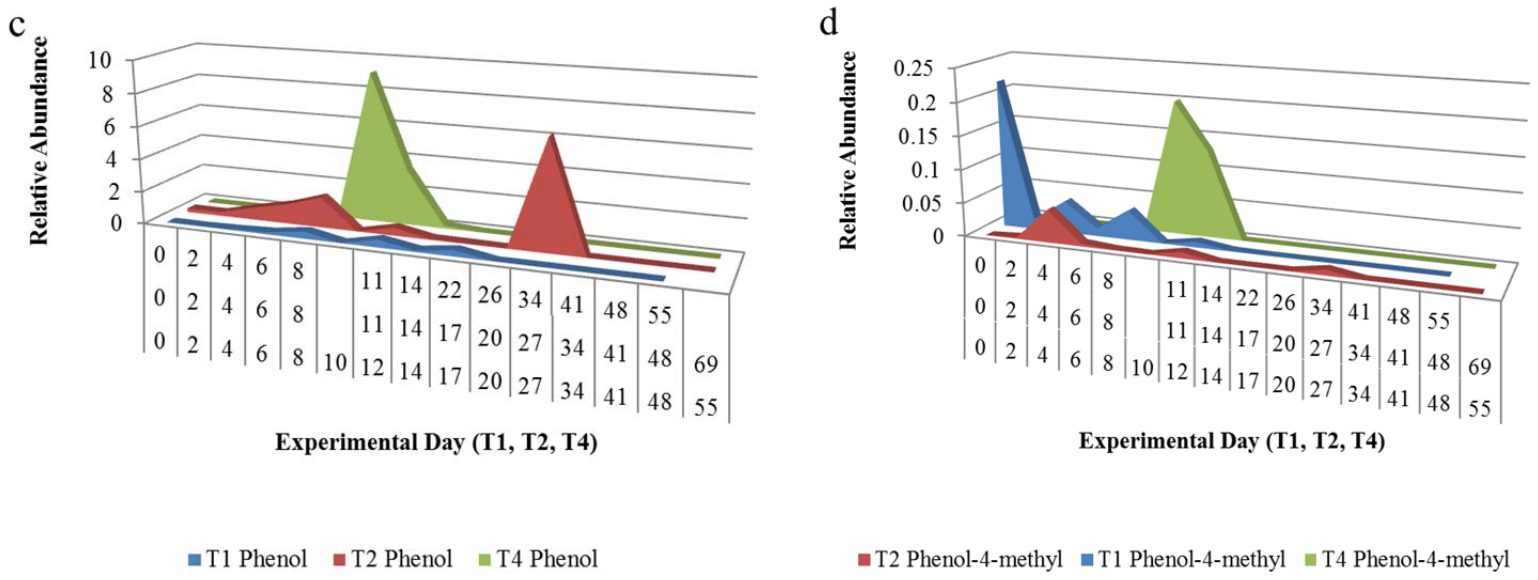


Figure 3.5: The relative abundances for the aromatics across decomposition for Trials 1, 2 and 4. The x-axis displays the experimental days for Trials 1, 2 and 4 respectively c) phenol d) phenol-4-methyl.

The aldehydes identified in the outdoor studies (hexanal, heptanal and octanal) were dominant during the later stages of decay. Although low levels of these compounds were detected in the earlier stages of decomposition particularly in Trials 1 and 2, the onset of their major trend occurred during the advanced decay stage. This occurred on experimental day 11 (ADD 179.5) for Trial 1 and on experimental day 14 (ADD 237.7) for Trial 4 (Figure 3.6). During Trial 2 the aldehydes appeared in the advanced decay stage during transition to the dry remains stage (experimental day 17, ADD 312.3) (Figure 3.6). The levels of the aldehydes during Trial 2 were also distinctly higher than those observed during the other outdoor trials.

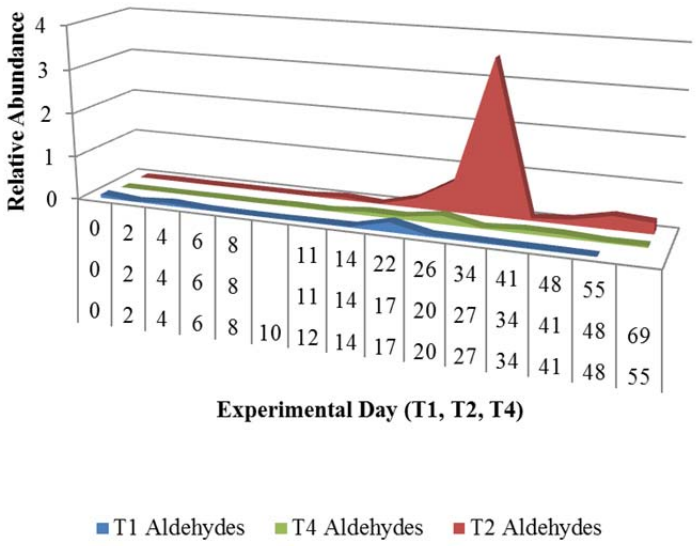


Figure 3.6: The relative abundances for the aldehydes; hexanal, heptanal and octanal across decomposition for Trials 1, 2 and 4. The x-axis displays the experimental days for Trials 1, 2 and 4 respectively.

3.3.1 *Chromatography*

Compound identification was potentially hindered by chromatographic co-elution and convoluted mass spectra. This was evidenced by the early eluting compounds as well as those being masked by the dominant compounds, such as the polysulfides. The first 4

minutes of the GC oven temperature program was an isothermal hold of 35 °C. During this period the more volatile compounds that were not retained by the column eluted together (Figure 3.7). Their co-elution and similar mass spectra did not produce library matches with satisfactory match factors.

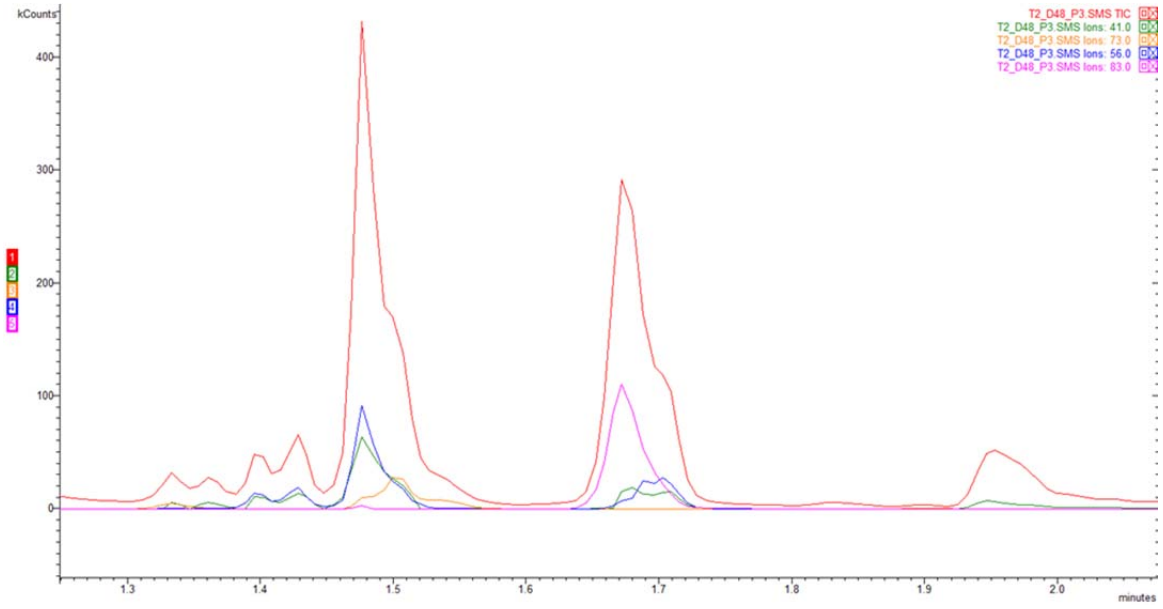


Figure 3.7: Representative chromatogram of compound co-elution during the isothermal period at the start of the oven ramp (~1.3 – 2.0 minutes). The y-axis indicates the abundance in kCounts. Red trace is the total ion chromatogram (TIC). Additional traces are extracted ion counts for m/z 41.0 (green), 56.0 (blue), 73.0 (yellow) and 83.0 (purple).

A similar issue occurred in the presence of the high level compounds such as DMDS. The sample chromatogram shown in Figure 3.8 was collected on experimental day 6, when an increased split flow was utilized to reduce the overloading and tailing of the dominant compounds while attempting to preserve detection of the lower level compounds. Figure 3.8a shows the tailing of DMDS and its corresponding spectra (94, 79 and 45 m/z) within the baseline following its elution, and Figure 3.8b shows the convoluted spectra of subsequent peaks. The tailing of DMDS and the presence of the 94, 79 and 45 ions within the spectra of subsequent peaks precluded their identification.

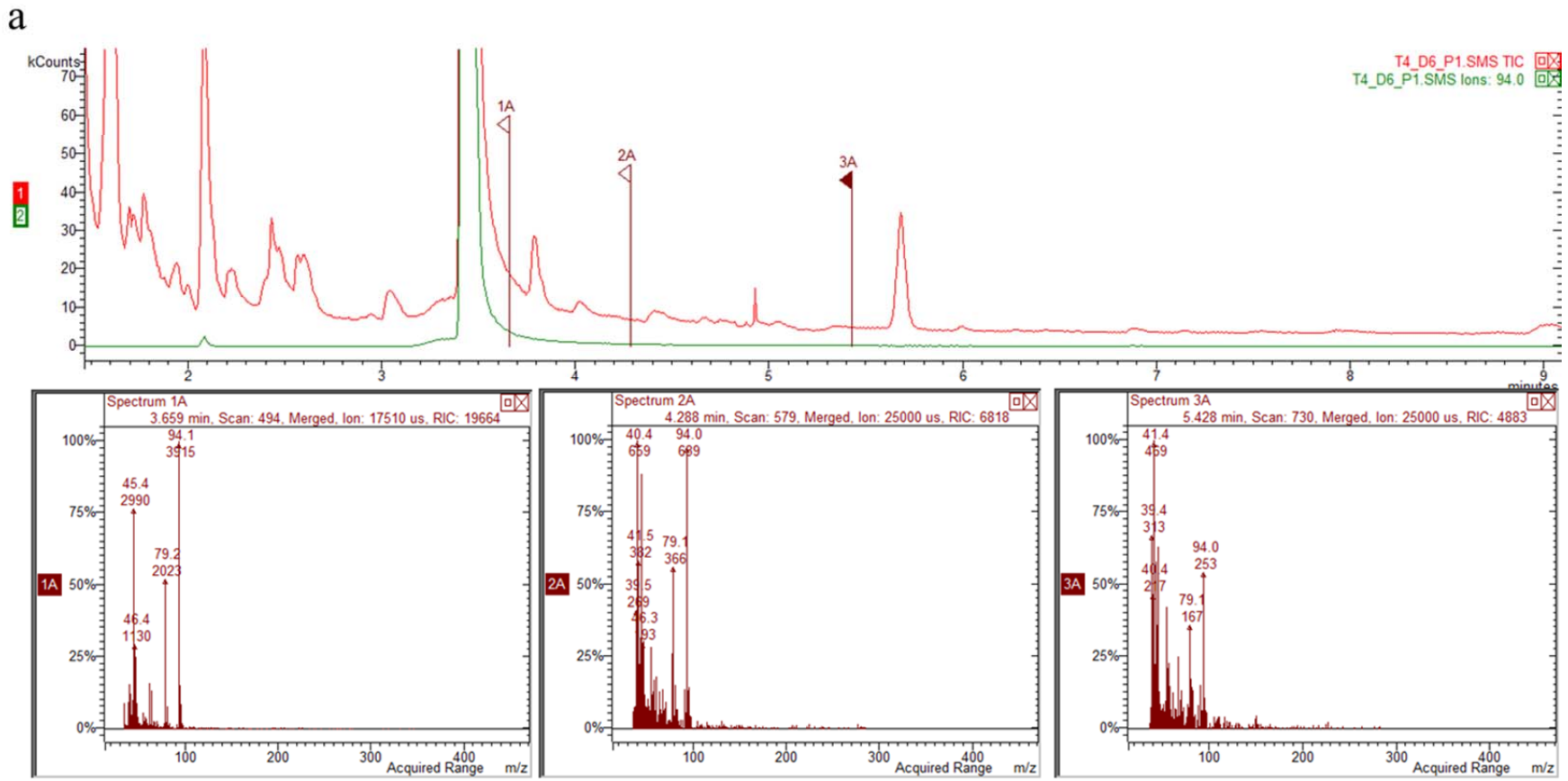


Figure 3.8: Representative chromatogram of compound co-elution (top panel) and resulting convoluted mass spectra (bottom panels) from DMDS. Top panel: x-axis: retention times 1.5 – 9 minutes, y-axis: abundance in kCounts. Red trace is TIC, green trace is extracted ion count for m/z 94.0 the quantitation ion for DMDS. Bottom panel: Tailing of DMDS and the presence of its mass spectra (94, 79, 45 m/z) within the baseline at RT 3.659 min, 4.288 min and 5.428 min.

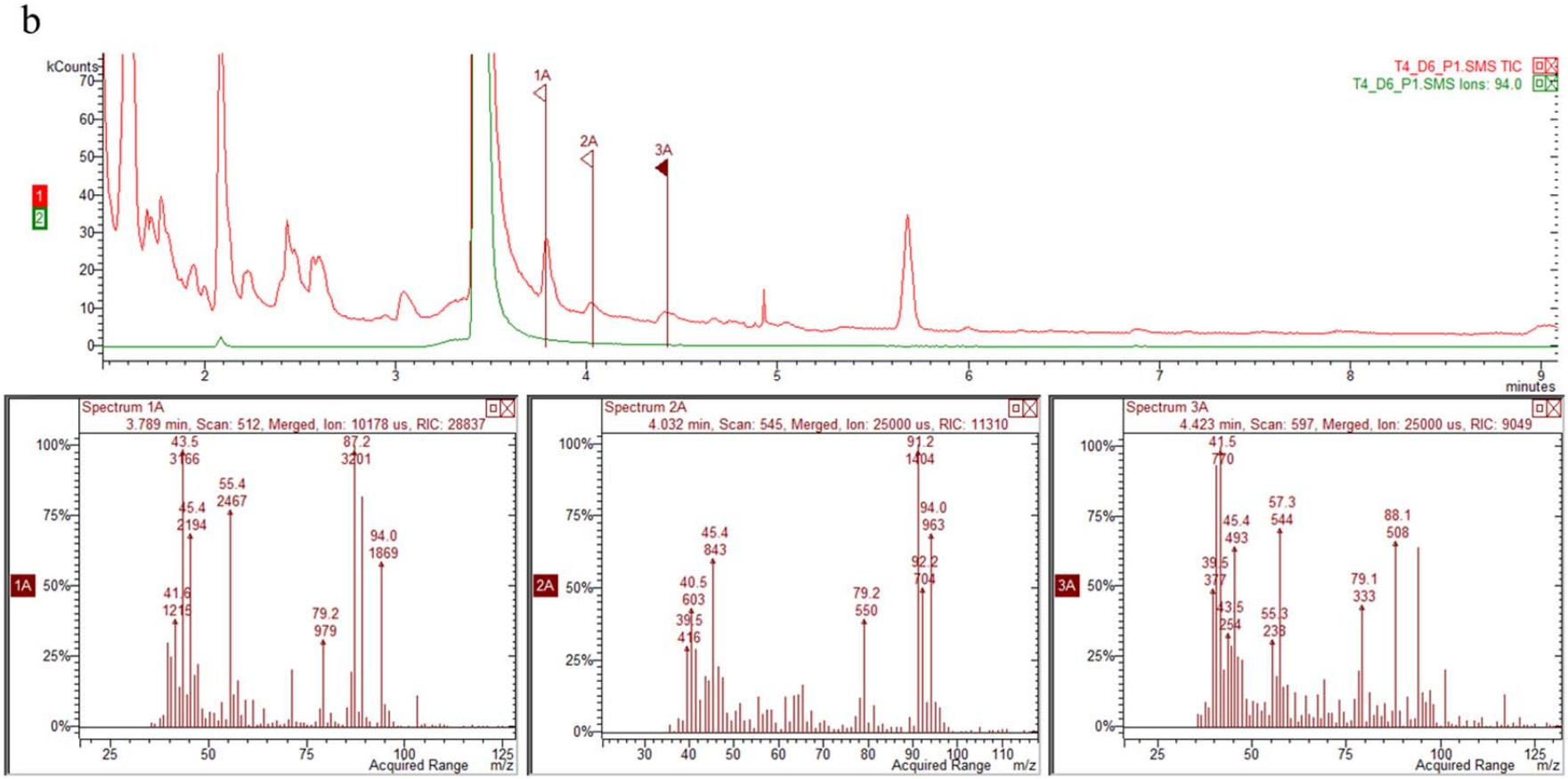


Figure 3.8b: Representative chromatogram of compound co-elution (top panel) and resulting convoluted mass spectra (bottom panels) from DMDS. Top panel: x-axis: retention times 1.5 – 9 minutes, y-axis: abundance in kCounts. Red trace is TIC, green trace is extracted ion count for m/z 94.0 the quantitation ion for DMDS. Bottom panel: Convoluted mass spectra of later eluting peaks at RT 3.789 min, 4.032 min and 4.423 min.

3.4 *Discussion*

The slowed decomposition process observed during the microcosm trial was likely the result of the closed environment. Few published studies have discussed the rates of decomposition within closed environments and most of the literature that is available investigate remains located indoors or within vehicles (Hyder 2007; Voss et al., 2008; Anderson 2011). However in most closed environments access to the remains by entomological fauna is restricted and/or delayed (Anderson 2011). In this study, although small flies were able to gain access to the remains, the small larvae did not appear to contribute to any loss in soft tissue. This lack of maggot activity was likely the main factor in the decreased rate of decomposition observed (Mann et al., 1990; Catts 1992). The intermediate temperatures (19 °C) and retention of decomposition fluid within the system prevented mummification and caused the carcasses to remain in a state of liquefaction. The resultant mass of putrefied tissue and decomposition fluid is similar to what has been observed in sealed coffins (Dent et al., 2004).

Soft tissue decomposition is a variable process from fresh remains to skeletonization. The onset of the early stages, particularly the bloat stage appears to be more consistent. This was observed during the outdoor trials as well as the microcosm, when the carcasses all entered the bloat stage on experimental day 2. During these early stages, the intrinsic processes of early decomposition are likely to be less influenced by external factors (Clark et al., 1997). However as decomposition progresses it becomes more variable and during this research, the onset and duration of decomposition stages, even when standardized as ADD, were no longer consistent between trials.

Decomposition studies have conventionally used days as the temporal measurement, however this method was not conducive to the comparison of studies, which led to the development of the standardized measurement of Accumulative Degree Days (ADD). Temperature is believed to be the most significant factor affecting decomposition (Mann et al., 1990) and ADD represents the heat energy units required for biological processes to proceed including bacterial and larval growth as well as the breakdown of soft tissue (Vass et al., 2002; Megyesi et al., 2005; Simmons et al., 2010[b]). Therefore the ADD required to complete a process should remain constant.

Temperature is not the only factor affecting decomposition and the rate at which the biological process of decomposition occurs in relation to ADD may be accelerated due to the presence of a 'catalyst' such as insects (Simmons et al., 2010[b]) or the microbiological community. In the absence of such catalysts decomposition may progress at a slower rate. This is potentially the reason for the elongated bloat stage observed in Trial 4. During this trial, reduced insect activity was observed during the first few days, likely due to the cooler temperatures (~13°C). Anderson (2011) identified a similar deceleration of decomposition when there was a delay in insect colonization. Remains that were located indoors and experienced a delay in colonization showed signs of bloat until day 10 whereas those that were readily colonized had entered the active decay stage by day 7 (Anderson 2011). As decomposition progresses the presence of catalysts, such as insects or bacteria, may have a greater influence on decomposition and make characterizing it in terms of ADD or qualitative stages difficult.

Many researchers have recognized the role of insect activity on the rate of decomposition and have shown that, in their absence, the rate slows significantly (Mann et al., 1990; Catts 1992; Simmons et al., 2010[a;] Simmons et al., 2010[b;] Anderson 2011). The presence of blowfly larvae increases the rate of decomposition by the mechanical removal of tissue via feeding and if present in large masses they can also generate temperatures in excess of 20 °C above ambient (Catts 1992). This increase in temperature is not taken into consideration when calculating ADD using ambient temperature loggers however it may play a role in decomposition processes by increasing the rate of enzymatic reactions or by facilitating microbial action.

Once blowfly larvae have completed feeding they enter a migratory phase in preparation for their next life stage (Anderson 2011). Typically when they enter the migratory phase, individual larvae or small aggregates migrate outward from the remains in a 360° radius (Lewis and Benbow 2011). In rare instances, it has been reported that larvae can migrate in mass events as was observed in this work during Trial 2. Lewis and Benbow (2011) defined an en mass dispersal as greater than 90 % of all larvae migrating in the same direction as one or two collective groups. This migratory pattern was observed in Trial 2 with the majority of the larvae migrating as a large group in one direction from all four

replicate carcasses. A possible explanation for this rare migration pattern is the accumulation of rainfall and subsequent soil saturation. Similar to Lewis and Benbow (2011) the migration event observed during this project occurred following a rainfall. The rain may have saturated the soil surrounding the remains causing poor conditions for the larvae (Lewis and Benbow 2011). Additionally, the moist conditions following a rainfall can improve larvae motility. The stimulus for this type of en mass migration is still unclear and it may have been propagated by other individual larvae who had commenced migration (Lewis and Benbow 2011). The large numbers of maggots potentially leaving the carcass prematurely left excess soft tissue on the carcasses. This soft tissue persisted for a considerable length of time and explains the elongated advanced decay stage observed in Trial 2.

3.4.1 *Decomposition VOCs*

Several volatile organic compounds were detected throughout the research trials, thirteen of which were identified as products of the decomposition process. Some of these compounds were detected within the controls however this does not exclude them from being compounds of interest as their amounts were higher within the experimental samples and they showed trends that were distinctly different from the control samples. Compounds that were present at similar levels between the control and experimental treatments throughout decomposition were not included as target compounds. This selective process ensured that background environmental VOCs were not reported as decomposition products.

The preliminary investigation into decomposition odour identified ten compounds, predominantly polysulfides and aromatics, within the decomposition headspace of a controlled environment. These compounds displayed an increasing trend through the bloat and active decay stage. This correlated with observations of the progressive liquefaction of soft tissue and the distinct decomposition odour released from the microcosms during sampling. The absence of aldehydes within the microcosm study could have been the result of either the slowed decomposition process or the enclosed decomposition environment. Fewer fatty acids and therefore aldehydes may have been present within the microcosm as the carcasses only reached the active decay stage, not

the later decay stages in which fatty acids are likely released from remains (Larizza 2010; Swann et al., 2010[b]). Alternatively, the closed system may have caused a reduction in the microbial load and oxygen availability within the microcosm thereby hindering the oxidation of unsaturated fatty acids to aldehydes and ketones (Dent et al., 2004; Mohan Kumar et al., 2009). The production of these ten volatiles within the closed microcosm may indicate that they are produced by intrinsic soft tissue decomposition independently of some environmental factors.

Although there is a large amount of variability in the number and type of compounds reported within the literature, the polysulfides (DMDS, DMTS and DMQS) are the most commonly reported compounds for decomposition VOC profiles (Vass et al., 2004; Statheropoulos et al., 2005[a;] Statheropoulos et al., 2007; Vass et al., 2008; Dekeirsschieter et al., 2009; Hoffman et al., 2009; Statheropoulos et al., 2011; DeGreeff and Furton 2011[b;] Brasseur et al., 2012; Dekeirsschieter et al., 2012). The polysulfides are believed to be the result of the putrefactive breakdown of the sulfur containing amino acids, cysteine and methionine (Statheropoulos et al., 2005[a;] Higgins et al., 2006; Paczkowski and Schutz 2011). However, the polysulfides are not likely a direct product of amino acid metabolism but rather the result of methanethiol (MeSH) oxidation (Higgins et al., 2006; Statheropoulos et al., 2011). This reaction readily occurs in the presence of oxygen and as a result may decrease the amount of other thiols within the headspace of decomposition.

The polysulfides, particularly DMDS, are potential markers for soft tissue decomposition (Statheropoulos et al., 2011). Investigations of cadaveric VOCs have consistently identified these compounds as major components of decomposition odour. This class have been detected in a variety of decomposition scenarios from both human and porcine remains (Vass et al., 2004; Statheropoulos et al., 2005[a;] Statheropoulos et al., 2007; Vass et al., 2008; Dekeirsschieter et al., 2009; Hoffman et al., 2009; Statheropoulos et al., 2011; DeGreeff and Furton 2011[b;] Brasseur et al., 2012; Dekeirsschieter et al., 2012). During the three outdoor trials (1, 2 and 4), DMDS was the most abundant compound and was consistently first detected during the early stages of decomposition at approximately 30 ADD. These volatile sulfur compounds are known signalling

molecules for carrion insects (Kalinova et al., 2009; LeBlanc and Logan 2010; Statheropoulos et al., 2011; van der Niet et al., 2011) and a recent study showed that the early production of the sulfur VOCs played a role in the attractiveness of remains to carrion beetles during the early stages of decomposition (Kalinova et al., 2009). These polysulfides are key components of the decomposition odour and may also be the key compounds utilized for scent recognition by HRD canines.

The aromatic compounds identified in this study are also thought to be products of amino acid degradation. The indolic compounds, indole and skatole, are likely resulting from the amino acid tryptophan whereas the phenolic compounds, phenol and phenol-4-methyl, are potentially produced during the breakdown of tyrosine (Swann et al., 2010[d]; Paczkowski and Schutz 2011). Of the aromatics, phenol is one of the more commonly reported compounds in decomposition VOC profiles (Statheropoulos et al., 2007; Dekeirsschieter et al., 2009; Statheropoulos et al., 2011; DeGreeff and Furton 2011[b]; Brasseur et al., 2012; Dekeirsschieter et al., 2012). Conversely, compounds such as indole and skatole that are reported breakdown products (Vass et al., 2002; Dent et al., 2004) have been less frequently identified (Hoffman et al., 2009; Brasseur et al., 2012; Dekeirsschieter et al., 2012). Additional benzoic compounds typically reported within decomposition headspace such as benzaldehyde and benzonitrile were not consistently detected in this study (Vass et al., 2004; DeGreeff and Furton 2011[b]; Brasseur et al., 2012).

The aldehydes identified in this study; hexanal, heptanal and octanal, along with additional short chain aldehydes have previously been identified within the headspace of decomposition (Statheropoulos et al., 2005[a]; Dekeirsschieter et al., 2009; Hoffman et al., 2009; Dekeirsschieter et al., 2012) and are likely the result of fatty acid degradation (Dent et al., 2004; Boumba et al., 2008; Janaway et al., 2009; Paczkowski and Schutz 2011). Under aerobic conditions the oxidation of the unsaturated free fatty acids by fungi, bacteria and atmospheric oxygen will produce a variety of aldehydes and ketones (Dent et al., 2004; Mohan Kumar et al., 2009). The aerobic conditions necessary for oxidation would be typical of surface decomposition in contrast to a burial where the anaerobic conditions would facilitate transformation of the fatty acids to adipocere (Dent

et al., 2004). Studies that have utilized porcine remains in surface decomposition have shown that free fatty acids including the unsaturated fatty acids, are detectable in decomposition soil during the later stages of soft tissue decomposition (Larizza 2010; Swann et al., 2010[b]). Upon release into the environment, likely through the purging of decomposition fluid, fatty acids are exposed to oxygen and are available as substrates for microbiological activity. Larizza (2010) (unpublished thesis) identified peaks of unsaturated fatty acids from ADD 123.0 – 396.6 in a similar southern Ontario environment. Whereas Swann et al. (2010[b]) identified an increasing trend of free fatty acids with a maximum at an ADD of 310-359 in the same environment as this study. Both sets of results correlate with the trend observed in this study where the aldehydes were seen to peak during the transition from the advanced decay to dry remains stage (ADD of 395.1, 513.8 and 364.8 for Trials 1, 2 and 4, respectively).

Trial 2 displayed distinctly higher levels of aldehydes compared to Trials 1 and 4 with a later peak at ADD 513.8. This later peak could be the result of the mass larval migration. The moist tissue and subsequent fatty acids that remained following the migration would not have been consumed by the larvae but rather degraded slowly by enzymatic or microbial action. This could have led to an increase in the oxidation of fatty acids and the resultant increase in aldehydes present within the decomposition headspace.

The VOC profile changed throughout decomposition in both the number and types of compounds detected. Overall there was an increasing trend in the diversity of the VOCs present within the decomposition headspace through bloat to the active decay stage. This is congruent with the observations of decomposition. During this period of decomposition the carcasses lost the majority of soft tissue and a distinct decomposition odour was associated with the remains. The outdoor studies were terminated during the dry remains stage when the number and relative concentration of VOCs within the profile decreased to basal levels. The late stage of decomposition and the lack of soft tissue likely contributed to the lower levels of compounds detected, however cadaver dogs are known to be able to detect skeletonised remains over extended post-mortem intervals (Komar 1999; Rebmann et al., 2000; Lasseter et al., 2003). Therefore this VOC profile may be limited to soft tissue decomposition and further research into the VOCs produced

from skeletonised remains and over extended post-mortem intervals is required. This may include further examination into the VOCs within decomposition soil. As decomposition progresses and there is a transfer of decomposition products to the surrounding environment, the soil may act as a scent reservoir for decomposition odour and facilitate detection by cadaver dogs (Brasseur et al., 2012).

3.4.2 *Chemical Profiling of Decomposition Odour*

The variety of VOCs found within decomposition headspace is the result of soft tissue degradation and the breakdown of macromolecules. These processes are facilitated by the intrinsic bacteria of the gastrointestinal and respiratory tract, as well as by the microorganisms and entomological fauna that colonize the remains (Gill-King 1997; Boumba et al., 2008; Janaway et al., 2009; Paczkowski and Schutz 2011; Statheropoulos et al., 2011). However, the exact origin of these compounds within this complex system is unclear. The compounds may result from the metabolism of several bacteria or may be products of a sequential food chain with one organism working on the products of the next (Boumba et al., 2008; Paczkowski and Schutz 2011). Additionally, many decomposition VOCs have also been identified within the headspace of isolated blowfly larvae and pupae (Frederickx et al., 2012). As individual compounds these VOCs are not unique to decomposition and can be found in a variety of environments and from numerous substrates (Vass et al., 2008). However, HRD dogs are able to utilize this odour profile to locate human remains. The uniqueness of decomposition odour may be the result of the entire death assemblage; a combination of odorants from intrinsic soft tissue decomposition and those resulting from the microbial and entomological community that colonize the remains. It is currently unknown which compounds or ratio of compounds constitutes a recognizable profile for the HRD canines, however in order to be effective their training needs to include the entire profile of potential odorants (DeGreeff et al., 2012).

To achieve a better understanding of decomposition odour and canine training a detailed non-target analysis of decomposition VOCs is required; where the goal is to detect and identify all components of the samples (Dalluge et al., 2003; Hernandez et al., 2011). However the complex mixture and dynamic nature of the VOCs within decomposition

odour presents a challenge for this type of analysis. A non-target method requires a sample collection or preparation technique that is non-selective and compatible with a wide range of compounds, an efficient GC separation and automated peak find and mass spectral deconvolution (Hernandez et al., 2011).

The sample collection technique utilized in this work, thermal desorption, is compatible with a wide range of compounds especially when multiple sorbents are utilized (Harper 2000). However separation and identification of compounds utilizing GC-ion trap MS was problematic. The limited resolution power of conventional GC can cause compound co-elution and contaminated mass spectra. These instances of compound masking or co-elution make manual peak finding and compound identification via library search difficult (Hernandez et al., 2011). This was particularly an issue for the more volatile compounds and those being masked by compounds present in high amounts, such as the polysulfides. Compound masking could have been resolved prior to chromatographic separation by decreasing the amount of sample injected via an increase in the split flow. However this would risk the loss of trace-level compounds which may be key odorants of decomposition. Similarly, selection of a column better suited for the separation of very volatile compounds would have biased the analysis and likely caused a reduction in information gained on larger, less volatile compounds.

Chromatographic co-elution can be resolved using time-of-flight mass spectrometry (TOFMS). The use of TOFMS and spectral deconvolution is valuable in non-target analysis (Ibanez et al., 2008). The full spectra collected by the TOFMS along with sophisticated software allow for automated mass spectral deconvolution and peak finding (Cochran 2002; Ibanez et al., 2008). By analysing the spectral data the software identifies all sample components and extracts the pure spectra for each. The pure spectra can then be searched against a spectral library for compound identification. Application of this non-target analysis provides complete characterization of a sample which is useful in discriminating compounds that are specific to the headspace of decomposition. By comparing all components of control and experimental samples, or those from human and porcine remains, a comprehensive odour profile can be generated.

Chapter 4: *TD – GC x GC – TOFMS analysis of decomposition VOCs from Human Analogues and Human Remains*

Thermal desorption – Two Dimensional Gas Chromatography – Time-of-Flight Mass Spectrometry (TD-GC×GC-TOFMS) was utilized to analyze VOCs collected from the decomposition of human analogues (S*us scrofa domesticus*) and human remains.

This novel technique was first applied to the analysis of VOCs from the decomposition of pig carcasses collected during Trial 3 at the UOIT-GRF. The results of this project were published in Analytical Chemistry. 2013, 85, 998-1005 (Stadler et al., 2013).

The decomposition headspace of pig carcasses and human remains within the same environment at the Forensic Anthropology Research Facility during human trials 1, 2 and 3 were also analysed with this technique. These samples were collected in collaboration with the recipients of a US Department of Justice research grant (Hamilton et al., 2010).

4.1 *Climatic Conditions & Carcass Decomposition*

Trial 3 commenced during the third week of July 2011 and continued until the remains reached skeletonization in August. The average daily temperature throughout Trial 3 was 21.1 °C, with an absolute minimum of 8.4 °C and an absolute maximum of 41.9 °C (Figure 4.1). The total rainfall received during this trial was 160mm.

The decomposition was characterized following the same stages outlined previously in Table 3.1. The carcasses progressed through all decomposition stages. The onset and duration of the stages are provided in Figure 4.2 (ADD values are provided in Appendix E) and photographs of each stage can be found in Figure 4.3. The carcasses were characterized to be in the bloat stage on experimental day 2 (ADD 55) until experimental day 4 (ADD 107.2) when they entered the active decay stage. During active decay (experimental day 6, ADD 150) large amounts of maggots were observed migrating from the carcasses. This event took place following rainfall of approximately 4mm. Although a large migration event was observed, some maggots and considerable amounts of soft tissue remained on the carcass. On experimental day 11 (ADD 261) the carcasses had entered the advanced decay stage. During this stage the skin had begun to mummify however the enveloped tissues retained moisture and persisted. Once the carcasses consisted of only desiccated skin and bones they were characterized as being in the dry remains stage. This occurred on experimental day 40 (ADD 845.71).

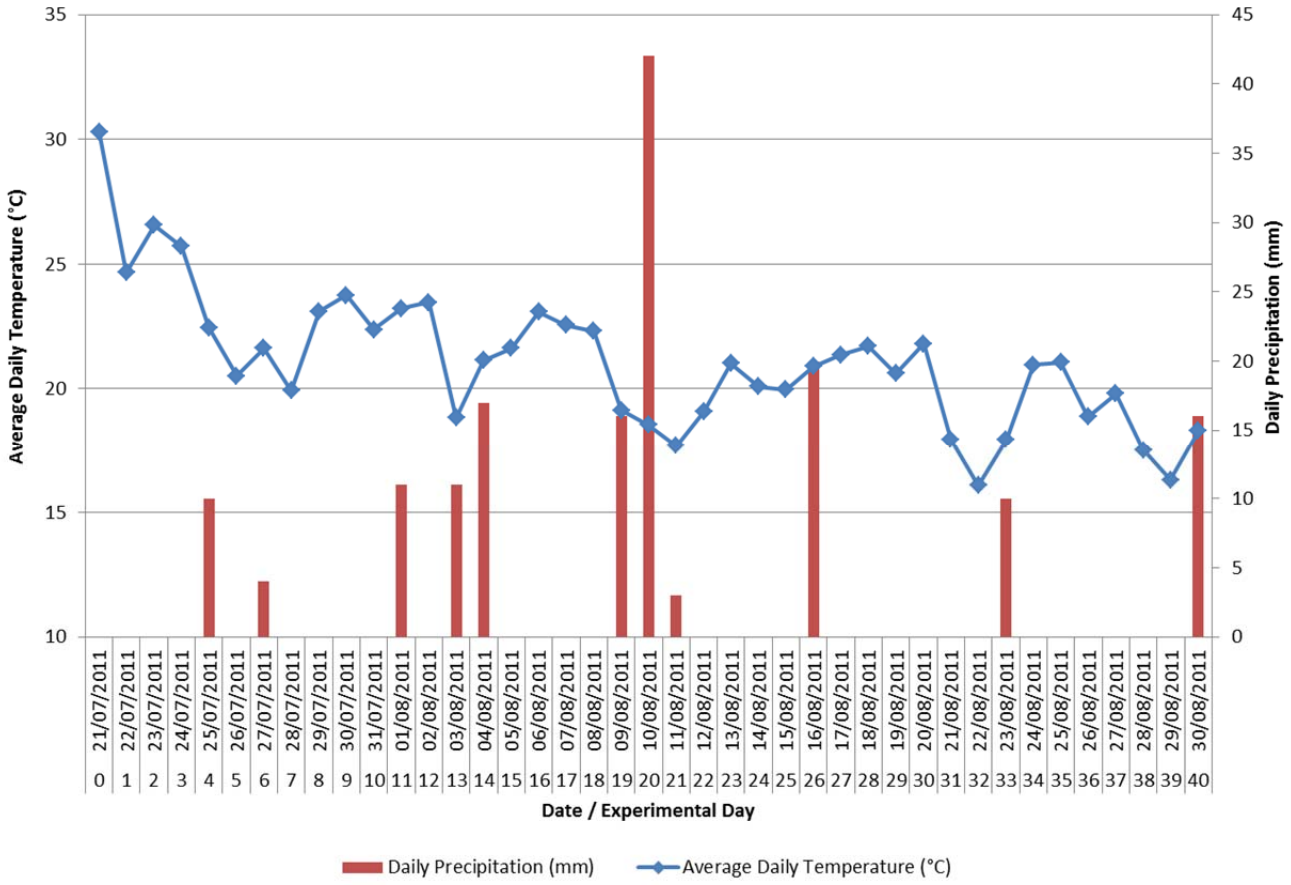


Figure 4.1: Climatic data for Trial 3 at the UOIT-GRF in Oshawa, Ontario, Canada. The average daily temperature is plotted on the primary y-axis and the daily precipitation is plotted on the secondary y-axis.

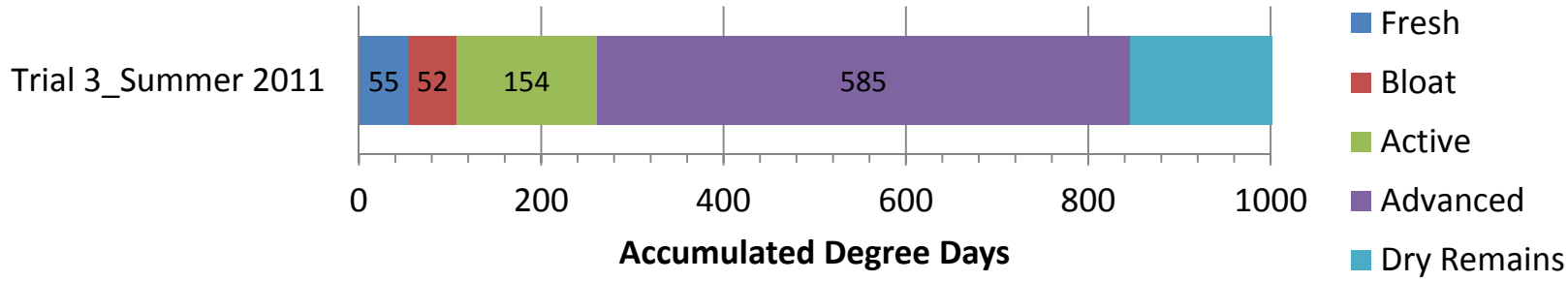


Figure 4.2: Onset and progression of decomposition by accumulated degree days (ADD) for Trial 3 at the UOIT-GRF in Oshawa, Ontario, Canada. Values indicate duration of the decomposition stage in ADD

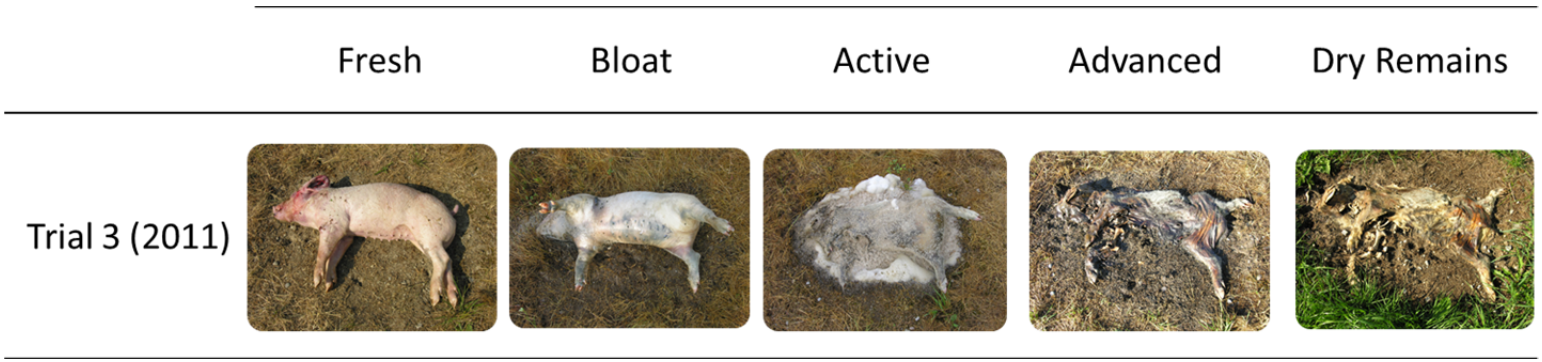


Figure 4.3: Photographs of each decomposition stage observed during Trial 3 at the UOIT-GRF in Oshawa, Ontario, Canada.

4.2 *Climatic Conditions of Human Remains Trials*

Human remains trial 1 commenced the first week of November 2011 and samples were collected for 4 experimental days (0, 1, 2, 3). During the four days the temperatures were cooler with a daily average temperature of 15.2 °C, an absolute minimum of -5 °C and an absolute maximum of 36.5 °C.

The human remains trial 2 commenced the third week of May 2012 and samples were collected for 6 experimental days (0, 1, 2, 3, 4, 5). This trial took place under warmer conditions, with a daily average temperature of 27.5 °C, an absolute minimum of 15 °C and an absolute maximum of 40.4 °C.

Human remains trial 3 commenced during the first week of November 2012 and samples were collected for 6 experimental days (0, 1, 2, 3, 4, 5). The average daily temperature of this trial was 18.9 °C, with an absolute minimum of 5 °C and an absolute maximum of 40 °C.

Figure 4.4 displays the average daily temperatures of each treatment group for human remains trials 1, 2 and 3. Within each trial, no distinct differences were observed in the temperatures of the different treatment groups (insect inclusion, insect exclusion, pig carcass).

The short duration of the human remains trials was governed by access to the Forensic Anthropology Research Facility (FARF) which was set out in the awarded grant proposal (Hamilton et al., 2010). This represented a major restriction to the overall process of decomposition.

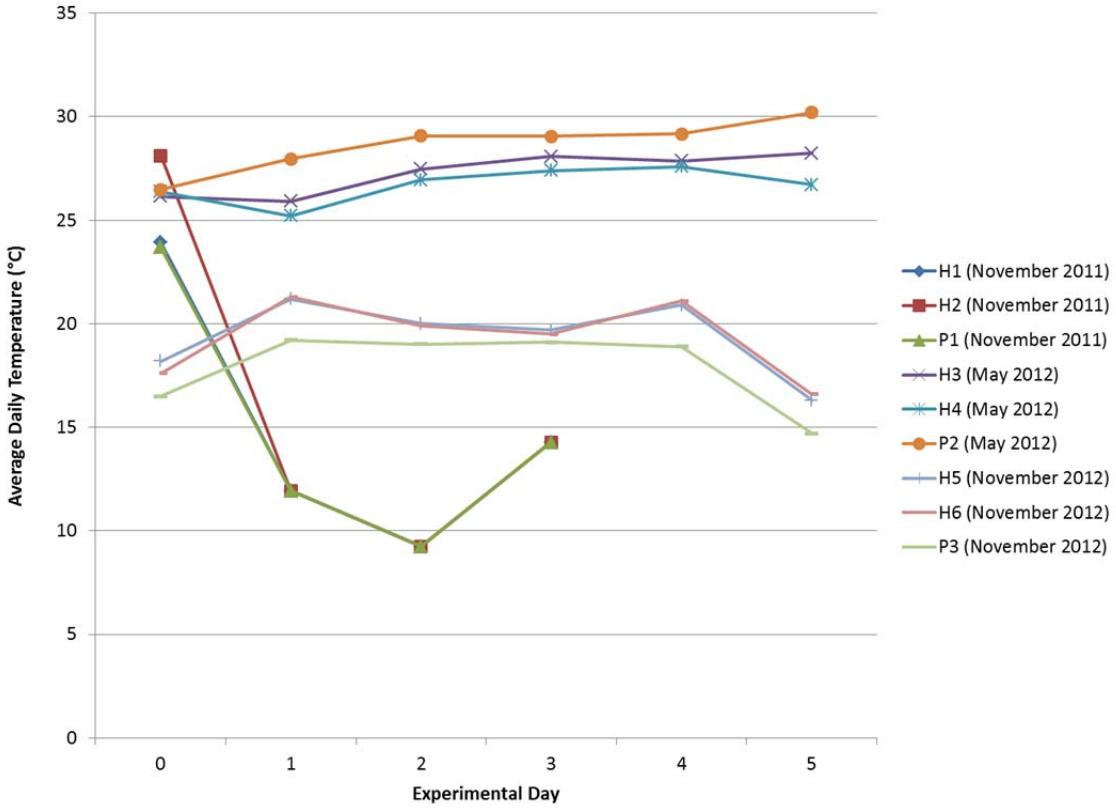


Figure 4.4: The average daily temperatures for each treatment group during the human remains trials 1, 2 and 3 at the Forensic Anthropology Research Facility in San Marcos, Texas, United States. H1, H3 and H5 were part of the insect inclusion treatment and H2, H4 and H6 were part of the insect exclusion treatment.

4.3 *Human Remains Decomposition*

The decomposition stage of the remains was evaluated using photographs and observations provided by the researchers present at the research facility during the human remains trials. Overall, minimal soft tissue decomposition was observed throughout the three trials.

During the human remains trial 1 the insect inclusion remains (H1) displayed some early autolytic changes with localized areas of skin sloughing and slight putrefactive colour changes. The insect exclusion remains (H2) only displayed signs of desiccation on the fingertips, forearms and face. The pig carcass (P1) displayed early signs of decomposition characterized by livor mortis and the extension of limbs due to the initial accumulation of gases within the tissues. Due to the graphic nature of these images, photographs from each sampling day are included in Appendix F rather than in text.

Soft tissue decomposition was more extensive during the human remains trial 2. The insect inclusion remains (H3) and pig carcass (P2) displayed a similar trend in their decomposition. Both displayed differential decomposition at sites of insect colonization. These localized areas displayed skin sloughing, and the insect larvae were present under this thin layer of skin. As they progressed across the remains the underlying skin tissue began to mummify, turning red-brown-black in colour with a leathery texture. Additional signs of soft tissue decomposition in H3 included the purging of fluids from the facial orifices and the tracheotomy wound as well as the distension of the abdomen (end of experimental day 5). As the skin mummified, blowfly larvae continued to be observed on the remains, suggesting that they may have been feeding under the mummified skin tissue. P2 entered the bloat stage on experimental day 2 and by experimental day 4 the carcass showed differential decomposition; with the head and upper torso in active decay and the lower torso in the bloat stage. P2 remained in this stage on experimental day 5. The insect exclusion remains of the human remains trial 2 (H4), displayed minimal visible signs of soft tissue decomposition. However the nose, lips, cheekbones and forehead showed signs of mummification. Photographs from each sampling day can be found in Appendix G.

Observations recorded by the researchers attending trial 2 noted that the human remains (H3 & H4) were "cool to the touch" until the end of experimental day 2; perhaps indicating that the remains were partially frozen at the time of placement.

The research subjects in the human remains trial 3 also displayed minimal soft tissue decomposition. The insect inclusion remains (H5) showed early putrefactive changes evidenced by a small area of marbling on the left shoulder. This was first observed on experimental day 2 and became more pronounced during the trial. The nose and lips of H5 also showed signs of desiccation across the experimental days. On the night of experimental day 5 (following collection of volatile samples in the morning), the remains began to show the onset of bloat and distension of the torso. Throughout this trial, the insect exclusion remains (H6) displayed some early putrefactive changes with a green colouration of the abdomen. No visible signs of decomposition were observed for the pig carcass (P3) in this trial. Photographs from each sampling day can be found in Appendix H.

4.4 *VOCs from Carcass Decomposition*

The raw data generated from the GC×GC-TOFMS analysis initially underwent spectral deconvolution and MS library identifications with the ChromaTOF® software. This generates a hit table of all components in each sample. Across the experimental chromatograms this amounted to approximately 10 000 hits. In addition to the VOCs from decomposition, these tables included environmental VOCs, instrumental signals such as column bleed and artifact hits resulting from various levels of peak tailing. In order to identify the compounds that are specific to decomposition headspace, the peak tables of the experimental samples were compared to those of the controls for each experimental day using the statistical comparison tool within the ChromaTOF® software, *Statistical Compare*. Using a mass spectral match criterion of 60 % to align multiple chromatograms, statistical compare is able to identify the meaningful differences between treatment groups. This data treatment strategy yielded a data matrix of greater than 300 compounds found to be specific to decomposition.

The resulting data sets were exported to Microsoft Excel where the relative peak area ratios were calculated against the internal standard (IS) and the compounds were classified into one of 11 chemical classes: alcohols, aldehydes, aromatics, carboxylic acids, esters, halogens, hydrocarbons, ketones, nitrogens, sulfides, and others. A list of the major decomposition VOCs within each chemical class is presented in Table 4.1. This matrix was also used to perform principal components analysis (PCA) to identify relationships between the decomposition process and the classes of VOCs produced. A scatter plot of the calculated principal component scores for each pig is shown in Figure 4.5 a & b.

Each stage of soft tissue decomposition is separated across the four quadrants of the plot. The vectors of the diagram indicate the variables, i.e. chemical class, that explain the distribution of the points, however only the major vectors have been shown for clarity. These vectors are the visual representation of the principal component loadings (coefficients) that describe the relationship between the original variable and the new principal component (Appendix I). In Figure 4.5a the loadings for component 1 show a positive relationship with sulfides (0.8469) and carboxylic acids (0.5148) indicating that the points in the quadrants to the right of the y axis have higher levels of these compounds compared to the those on the left. The loadings for component 2 of Figure 4.5a have positive coefficients for carboxylic acids (0.6015) and aromatics (0.3974) and a negative relationship with alcohols (-0.5082) and sulfides (-0.4557). Thus points on top of the x-axis have higher levels of carboxylic acids and aromatic compared to those below which have higher levels of alcohols and sulfides. Figure 4.5b for pig 2 component 1 had a positive relationship with carboxylic acids (0.8719) and aromatics (0.4728) whereas component 2 had a positive relationship with sulfides (0.8213) and alcohols (0.4647).

Both carcasses showed the same relative trends: the bloat stage was characterized by higher levels of alcohols and sulfides; active decay demonstrated high levels of alcohols, sulfides, carboxylic acids and aromatics; whereas advanced decay exhibited higher levels of carboxylic acids and aromatic compounds. However, in addition to the above, pig 2

displayed higher levels of nitrogen compounds during the bloat stage and higher amounts of aldehydes and ketones in the advanced decay stage.

On average during the bloat stage (experimental day 2, ADD 54.9), alcohols made up over 10 % of the profile whereas the sulfides were over 80 % (Table 4.2). The predominant alcohols identified across decomposition were short chain alcohols of three to eight carbons in length. As a class the alcohols demonstrated a three peak trend with maximums on experimental days 2, 6, and 11 (ADD 54.9, 150.2, 260.9 respectively) (Figure 4.6a). The early stages of putrefactive decay exhibited high levels of 1-propanol and 1-butanol whereas later in decomposition, longer chain alcohols such as 1-octen-3-ol were also present. The sulfide class was dominated by the polysulfide compounds DMDS, DMTS and DMQS. The sulfides peaked during the bloat stage (experimental day 2, ADD 54.9), and continued to be components of the decomposition profile for the remainder of the study (Figure 4.6b).

Table 4-1: The presence or absence of the major decomposition VOCs in each chemical class throughout the decomposition process in Trial 3. Citations indicate published studies that have reported the same compound.

Compound Class	Compound Name	Decomposition Stage								Literature Report
		Fresh	Bloat	Active			Advanced		Dry Remains	
Alcohols	1-Propanol									(Dekeirsschieter et al., 2012)
	1,2-Propanediol									(Statheropoulos et al., 2011)
	1-Butanol									(Statheropoulos et al., 2005[a]; Dekeirsschieter et al., 2009; Statheropoulos et al., 2011; Dekeirsschieter et al., 2012)
	1-Butanol,3-methyl									(Dekeirsschieter et al., 2009)
	2-Butanol									(Dekeirsschieter et al., 2009; Statheropoulos et al., 2011)
	1-Pentanol									(Statheropoulos et al., 2005[a]; Dekeirsschieter et al., 2009; Hoffman et al., 2009; Dekeirsschieter et al., 2012)
	1-Hexanol									(Statheropoulos et al., 2005[a]; Hoffman et al., 2009; Statheropoulos et al., 2011; Dekeirsschieter et al., 2012)
	1-Heptanol									(Dekeirsschieter et al., 2012)
	1-Octanol									(Hoffman et al., 2009; DeGreeff and Furton 2011[b]; Dekeirsschieter et al., 2012)
	1-Octen-3-ol									(Hoffman et al., 2009; Statheropoulos et al., 2011)
		0	2	4	6	8	11	14	40	
		Experimental Day								
		0	54.9	107.2	150.2	191.7	260.9	326.5	845.7	
		Accumulated Degree Days (ADD)								

Table 4-1 continued:

Compound Class	Compound Name	Fresh	Bloat	Active			Advanced		Dry	Literature Report
Aldehydes	Butanal, 3-methyl									(Statheropoulos et al., 2007; Statheropoulos et al., 2011)
	Pentanal									(Statheropoulos et al., 2005[a]; Dekeirsschieter et al., 2012)
	Pentanal, 2-methyl									
	Hexanal									(Statheropoulos et al., 2005[a]; Dekeirsschieter et al., 2009; Hoffman et al., 2009)
	Heptanal									(Hoffman et al., 2009; Dekeirsschieter et al., 2012)
	2-Heptenal									(Hoffman et al., 2009)
	Octanal									(Hoffman et al., 2009; Dekeirsschieter et al., 2012)
	2-Octenal									(Hoffman et al., 2009; Dekeirsschieter et al., 2012)
	2-Decenal									(DeGreeff and Furton 2011[b])
Aromatics	Benzaldehyde									(Vass et al., 2004; Hoffman et al., 2009; Statheropoulos et al., 2011; DeGreeff and Furton 2011[b]; Brasseur et al., 2012; Dekeirsschieter et al., 2012)
	Benzonitrile									(Vass et al., 2004; DeGreeff and Furton 2011[b]; Brasseur et al., 2012)
	Benzenesulfonic acid,4-hydroxy									(Dekeirsschieter et al., 2012)
		0	2	4	6	8	11	14	40	
		Experimental Day								
		0	54.9	107.2	150.2	191.7	260.9	326.5	845.7	
		Accumulated Degree Days (ADD)								

Table 4-1 continued:

Compound Class	Compound Name	Fresh	Bloat	Active			Advanced		Dry	Literature Report
Aromatics	Indole									(Hoffman et al., 2009; Brasseur et al., 2012; Dekeirsschieter et al., 2012)
	Indole,3-methyl									(Dekeirsschieter et al., 2012)
	Phenol									(Statheropoulos et al., 2007; Dekeirsschieter et al., 2009; Statheropoulos et al., 2011; DeGreeff and Furton 2011[b;] Brasseur et al., 2012; Dekeirsschieter et al., 2012)
	Phenol,4-methyl									(Statheropoulos et al., 2007; Dekeirsschieter et al., 2012)
Carboxylic acids	Acetic acid									(Statheropoulos et al., 2011; DeGreeff and Furton 2011[b])
	Propanoic acid									(Dekeirsschieter et al., 2009; Hoffman et al., 2009)
	Propanoic acid,2-methyl									(Dekeirsschieter et al., 2009; Dekeirsschieter et al., 2012)
	Butanoic acid									(Dekeirsschieter et al., 2009; Hoffman et al., 2009; Dekeirsschieter et al., 2012)
	Butanoic acid,2-methyl									(Dekeirsschieter et al., 2009; Dekeirsschieter et al., 2012)
	Butanoic acid,3-methyl									(Dekeirsschieter et al., 2009; Statheropoulos et al., 2011; Dekeirsschieter et al., 2012)
	Pentanoic acid									(Dekeirsschieter et al., 2009; Hoffman et al., 2009; Dekeirsschieter et al., 2012)
	Hexanoic acid									(Dekeirsschieter et al., 2009; Hoffman et al., 2009; DeGreeff and Furton 2011[b;] Dekeirsschieter et al., 2012)
		0	2	4	6	8	11	14	40	
		Experimental Day								
		0	54.9	107.2	150.2	191.7	260.9	326.5	845.7	
		Accumulated Degree Days (ADD)								

Table 4-1 continued:

Compound Class	Compound Name	Fresh	Bloat	Active			Advanced		Dry	Literature Report
Hydrocarbons	1-Octene									
	Octane									(Statheropoulos et al., 2007; Statheropoulos et al., 2011; Dekeirsschieter et al., 2012)
	Nonane									(Statheropoulos et al., 2011; Dekeirsschieter et al., 2012)
	1,11-Dodecadiene									
	1-Undecene									(Statheropoulos et al., 2007)
Ketones	2-Butanone									(Statheropoulos et al., 2005[a]; Statheropoulos et al., 2007; Statheropoulos et al., 2011)
	2-Butanone,3-methyl									(Statheropoulos et al., 2011)
	2-Pentanone									(Statheropoulos et al., 2005[a]; Statheropoulos et al., 2011)
	2-Heptanone									(Statheropoulos et al., 2005[a]; Hoffman et al., 2009; Dekeirsschieter et al., 2012)
	2-Octanone									(Dekeirsschieter et al., 2012)
	1-Octen-3-one									(Dekeirsschieter et al., 2012)
	2-Nonanone									(Dekeirsschieter et al., 2012)
	2-Decanone									(Dekeirsschieter et al., 2012)
	2-Undecanone									(Dekeirsschieter et al., 2012)
	3-Octanone									(Dekeirsschieter et al., 2012)
	3-Octen-2-one									
		0	2	4	6	8	11	14	40	
		Experimental Day								
		0	54.9	107.2	150.2	191.7	260.9	326.5	845.7	
		Accumulated Degree Days (ADD)								

Table 4-1 continued:

Compound Class	Compound Name	Fresh	Bloat	Active			Advanced		Dry	Literature Report
Nitrogens	Trimethylamine									(Dekeirsschieter et al., 2009; Statheropoulos et al., 2011; Dekeirsschieter et al., 2012)
	Azidrine									
	Ethanamine,N-methyl									
	Ethylenimine									
	Octodrine									
	Hexanitrile									
	Pyrazine,2,6-dimethyl									(Dekeirsschieter et al., 2012)
Sulfides	Dimethyl Disulfide									(Vass et al., 2004; Statheropoulos et al., 2005[a]; Statheropoulos et al., 2007; Vass et al., 2008; Dekeirsschieter et al., 2009; Hoffman et al., 2009; Statheropoulos et al., 2011; DeGreeff and Furton 2011[b]; Brasseur et al., 2012; Dekeirsschieter et al., 2012)
	Dimethyl Trisulfide									(Vass et al., 2004; Statheropoulos et al., 2005[a]; Statheropoulos et al., 2007; Vass et al., 2008; Dekeirsschieter et al., 2009; Statheropoulos et al., 2011; DeGreeff and Furton 2011[b]; Brasseur et al., 2012; Dekeirsschieter et al., 2012)
	Dimethyl Tetrasulfide									(Statheropoulos et al., 2011)
		0	2	4	6	8	11	14	40	
		Experimental Day								
		0	54.9	107.2	150.2	191.7	260.9	326.5	845.7	
		Accumulated Degree Days (ADD)								

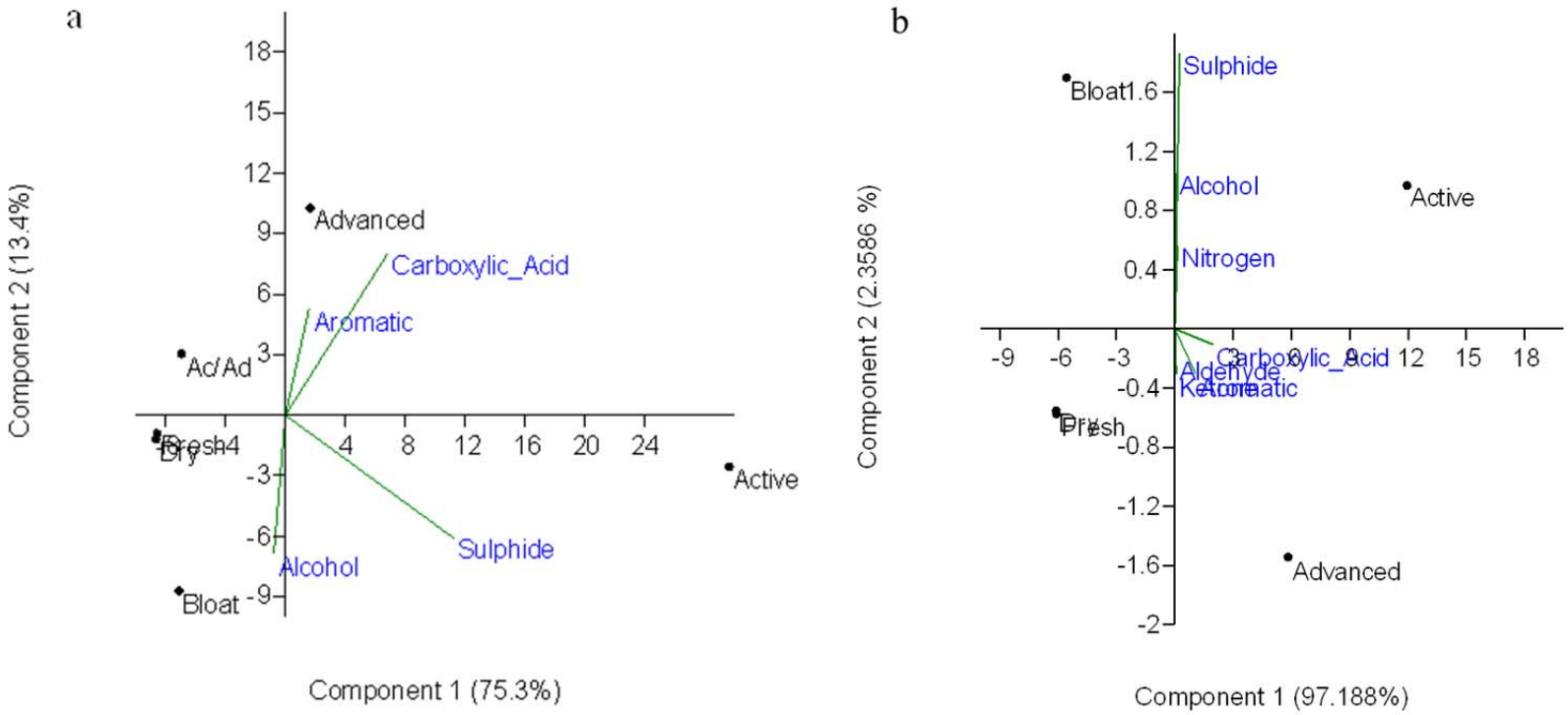


Figure 4.5: Principal component analysis scatter plot of the calculated PCA scores for Trial 3. Vectors for major chemical components are displayed. Data set: total relative peak abundances for each chemical class across decomposition stages a) Pig 1 loadings for component 1: sulfides 0.8469, carboxylic acids 0.5148; loadings for component 2: carboxylic acids 0.6015, aromatics 0.3974, alcohols -0.5082, sulfides -0.4557 b) Pig 2 loadings for component 1: carboxylic acids 0.8719, aromatics 0.4728; loadings for component 2: sulfides 0.8213 and alcohols 0.4647.

The carboxylic acids identified during this study were two to six carbons in length and are also referred to as volatile fatty acids (VFAs) in particular; acetic acid, 3-methyl and 2-methyl butanoic acid and hexanoic acid. During the active and advanced decay stages the carboxylic acids display a two peak trend with maximums occurring on experimental day 6 and 11 (ADD 150.2 and 260.9) shown in Figure 4.6b. During these maxima this class of compounds represented over 50 % of the decomposition VOC profile (Table 4.2).

Aromatics represented the other major class of compounds identified by PCA. The main compounds within this class were phenol, indole, skatole, benzonitrile and benzaldehyde. Although present throughout decomposition, the high level of aromatics observed during the end of the active decay stage through the advanced decay stage (experimental day 6 – 14, ADD 150.2 – 326.5) characterizes these later post-mortem processes (Figure 4.6a).

In addition to the main chemical classes discussed above the aldehydes, ketones, and nitrogen compounds appear to be integral to the overall VOC profile. Dominant compounds within these classes included 3-methyl butanal, octanal, 3-octanone, 1-octene-3-one and trimethylamine. The aldehydes and ketones had higher levels in the later stages of decomposition with the maximum for aldehydes observed on experimental day 8 (ADD 191.7) and the maximum for ketones observed on experimental day 11 (ADD 260.9) (Figure 4.6a). These two classes represented over 60 % of the profile in the dry remains stage (Table 4.2). The nitrogen class of compounds was highest during the active decay stage (experimental day 4 and 6, ADD 107.2 and 150.2).

Table 4.2: Percent composition of each chemical class for each experimental day in Trial 3

T3 % Composition of VOC Profile												
Experimental Day	ADD	Alcohol	Aldehydes	Aromatics	Carboxylic Acids	Esters	Halogens	HC	Ketones	Nitrogens	Sulfides	Other
0	0	0	4.11	85.70	0	0.58	0	0	6.29	2.73	0	0.60
2	54.9	13.36	0.43	1.80	0.77	0.38	0.02	0.04	0.63	1.48	81.01	0.07
4	107.2	1.12	0.83	7.06	0	0	0.05	0.24	1.42	9.87	79.37	0.05
6	150.2	3.90	1.95	23.41	62.96	0.09	0.05	0.76	1.77	2.88	2.05	0.18
8	191.7	2.74	12.85	64.26	10.72	0.10	0	0	5.30	1.28	2.33	0.42
11	260.9	4.28	2.88	30.54	50.13	0.24	0.54	0.19	6.49	0.44	3.00	1.02
14	326.5	7.31	8.20	21.96	53.36	0.17	0.02	0.07	5.46	0.79	2.34	0.31
40	845.7	17.57	25.30	3.70	0	2.39	0	8.10	38.98	0.28	1.47	2.20

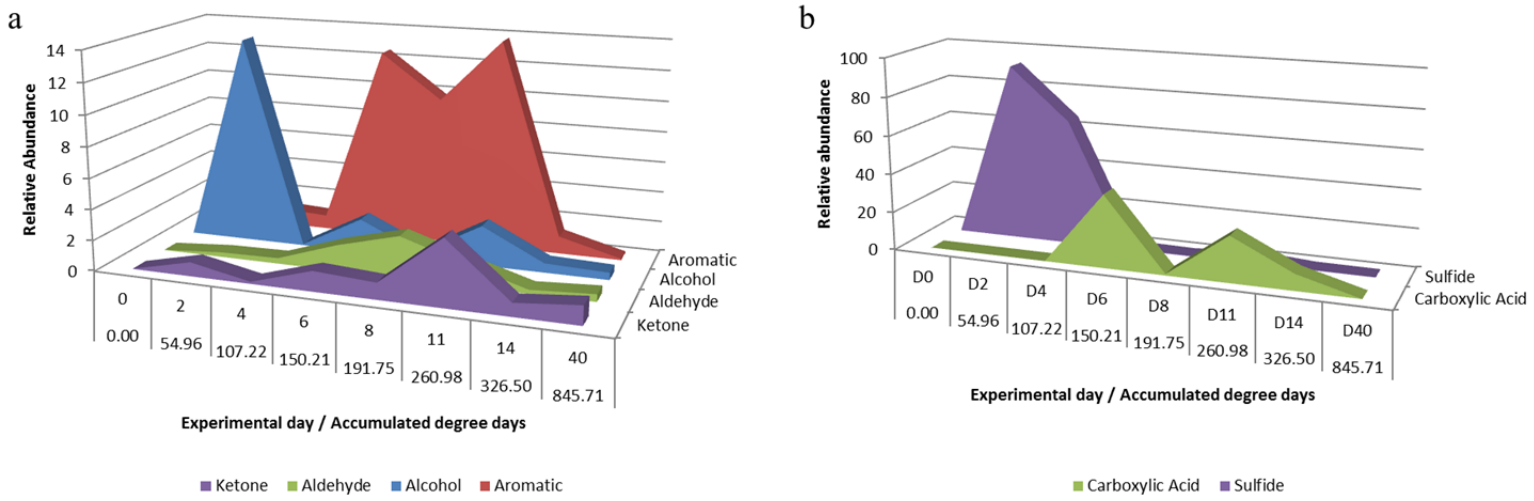


Figure 4.6: The relative abundances for compounds within the dominant chemical classes for Trial 3 a) ketones, aldehydes, alcohols, and aromatic compounds and b) carboxylic acids and sulfide compounds

4.5 *VOCs from the Early Post-Mortem Period of Pig Carcasses & Human Remains*

The data from the human trials 1, 2 and 3 underwent the same processing sequence as previously outlined. Following the initial processing with the ChromaTOF® software, the statistical comparison tool was utilized to identify any meaningful differences between treatment groups. The resulting data sets were exported to Microsoft Excel where the compounds were classified into one of 11 chemical classes: alcohols, aldehydes, aromatics, carboxylic acids, esters, halogens, hydrocarbons, ketones, nitrogens, sulfides, and others. The peak areas for each chemical class were normalized prior to principal component analysis (PCA).

For the human remains trials 1 and 3 (November 2011 & 2012 respectively) the PCAs did not identify any meaningful variation between the treatments groups (Figure 4.7 & 4.8). For both human remains trials 1 and 3 the first component extracted less than 50% of the variation within the data set and distinct clustering of points was not achieved. A number of compounds from a variety of the chemical classes were identified within the samples, however no trends were apparent. During the human remains trial 3 the number of experimental days was extended from four to six and the remains were exposed to slightly warmer temperatures (average 18.9°C vs. 15.2 °C). During this trial, decomposition VOCs such as the polysulfides (DMDS, DMTS, DMQS), phenol and pentanal were identified within the headspace of decomposition and were more evident within the pig carcass (P3) sample.

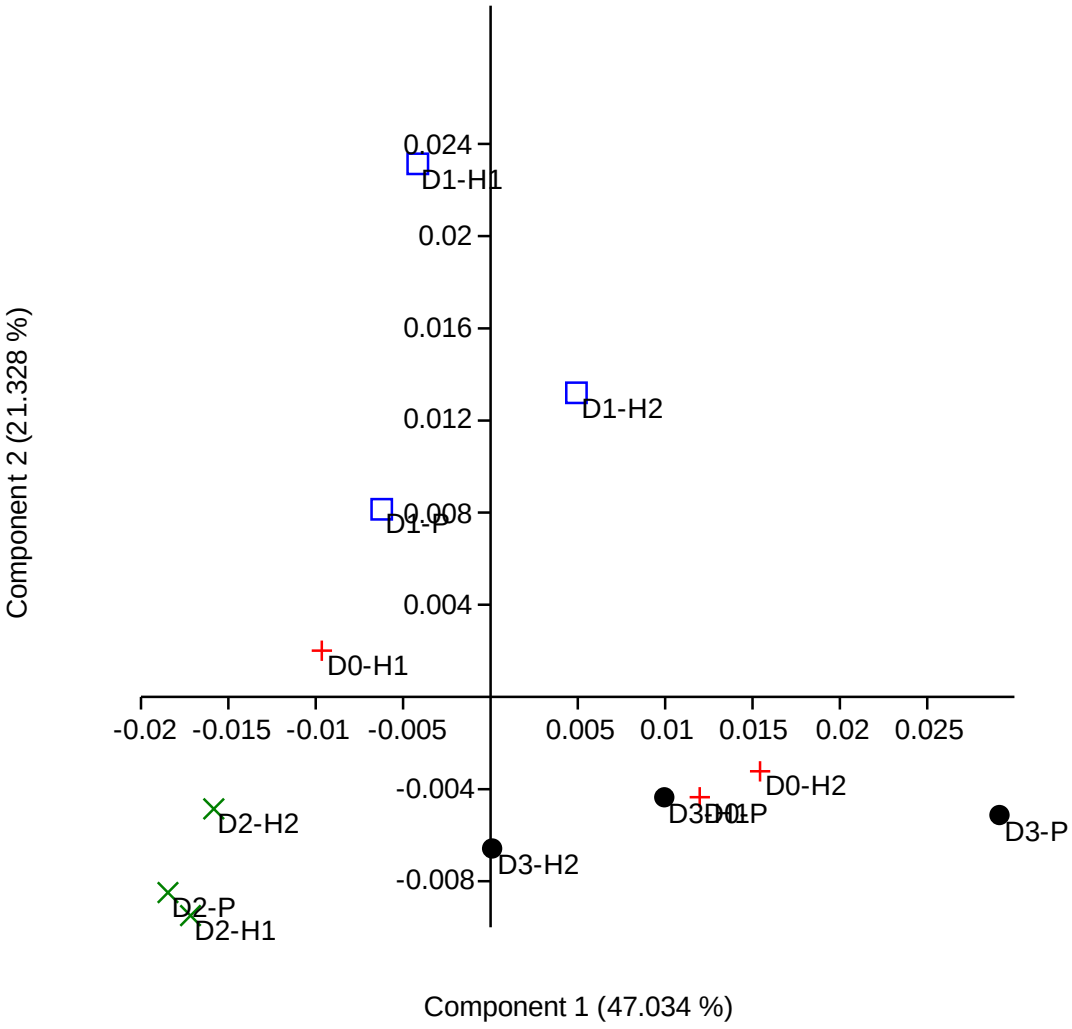


Figure 4.7: Principal component analysis scatter plot of the calculated PCA scores for human remains trial 1: (+) experimental day 0 (□) experimental day 1 (×) experimental day 2 (•) experimental day 3. Data set: total relative peak abundances for each chemical class for each experimental day. H1: insect inclusion remains, H2: insect exclusion remains, P: pig carcass. Loadings for component 1: aromatic 0.6589, ester -0.2418; loadings for component 2: HC 0.8088, ester -0.3634

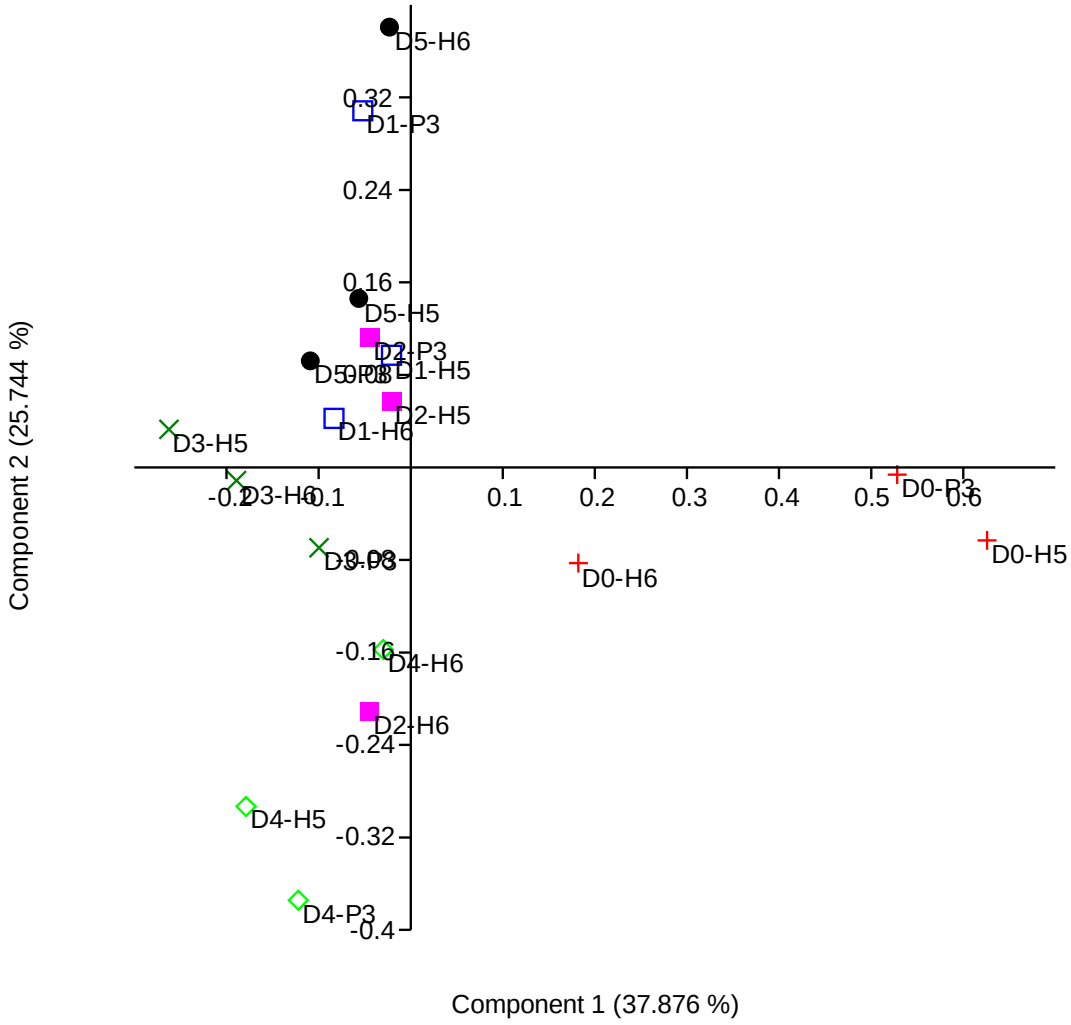


Figure 4.8: Principal component analysis scatter plot of the calculated PCA scores for human remains trial 3: (+) experimental day 0 (□) experimental day 1 (■) experimental day 2 (×) experimental day 3 (◊) experimental day 4 (•) experimental day 5. Data set: total relative peak abundances for each chemical class for each experimental day. H5: insect inclusion remains, H6: insect exclusion remains, P3: pig carcass. Loadings for component 1: carboxylic acid 0.8571; loadings for component 2: aromatics 0.7828.

The PCA of the data from the human remains trial 2 (May 2012) was able to differentiate the later experimental days based on the VOCs present within the headspace of the pig carcass and the insect inclusion remains (Figure 4.9). This trend was not observed for the insect exclusion remains (H4), which could not be differentiated based on the chemical composition of the headspace. Both the pig carcass (P2) and the insect inclusion remains (H3) were characterized by the alcohols and the sulfides present on experimental days 4 and 5. Although the PC loadings for the alcohols and sulfides are lower (component 1: sulfide -0.3567, alcohol -0.2006; component 2: sulfide -0.6838, alcohol -0.3050), due to the relationship with both components distinct separation of these data points is achieved in the lower left quadrant. A table of the dominant compounds identified within these samples is shown in Table 4.3. These compounds appeared earlier in the pig carcass (P2), with DMDS first appearing on experimental day 2 (ADD 54.4) for the pig carcass and on experimental day 3 (ADD 79.5) for the insect inclusion remains (H3). Overall the chemical profile from the insect inclusion remains (H3) appeared to lag behind that of the pig carcass, with P2 displaying a greater variety and increased levels of compounds earlier than H3 (Figure 4.10).

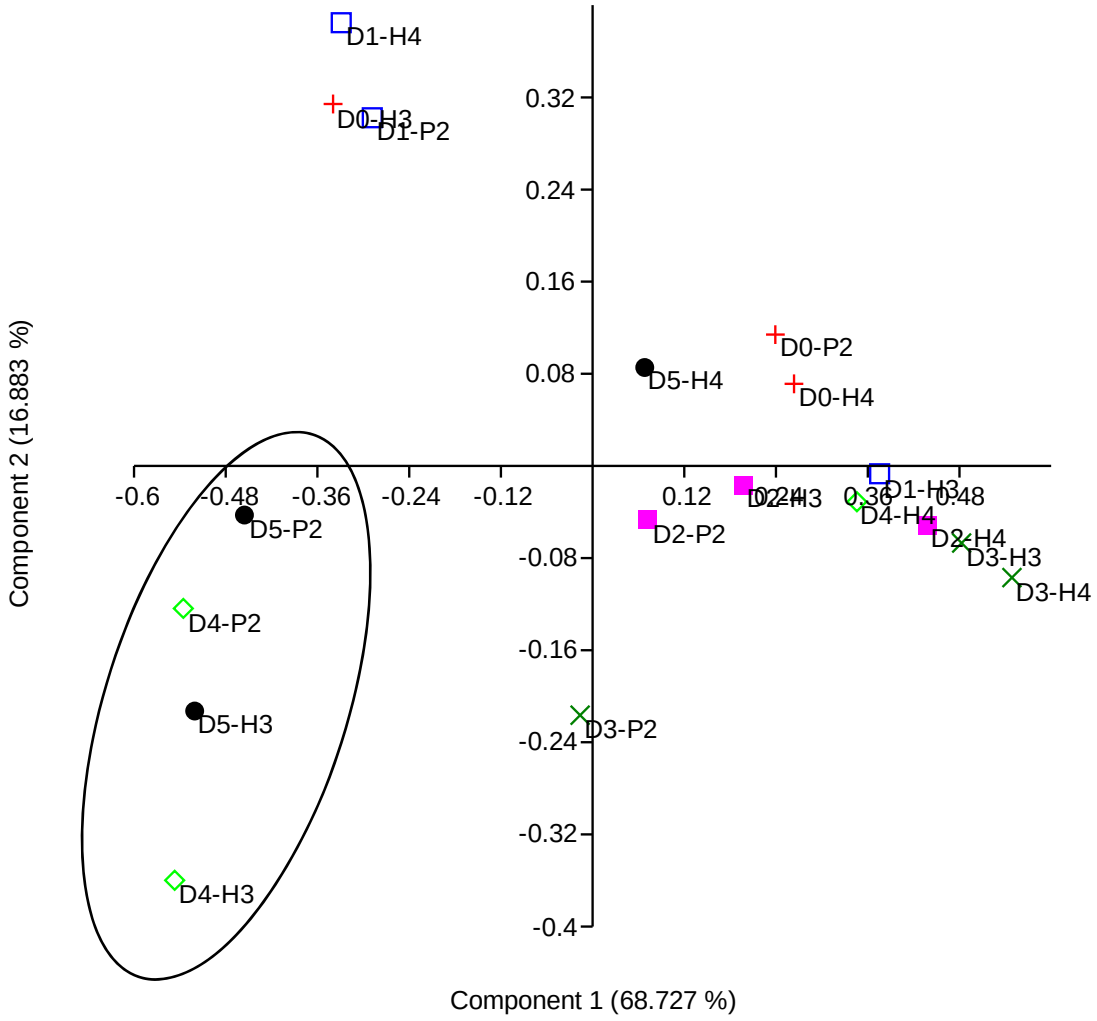


Figure 4.9: Principal component analysis scatter plot of the calculated PCA scores for human remains trial 2: (+) experimental day 0 (□) experimental day 1 (■) experimental day 2 (×) experimental day 3 (◊) experimental day 4 (•) experimental day 5. Data set: total relative peak abundances for each chemical class for each experimental day. H3: insect inclusion remains, H4: insect exclusion remains, P2: pig carcass. Loadings for component 1: carboxylic acid 0.8986, sulfide -0.3567, alcohol -0.2006; loadings for component 2: aromatic 0.4621, sulfide -0.6838, alcohol -0.3050. Ellipse is for illustrative purposes only.

Table 4.3: The presence or absence of the major decomposition VOCs in each chemical class in the human remains trial 2

Compound Class	Compound Name	Experimental day 4		Experimental day 5	
		H3	P2	H3	P2
Alcohols	Ethanol				
	Ethenol				
	1-Propanol				
	1-Propanol,2-methyl				
	1-Butanol, 2-methyl-				
	1-Butanol, 3-methyl-				
	2-Pentanol				
Aldehydes	Propanal, 2-methyl-				
	2-Butenal				
	Butanal, 3-methyl-				
Aromatics	Indole				
	Indole-3-methyl				
	Phenol				
	Phenol, 4-methyl-				
Carboxylic acids	Acetic acid				
	Propanoic acid				
	Butanoic acid, 3-methyl-				
	Pentanoic acid				
Nitrogens	1,2-Ethanediamine				
	Acetamide				
	Acetamide, N,N-dimethyl-				
	Trimethylamine				
	Methanediamine, N,N,N',N'-tetramethyl-				
	Ethylenimine				
Sulfides	2,4-Dithiapentane				
	Methanethiol				
	Dimethyl Disulfide				
	Dimethyl Trisulfide				
	Dimethyl Tetrasulfide				

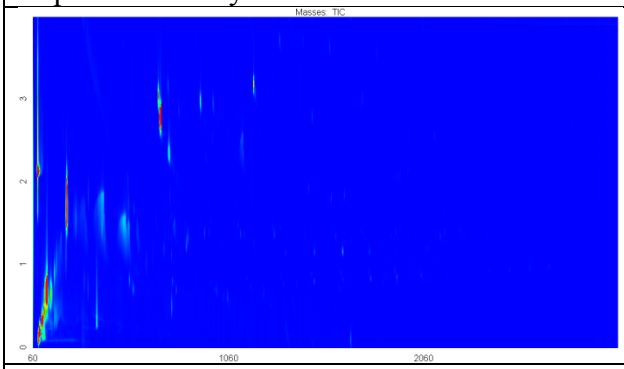
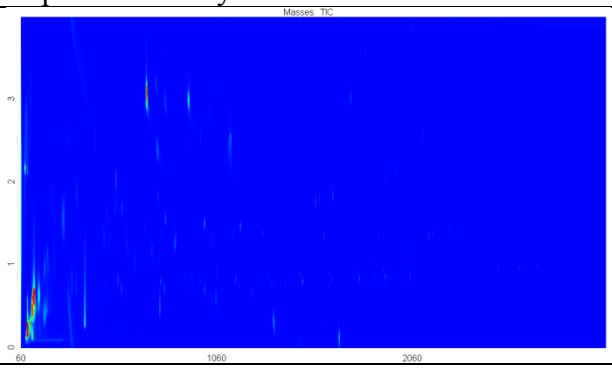
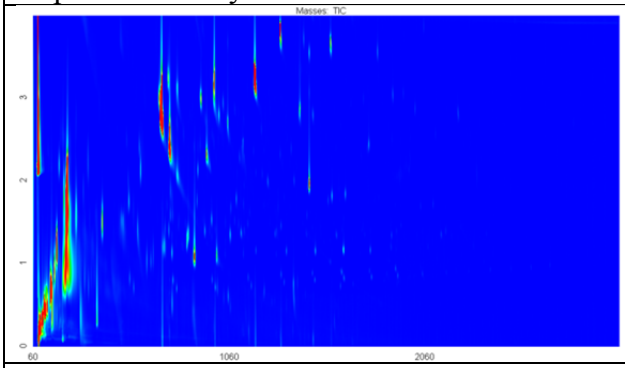
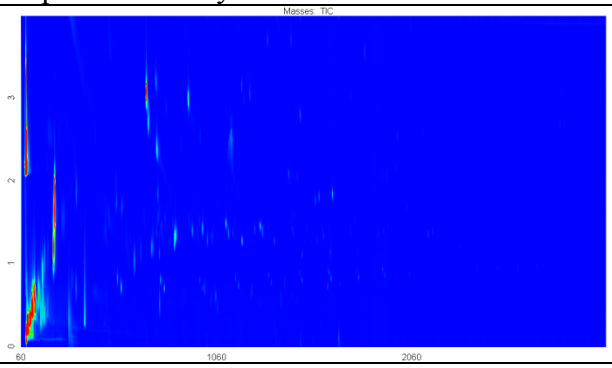
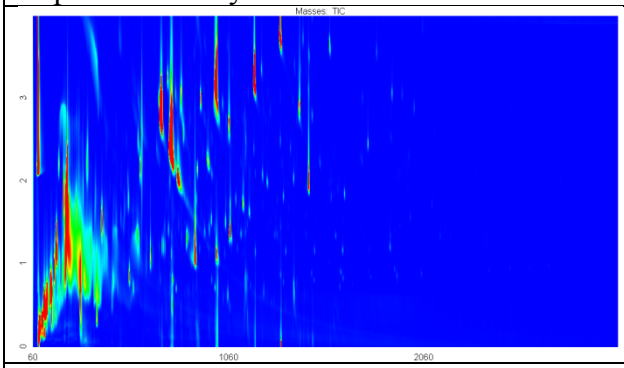
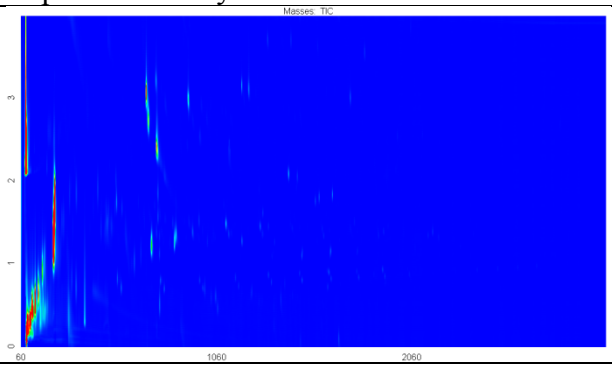
Pig carcass (P2)	Insect inclusion remains (H3)
Experimental day 3	Experimental day 3
	
Experimental day 4	Experimental day 4
	
Experimental day 5	Experimental day 5
	

Figure 4.10: Two dimensional chromatograms of the pig carcass (P2) and insect inclusion remains (H3) from experimental days 3,4 and 5 of the human remains trial 3.

4.6 *Discussion*

4.6.1 *VOCs from Carcass Decomposition*

The application of TD-GC×GC-TOFMS to the analysis of decomposition odour provided a distinct advantage in the non-target analysis of these complex samples. The additional peak capacity, sophisticated mass spectral deconvolution software and data comparison tools allowed for the identification of over 300 decomposition specific compounds. This allowed for the characterization of the decomposition process and the more detailed profile facilitated comparisons between varying environments.

One class of compounds not evident within the 1D analysis of decomposition odour was the alcohols. Using TD-GC×GC-TOFMS this class of compounds was shown to be a major contributor to the characterisation of the bloat and active decay stages. The alcohols likely originate from a variety of sources including the bacterial degradation and fermentation of amino acids, fatty acids and carbohydrates (Boumba et al., 2008; Paczkowski and Schutz 2011). The shift in the predominant alcohols from the shorter chain alcohols such as 1-propanol and 1-butanol to the longer chain alcohols such as 1-octen-3-ol could be explained by the change in the availability of oxygen within the remains and the resultant bacterial metabolism (Janaway et al., 2009). The metabolism of threonine by *Clostridium sp.*, an anaerobic organism, produces 1-propanol (Boumba et al., 2008) whereas the oxidation of fatty acids such as linoleic acid by fungi can produce 1-octen-3-ol (Combet et al., 2006; Paczkowski and Schutz 2011). The aldehydes and ketones are also believed to result from aerobic degradation of fatty acids (Dent et al., 2004; Janaway et al., 2009; Paczkowski and Schutz 2011). These oxygen containing compounds identified with TD-GC×GC-TOFMS have also been identified as components of decomposition VOCs profile within other studies (Statheropoulos et al., 2005[b;] Statheropoulos et al., 2007; Hoffman et al., 2009; Statheropoulos et al., 2011; DeGreeff and Furton 2011[b;] Dekeirsschieter et al., 2012).

The aromatics were also a predominant class identified with TD-GC×GC-TOFMS. The benzoic compounds such as benzonitrile and benzaldehyde were identified whereas these were not seen using 1D GC-MS. Swann et al. 2010[a,c] identified selected biogenic amines

and amino acids within purged decomposition fluid over the active and early advanced stages of decay (Swann et al., 2010[a]; Swann et al., 2010[c]). The aromatic compounds identified in the current study were predominant across the same stages (ADD 107.2 – 260.9), as shown in Figure 4.6a. It is likely that these compounds are released from the carcass in the fluid matrix and, upon release into the environment, volatilize into components of the decomposition headspace.

Predominant carboxylic acids identified during this study were two to six carbons in length, and are also known as volatile fatty acids (VFAs). In particular, acetic acid, 3-methyl and 2-methyl butanoic acid, and hexanoic acid were major components of the carboxylic acid profile. Decomposition research has identified VFAs, including those listed above, within decomposition fluid-soil solutions (Vass et al., 1992; Swann et al., 2010[a]; Swann et al., 2010[b]; Swann et al., 2010[d]) and within decomposition headspace (Dekeirsschieter et al., 2009; Hoffman et al., 2009; Statheropoulos et al., 2011; DeGreeff and Furton 2011[b]; Dekeirsschieter et al., 2012). Swann et al.(2010[b]) identified the temporal trends of VFAs within decomposition fluid from pig carcasses during the summer months in Southern Ontario, Canada. The authors identified a cyclic trend with two maxima, the first and most abundant occurring at ADD 126.0 and the second upward trend occurring at ADD 310.0 (Swann et al., 2010[b]). A similar temporal trend was also observed during the current study with a maximum at ADD 150.2 and a second peak at ADD 260.9 (Figure 4.6b). The formation of VFAs from amino acids and/or carbohydrate degradations is an anaerobic process, facilitated by the carcass's intrinsic bacteria (Vass et al., 1992; Boumba et al., 2008; Paczkowski and Schutz 2011). However, the second peak of VFAs occurred around the same time as some aerobic compounds (e.g. 1-octen-3-ol and 3-octanone), illustrating that the shift from a predominately anaerobic system to an aerobic one is not discrete but a gradual transition.

A recent study by Dekeirsschieter et al. (2012) investigated VOCs from pig carcass surface decomposition. Despite the decomposition occurring in different environments they reported a similar VOC profile to this study. In both studies, the predominant compound classes included alcohols, carboxylic acids, aromatics and sulfides along with aldehydes and ketones as major contributors to the overall profile. Additionally, the

major compounds from within these classes were common to both studies e.g. 1-butanol, 2-and 3-methyl butanoic acid, trimethylamine, 2-octanone, 1H-indole, DMDS and DMTS. Notably, Dekeirsschieter et al. (2012) conducted their study within a forested environment in Belgium during the spring season (March-May). However, the current study took place in an open field in Southern Ontario, Canada during the summer months (July-August). Despite differences in location (Walloon Brabant, Belgium vs. Southern Ontario, Canada), decomposition environment (forest vs. open field), and average temperature during the study (13.1 °C vs. 21.1 °C), the analysis of decomposition headspace via GC×GC-TOFMS demonstrated a consistent VOC profile for pig carcass decomposition. This suggests that although the decomposition environment clearly impacts the process of decomposition and subsequently the VOC profile, (Mann et al., 1990; Dekeirsschieter et al., 2009; Paczkowski and Schutz 2011) a consistent profile of decomposition VOCs can be identified across different geographical locations when utilizing the advanced capabilities of GC×GC-TOFMS.

4.6.2 *Decomposition of Human Remains*

The limited decomposition observed during the human remains trials likely resulted from the short time frame of each study as well as the combination of various intrinsic and extrinsic factors. The short time frame of the human remains trials was governed by access to the Forensic Anthropology Research Facility (FARF) which was set out in the awarded grant proposal (Hamilton et al., 2010). Other factors in these studies that could have negatively influenced the rate of decomposition include body type (i.e. obesity and emaciation), ante-mortem treatments (i.e. antibiotics and chemotherapy), potential storage conditions of remains (i.e. refrigeration or frozen) and cool environmental temperatures. The storage conditions of the remains from the time of death to arrival at the FARF are unclear. Observations provided by the field researchers indicate that the remains may have been frozen prior to refrigeration at approximately 4 °C at the FARF. Cold temperatures (<4 °C) hinder the decomposition process by inhibiting or killing the bacteria present (Micozzi 1986; Vass et al., 1992; Janaway et al., 2009). Without the activity of the internal gut flora to drive the initial putrefaction, the processes of decomposition are slowed (Micozzi 1986). Remains that are frozen may undergo more

degradation or aerobic decomposition as the remains get colonized by insects and microbial flora from the surrounding environment (Micozzi 1986). This type of decomposition is in contrast to the anaerobic putrefactive decay that is initiated internally and progresses outward (Micozzi 1986). Another factor that would have contributed to a lower bacterial load within the body are the medical treatments given to individuals prior to death, such as chemotherapy and antibiotics (Bengmark 2012). Similar observations of delayed decomposition were observed at the same research facility, following storage of donated remains in a refrigerated environment (Parks 2011).

4.6.3 *Decomposition VOCs from the Early Post-Mortem Period*

The lack of soft tissue decomposition during these trials was evident in chemical profiles of the decomposition headspace. The production of decomposition VOCs is associated with the putrefactive breakdown of soft tissue. Putrefactive changes including decomposition odour are commonly first observed during the bloat stage (Anderson and VanLaerhoven 1996). In the human remains trials 1 and 3 the remains did not reach the bloat stage and therefore it is likely that only minimal putrefaction had occurred and therefore minimal changes in the decomposition headspace were observed.

The human remains trial 2 took place in May 2012 and during this trial the remains were exposed to higher temperatures (average 27.5°C). These higher temperatures likely facilitated the decomposition observed in this trial. However the presence of insects was also a factor in the progression of decomposition as evidenced by the lack of decomposition observed in the insect exclusion remains (H4). The onset of decomposition of the pig carcass and insect inclusion human remains was characterized by higher levels of alcohols and sulfides. Both their profiles were dominated by compounds from a variety of chemical classes such as 2-methyl and 3-methyl 1-butanol, 2-pentanol, phenol, phenol-4-methyl, acetic acid, 3-methyl butanoic acid, acetamide, trimethylamine and the polysulfides (DMDS, DMTS, DMQS). These compounds have been reported during the onset of decomposition and throughout the later stages (Statheropoulos et al., 2007; Dekeirsschieter et al., 2009; Statheropoulos et al., 2011; Dekeirsschieter et al., 2012). During this study the appearance of these compounds within the VOCs profile is consistent with the decomposition observed during this trial.

The first appearance of the polysulfides occurred on experimental day 2 for the pig carcass and on experimental day 3 for the insect inclusion remains (H3). This coincides with the onset of the bloat stage in the pig carcass and the localized decomposition of the face and neck of H3. The identification of these compounds during this period further supports the use of these compounds as markers for early decomposition (Statheropoulos et al., 2011).

When the VOC profile of the pig carcass and insect inclusion remains are compared for a single experimental day, there appears to be differences in the overall composition of the profile. The VOC profile of the insect inclusion remains appears to lag behind the pig; however a delay in the onset of putrefaction may have been caused by the different storage conditions of the remains prior to deposition at the research site. The pig was killed and then stored on ice prior to placement at the field site, whereas the human remains were refrigerated and potentially frozen for days. The elongated time in the cold conditions may have further reduced the bacterial flora within the human remains compared to the pig carcass, resulting in a delayed onset of putrefaction within the human remains.

The objective of this study was to compare the decomposition VOCs of pig carcasses and human remains in order to determine if pig carcasses could function as a human decomposition odour analogue. Due to the limitations in the duration of the study and factors contributing to the delay in decomposition only minimal information was obtained. However the profiles from the pig carcass and human remains generated from the onset of decomposition during the May 2012 study were similar. The PCA indicated that there was greater variation between the insect inclusion (H3) and insect exclusion (H4) remains than between H3 and the pig carcass. Further similarities in the decomposition odour of pig carcasses and human remains are exemplified by studies that have used pig carcasses as human body analogues to examine decomposition VOCs across the decomposition process.

The trials conducted at the UOIT-GRF during this study as well as other published studies (Dekeirsschieter et al., 2009; Statheropoulos et al., 2011; Brasseur et al., 2012; Dekeirsschieter et al., 2012) have used pig carcasses as human body analogues in order

to study volatiles from decomposition. In contrast others have published results obtained from human remains (Vass et al., 2004; Statheropoulos et al., 2005[a]; Statheropoulos et al., 2007; Vass et al., 2008; Hoffman et al., 2009; DeGreeff and Furton 2011[b]). Although pig carcasses are commonly used as human body analogues within decomposition chemistry and entomology research (Catts and Goff 1992; Anderson and VanLaerhoven 1996; Dent et al., 2004; Schoenly et al., 2006; Statheropoulos et al., 2011) their use as human decomposition odour analogues has been questioned (Cablk et al., 2012). The primary argument against utilizing pig carcasses as human decomposition odour mimics is that their profiles show few similarities to those produced by human remains (Cablk et al., 2012). Nevertheless, the variability of the decomposition VOC profiles found in the literature is high (Paczkowski and Schutz 2011) and few compounds have been consistently identified in all studies, with dimethyl disulfide being the most reported. A recent study (Cablk et al., 2012) compared volatiles from animal tissues and indicated that the volatile profile of pig tissue was the "least similar" to that of the human profile. However, a number of the compounds reported by Cablk et al. (Cablk et al., 2012) as missing from the pig profile have been recorded in this and other studies that analyzed the decomposition headspace of pig carcasses (Dekeirsschieter et al., 2009; Statheropoulos et al., 2011; Dekeirsschieter et al., 2012). Some of these same compounds which were found in the decomposition headspace of pig carcasses were additionally reported as "unique compounds not shared by any animal species" (Cablk et al., 2012) including propanoic acid, pentanoic acid, hexanoic acid, and cyclohexanone. The similarities in the decomposition VOCs of pig carcasses and human remains exhibited herein demonstrate that pig carcasses should not be dismissed as human odour analogues and that this alternative odour mimic warrants further investigation. The current variability in VOC profiles makes comparisons between individuals and between species difficult, however further investigation with high powered instrumentation such as TD-GC×GC-TOFMS will allow for more detailed comparisons and facilitate the characterization of the fundamental decomposition odour profile.

4.6.4 *Application of Decomposition VOCs*

In the United States a recent trial brought this area of research to the forefront. Decomposition odour was presented as evidence for the prosecution in the State of Florida v. Casey Marie Anthony (case no. 48-2008-CF-15606-O). During the trial the admissibility of the evidence was questioned and the defense filed a motion to exclude unreliable evidence. Specifically the defence sought to exclude "…any testimony or evidence concerning any alleged identification of the chemical composition of human decomposition odour…" (Perry 2011). As GC-MS is an accepted methodology the issue of admissibility centered on the interpretation of the results and whether the compounds identified by GC-MS could be said to result from a human decomposition event. The expert witness for the prosecution argued that the compounds identified, particularly carbon tetrachloride, were "consistent with an early decomposition event of human origin" (Perry 2011). However the expert for the defense stated that the "odour signatures of human decomposition were not generally accepted in the scientific community and [that] there are no scientifically valid methods capable of identifying the presence of human remains" (Perry 2011). Ultimately the judge determined that this disagreement in opinions was to be left to the trier of fact, the jury, to resolve and allowed the evidence to be presented in court. This case brought the question of a human decomposition odour signature to the forefront and indicates the need for further research to determine its composition and evaluate its uniqueness.

Currently, scent detection canines are the best detectors for human remains. Cadaver dogs are reported to differentiate human from animal remains and locate human remains in a variety of conditions including submerged, buried and those with extended PMIs (Komar 1999; Lasseter et al., 2003; Lorenzo et al., 2003; Oesterhelweg et al., 2008). However it remains unclear how these canines recognize a target odour.

Canines are capable of chromatographic separation of odorants within their nasal cavity (Lawson et al., 2012). Within the olfactory epithelium there are two types of olfactory receptor neurons (ORNs). Class I are sensitive to soluble or hydrophilic odorants and are found at the entrance to the olfactory structure within the nasal cavity (Lawson et al., 2012). Class II ORNs are sensitive to the more insoluble/hydrophobic odorants and are

distributed across the olfactory surfaces (Lawson et al., 2012). This distribution of odorant receptors allows for the separation of compounds and might facilitate odour recognition. In addition to mechanisms of odour recognition it is unclear to which components of a target odour the canines are responsive.

Olfaction requires that the compounds be present in the gas phase and if the compounds are not volatile enough, as is the case with components of plastic explosives, they will not be part of the odorants recognized (Moore et al., 2011). Conversely, compounds that are highly soluble may become absorbed upon entering the nasal cavity before reaching the olfactory epithelium and the Class I ORNs (Lawson et al., 2012). Research on the odour signatures of explosives have indicated that canines may be responding to the most abundant compounds in an odour profile (i.e. TNT) as opposed to other components of the explosive such as plasticizers (Johnston 1999; Lorenzo et al., 2003). Currently there is no consensus on what constitutes the human decomposition odour signature and few compounds have been consistently detected within the published literature. Current training aids for cadaver dogs, including natural scent sources, only represent a portion of the VOCs produced during decomposition (Hoffman et al., 2009; DeGreeff et al., 2012). Similarly, this study in conjunction with the published literature, has shown that the decomposition of pig carcasses also produces a number of compounds in common with human remains (Dekeirsschieter et al., 2009; Statheropoulos et al., 2011; Dekeirsschieter et al., 2012; Stadler et al., 2013) and could be used as supplements to current training regimes. Recognition of complicated target odours such as decomposition may require a variety of compounds in addition to the dominant ones. Characterization of decomposition odour utilising techniques such as TD-GC×GC-TOFMS will facilitate the development of a comprehensive profile and the identification of additional compounds that may be required for canine odour recognition.

Chapter 5: *Analysis of Synthetic Canine Training Aids*

Artificial scents are commonly employed for the training of cadaver dogs, however the composition of these solutions is not available and it is unclear if they are representative of decomposition odour. One set of commercially available synthetic training aids from Sigma-Aldrich® were analysed using GC×GC-TOFMS and the results were published in the Journal of Chromatography A, 1255 (2012) 202-206 (Stadler et al., 2012). In this study, the sample introduction was carried out via liquid injection onto the GC×GC-TOFMS system. This method was utilized for the investigation in order to minimize any modifications to its unknown composition.

5.1 *Compound Identification*

Both formulations within the Sigma Pseudo™ Corpse scent kit were analysed; *Sigma Pseudo™ Corpse Scent Formulation I- for early detection or below 0°C* and *Sigma Pseudo™ Corpse Scent Formulation II- for post putrefactive detection*. Two major components were identified in formulation I; 2-pyrrolidone and 4-aminobutanoic acid (GABA). In addition to the above compounds, 1,4-diaminobutane (putrescine) and 1,5-diaminopentane (cadaverine) were identified as major components of formulation II. A few additional compounds were found within the two pseudo scents at lower amounts; DBU, butyrolactone and tridecylamine (Table 5.1). These seven compounds were selected for confirmatory identification with standards due to their abundance, occurrence in replicate injections and strong library matches. Standards for GABA, putrescine, cadaverine, DBU, butyrolactone and tridecylamine were analysed. The first and second dimension retention times (^1t_R and ^2t_R), first dimension retention indices (^{1}DRI) and mass spectra of the standards were compared to the pseudo scents and provided confirmatory identifications for the seven compounds. Retention indices were calculated using the following formula and are shown in Table 5.1.

Table 5.1: Identification of compounds within pseudo scent formulations I and II (Sigma Pseudo™ Corpse Scent kit from Sigma Aldrich®). Match factors are obtained from the library search engine to the NIST and Wiley mass spectral libraries. Retention time deviations were obtained from triplicate injections. First dimension retention indices were calculated from n-alkane retention times.

Compound Name	Chemical Formula	Cas #	Library Match Factors			Retention Times						Retention Index (^{1}DRI) (n-alkanes)
			Forward	Reverse	Probability	Average (sec)		SD (sec)		RSD (%)		
						^1t_R	^2t_R	^1t_R	^2t_R	^1t_R	^2t_R	
Formulation I												
2-pyrrolidone	C_4H_7NO	616-45-5	964	964	9537	889	3.47	6	0.02	0.7	0.5	1099
4-aminobutanoic acid	$C_4H_9NO_2$	56-12-2	857	869	4700	1945	1.32	7	0.01	0.4	0.8	1807
Butyrolactone	$C_4H_6O_2$	96-48-0	972	972	7993	555	2.52	2	0.07	0.3	2.8	913
3-methyl pyridine	C_6H_7N	108-99-6	944	944	7756	481	2.03	4	0.25	0.7	12.3	866
DBU	$C_9H_{16}N_2$	6674-22-2	722	722	4884	1475	2.46	2	0.02	0.1	0.6	1456
Tridecanamine	$C_{13}H_{29}N$	2869-34-3	893	975	2779	1662	1.43	286	0.11	17.0	7.3	1587
Formulation II												
2-pyrrolidone	C_4H_7NO	616-45-5	952	952	9746	858	3.43	20	0.17	2.3	5.0	1080
4-aminobutanoic acid	$C_4H_9NO_2$	56-12-2	829	839	2809	1956	1.30	12	0.00	0.6	0.3	1821
1,4-diaminobutane	$C_4H_{12}N_2$	110-60-1	934	947	9391	508	1.70	3	0.01	0.5	0.4	882
1,5-diaminopentane	$C_5H_{14}N_2$	462-94-2	858	883	6998	688	1.75	3	0.01	0.4	0.6	986
Butyrolactone	$C_4H_6O_2$	96-48-0	972	972	9024	554	2.49	3	0.02	0.5	0.8	911
3-methyl pyridine	C_6H_7N	108-99-6	931	931	7898	486	1.84	0	0.02	0.0	1.0	867
DBU	$C_9H_{16}N_2$	6674-22-2	714	714	5038	1476	2.47	0	0.00	0.0	0.2	1456
Tridecanamine	$C_{13}H_{29}N$	2869-34-3	907	968	3200	1668	1.32	312	0.24	19.0	17.9	1591

$$^{1}DRI(target) = 100\left[\left(\frac{1tR(s) - 1tR(n)}{1tR(n+1) - 1tR(n)}\right) + n\right]$$

Where $^{1}t_R(s)$ = retention time of target,

$^{1}t_R(n)$ = retention time of alkane preceding target,

$^{1}t_R(n+1)$ = retention time of alkane after target,

n = number of carbon atoms of preceding alkane.

During the preparation and analysis of the standards, the solubility of GABA posed a problem. As an alternative the headspace of GABA was sampled to attain $^{1}t_R(s)$ for comparison. During headspace analysis of the pure GABA standard, large amounts of 2-pyrrolidone appeared in the chromatogram. It was hypothesized that the cyclization of GABA to produce 2-pyrrolidone was occurring under the high temperatures used for desorption and injection. It was found that at temperatures above 250 °C, GABA undergoes cyclization without a catalyst to produce high yields of 2-pyrrolidone (Lammens et al., 2010). However Lammens et al., (2010) did not test the cyclization of GABA at temperatures below 250 °C. In order to investigate this, direct thermal desorption of the pure GABA standard was carried out at temperatures of 120 °C; the injector inlet was also kept at 120 °C. At these lower temperatures, GABA became more evident in the chromatograms, indicating the thermal lability of GABA and its possible conversion to 2-pyrrolidone.

Despite the simplicity of the mixtures, GC×GC-TOFMS was originally used due to the expected complexity of the training aid solutions as decomposition odour is known to be complex with potentially hundreds of compounds being present within the headspace of decomposition (Vass et al., 2008; Statheropoulos et al., 2011; Dekeirsschieter et al., 2012). However, GC×GC produced two retention times ($^{1}t_R$ and $^{2}t_R$) for each compound which aided in their identification. The identification of compounds was further confirmed using high resolution time-of-flight mass spectrometry (HRTOFMS) (Stadler et al., 2012).

5.2 Discussion

The use of synthetic training aids is highly debated. Canines that have been trained with pseudo scents are able to locate human remains. However canines that are trained on the authentic samples using natural scent sources do not identify or alert on pseudo scents. As a result, some agencies and certification programs will not use synthetic odorants (Koenig 2001). This has also been the case with other Sigma Pseudo products for the training of drug detection dogs (Macias et al., 2008), indicating a disconnect between the two types of training aids.

The marketing of these products as a synthetic mimic of decomposition odour suitable for the training of cadaver dogs is questionable, as it does not appear to be representative of decomposition odour. The current literature on decomposition VOCs including our own studies has identified tens to hundreds of compounds (Vass et al., 2004; Statheropoulos et al., 2005[a;] Statheropoulos et al., 2007; Vass et al., 2008; Dekeirsschieter et al., 2009; Hoffman et al., 2009; Statheropoulos et al., 2011; Dekeirsschieter et al., 2012; Stadler et al., 2013), whereas this investigation identified only seven compounds between two formulations. Although it is possible that cadaver dogs only rely on a few chemicals to identify remains, it is not known currently which chemicals elicit a response in cadaver dogs. Therefore, this oversimplification of decomposition odour could cause poor odour imprinting during training (Rebmann et al., 2000; Macias et al., 2008; Oesterhelweg et al., 2008; Hoffman et al., 2009).

The purpose of a scent mimic is to produce an odour profile that is comparable to the parent source (Moore et al., 2011), and Sigma Pseudo Corpse Scent is supposed to be representative of decomposition odour (Koenig 2001). However none of the major components of decomposition odour were identified within the product, e.g. poly-sulfides. It is recognized that scent detection canines may not respond to the major compounds of an odour source but to the minor components present within the headspace (Macias et al., 2008). Regardless the compounds identified within this product do not appear to be major or minor components in decomposition odour. The seven compounds found in these products have not been reported as key odorants of decomposition nor have they been identified within the headspace of decomposition (Vass et al., 2004;

Statheropoulos et al., 2005[a;] Statheropoulos et al., 2007; Vass et al., 2008; Dekeirsschieter et al., 2009; Hoffman et al., 2009; Statheropoulos et al., 2011; Dekeirsschieter et al., 2012).

The diamines, 1,4-diaminobutane (putrescine) and 1,5-diaminopentane (cadaverine) are believed to be common components of artificial decomposition scents (DeGreeff et al., 2012). These compounds result from the putrefactive degradation of the amino acids ornithine and lysine, respectively (Gill-King 1997). Putrescine and cadaverine were thought to be representative of decomposition odour and the key odorant for the detection of remains by cadaver dogs (Gill-King 1997), but these compounds have not been detected within the headspace of decomposition (Vass et al., 2004; Statheropoulos et al., 2005[a;] Statheropoulos et al., 2007; Vass et al., 2008; Dekeirsschieter et al., 2009; Hoffman et al., 2009; Statheropoulos et al., 2011; Dekeirsschieter et al., 2012). 4-Aminobutanoic acid (GABA) is a neurotransmitter that is synthesized in the brain and is not generally known as a decomposition product. Vass et al., (2002) used GABA as a potential biomarker for post-mortem interval estimation by extracting it from cadaveric tissues following derivatization (Vass et al., 2002). Despite being associated with decomposition material, the absence of these compounds within the vapour phase required for olfaction indicates that they may not be suitable target odorants for scent detection canines (Furton and Myers 2001).

The Scientific Working Group for Dog and Orthogonal Detectors (SWGDOG) has outlined research areas that are of critical importance to the improvement of canine scent detection. A primary research interest is the identification of target odorants for HRD canines as well as investigation and development of canine training aids (SWGDOG 2010; DeGreeff et al., 2012). In order to address the need for improved training aids, DeGreeff et al. (2012) collected decomposition VOCs from human remains onto sorbent pads. The VOCs contained on the sorbent pads, as analysed by SPME-GC-MS, represented a subset of VOCs found within decomposition odour and showed potential as a HRD training aid (DeGreeff et al., 2012). Although this training aid reduces the biohazard risk and storage complications of other natural scent sources, it still requires access to human remains for its fabrication. SWGDOG has outlined that HRD canines

should be trained on the complete spectrum of odorants from all the decomposition stages and undergo training in a variety of different circumstances and environments (SWGDOG 2009; SWGDOG 2010). Canines presented with new or unfamiliar scenarios do not perform as well as canines who have been previously been exposed to similar situations during training (Komar 1999; Lasseter et al., 2003; DeGreeff et al., 2012). Therefore it is essential that the range of odorants presented to canines as well as a variety of possible search scenarios be incorporated into ongoing or maintenance training (Komar 1999; SWGDOG 2009; DeGreeff et al., 2012).

It is unlikely that the range of odorants required for ongoing training could be consistently collected from human remains. Similar to other natural scent sources, pig carcass decomposition odour is a subset of the complete human decomposition odour profile and utilization of pig carcasses to supplement canine training would be beneficial. These human analogues can be decomposed to a variety of end-points and can be placed in a number of situations and environments that would provide HRD canines with the holistic training they require for efficient searches.

Chapter 6: *Conclusion*

A comprehensive profile of decomposition odour is required in order to better understand how cadaver dogs are able to locate human remains. This study was able to gain an understanding of the VOCs from the decomposition of soft tissue by utilizing thermal desorption and conventional gas chromatography – mass spectrometry to analyse the headspace of pig carcasses. The microcosm study allowed for a preliminary investigation into decomposition odour in the absence of environmental VOCs. Ten compounds, mainly polysulfides and aromatics, were identified as components of decomposition odour within this controlled environment. Although full trends could not be observed due to the restricted decomposition within the microcosm, the dynamic nature of decomposition odour was evident.

Further examination of decomposition odour using TD-GC-MS was carried out by analysing the headspace of pig carcasses during surface decomposition within Oshawa, Ontario, Canada from three trials replicated over three years. The decomposition of soft tissue by the putrefactive breakdown of macromolecules produced a number of compounds from a variety of chemical classes namely, dimethyl disulfide, dimethyl trisulfide, dimethyl tetrasulfide, phenol, phenol-4-methyl, indole, hexanal, heptanal and octanal. Overall the greatest variety of compounds was evident during the active decay stage when the majority of soft tissue is degraded. However trends in the appearance of these compounds were observed; the sulfides were predominant during the earlier bloat stage, the aromatics were dominant during the active and advanced stages and the aldehydes represented a larger portion of decomposition odour during the later stages of advanced decay. These compounds were consistently detected in the three trials, however the exact origin of the compounds is unclear and variations in the environmental variables and the insect and microbial populations can alter the relative trends of these compounds. In order to achieve a better understanding of decomposition odour a detailed non-target analysis of decomposition VOCs was required.

The decomposition headspace of pig carcasses was analysed using thermal desorption and comprehensive two dimensional gas chromatography – time-of-flight mass spectrometry. This sophisticated instrumentation along with its data handling software provided the chromatographic and mass spectral resolution required for the

comprehensive identification of the chemical components of decomposition odour. By utilizing this novel technique, the complete process of decomposition was characterized in terms of the decomposition VOCs. The key chemical families, alcohols, sulfides, aromatics, and carboxylic acids characterized the continuous process of decomposition and the dominant compounds (e.g., 1-butanol, 2-and 3-methyl butanoic acid, DMDS, DMTS, phenol, and indole) were identified as potential target odorants of decomposition. In addition to these major chemical classes the aldehydes, ketones and nitrogen compounds further characterized the complete profile. GC×GC-TOFMS not only facilitated the identification of compounds within these complex samples but also generated consistent profiles of decomposition odour produced from distinct geographical locations. It is recommended that this methodology be applied to chemically profiling decomposition scent in different geographical locations and decomposition environments. This will facilitate a better understanding of the complete VOC profile and will assist in identifying the key compounds of decomposition odour.

Current training aids for cadaver dogs include natural scent sources and artificial scent sources such as pseudo scents. Due to the use of pig carcasses as human body analogues it has been proposed that they could function as human decomposition odour analogues. To date no study has conducted a direct comparison of the decomposition headspace of both pig carcass and human remains in the same environment. This study conducted a comparison of decomposition odour in three human remains trials in San Marcos, Texas, United States. However due to the short duration of the studies and the minimal soft tissue decomposition observed only initial comparisons can be made. This investigation into the decomposition VOCs between pig carcasses and human remains along with comparisons of the VOC profile generated by GC×GC-TOFMS to the literature has shown that these human analogues cannot be excluded as potential decomposition odour mimics.

In order to determine the odour signature of a commercially available synthetic canine training aid, the chemical composition of a Sigma Aldrich® product was analysed using GC×GC-TOFMS. The two pseudo scent products appear to be oversimplifications of decomposition odour and do not contain compounds that have been previously reported

within the headspace of decomposition. Furthermore, the comparison of pseudo scents and pig carcasses to human decomposition odour has shown that the VOC profile of pig carcass decomposition is a better representation of this complex target odour. There is a need for improved canine training aids that are capable of presenting the complete spectrum of odorants from all decomposition stages and can be utilized in a variety of different circumstances and environments. Pig carcasses fulfill these requirements and their use as an alternative canine training aid warrants further investigation.

6.1 *Future Considerations*

In order to determine the components of human decomposition odour, extensive research into the chemical composition of decomposition headspace needs to be conducted utilizing instrumentation capable of the non-target analysis of complex samples, such as TD-GC×GC-TOFMS. The non-target analysis of decomposition VOCs is essential to the identification of the core compounds of decomposition odour. A major challenge in the analysis of decomposition odour is the separation of environmental VOCs from those resulting from decomposition. It would be beneficial if future studies could incorporate control samples from not only the environmental air but also from surrounding soil and vegetation. By first identifying all chemical components within samples the resulting profile can be compared and the key differences can be identified. However this may be a challenge as the software required for the background subtraction of such complex samples is not readily available.

Non-target analysis will not only allow for the elimination of environmental VOCs, but will also facilitate the comparison of VOC profiles from different experimental treatments. Investigations into the decomposition odour from a variety of geographical locations and decomposition environments will facilitate the development of a comprehensive odour signature. Decomposition is a highly variable process that is influenced by a number of factors such as temperature, availability of oxygen, contact with soil and the presence of insects. As all these factors can affect how soft tissue is degraded, their influence on the production of decomposition VOCs needs to be investigated. The effect of insect larvae on the production and release of decomposition VOCs requires further investigation as it is unclear how the insects and their excretions

may alter the overall profile. Additionally the role of the microbial community in soft tissue decomposition requires further investigation. These organisms are a driving force in decomposition, however it remains unclear which organisms are involved in putrefaction and how they produce the various by-products of decomposition including VOCs. Studies isolating these variables and identifying the resultant changes in the VOCs produced would provide an expanded decomposition odour profile that would provide valuable information about this complex target odour and advance our knowledge of decomposition chemistry.

The characterization of decomposition odour along with current training aids is essential to understanding how cadaver dogs recognize this target odour. The potential for pig carcasses to function as an alternative canine training aid requires further examination. A major limitation in this area is the difficulties associated with accessing human remains however, research trials examining the whole process of decomposition of both pig carcasses and human remains within the same environment are essential in order to address this question. For these trials to be a success they must be conducted over extended PMIs and consideration into the storage of remains and the environmental variables affecting decomposition must be taken into account. As an alternative, the use of individual tissue types of both pig carcasses and human remains as training aids could be investigated. In addition to the analysis of soft tissue decomposition, research into skeletonised remains as well as potential scent reservoirs such as decomposition soil is required. Few studies have investigated these sources of decomposition odour; however cadaver dogs are commonly utilized after extended PMIs when soft tissue no longer remains. In order to ensure efficient training for these types of scenarios, characterization of the decomposition VOCs from these scent sources is required.

This comprehensive analysis of decomposition odour should be focused on the development of holistic training aids that are safe, accessible and provide an accurate representation of the target odour. In order for potential training aids to be successfully implemented, behavioural studies utilizing scent canines are required. These behavioural studies will aid in the identification of the key compounds required for scent recognition as well as elucidate the olfactory capabilities of cadaver dogs.

Chapter 7: *References*

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Chapter 8: *Appendices*

Appendix A: Select chemical structures of decomposition VOCs

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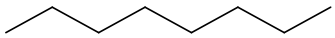
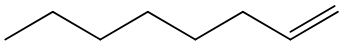
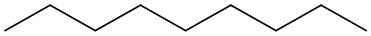
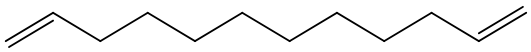
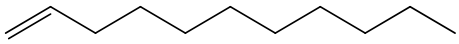
 Octane	 1-Octene	 Nonane	
 1,11-Dodecadiene	 1-Undecene		
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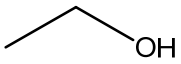
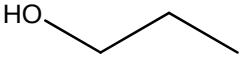
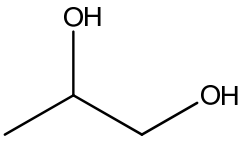
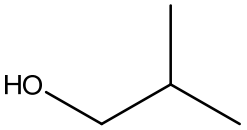
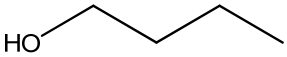
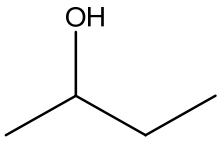
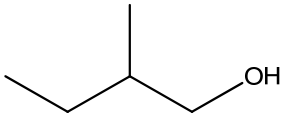
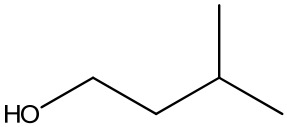
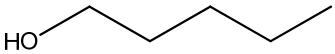
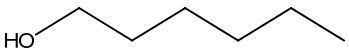
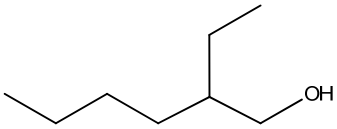
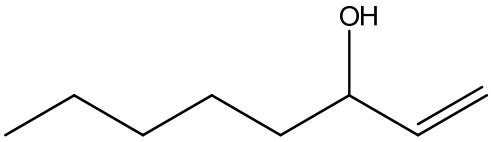
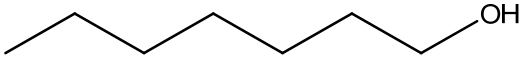
 Ethanol	 1-Propanol	 Propane-1,2-diol	 2-Methyl-propan-1-ol
 1-Butanol	 2-Butanol	 2-Methyl-butan-1-ol	 3-Methyl-butan-1-ol
 1-Pentanol	 1-Hexanol	 2-Ethyl-hexan-1-ol	
 1-Octen-3-ol	 1-Heptanol		
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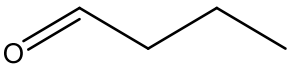
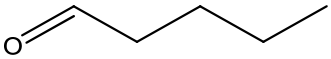
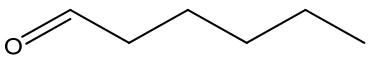
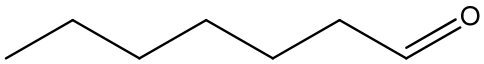
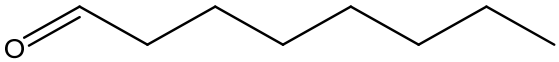
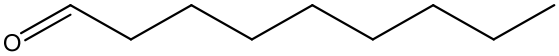
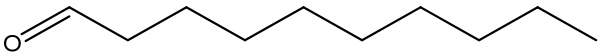
 Butanal	 Pentanal	 Hexanal	

 Heptanal	 Octanal
 Nonanal	 Decanal
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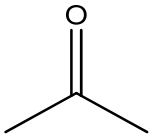
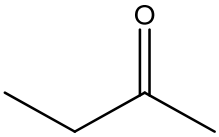
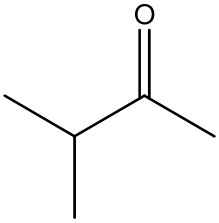
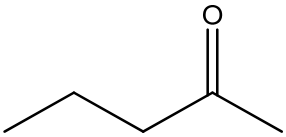
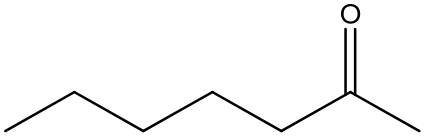
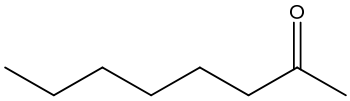
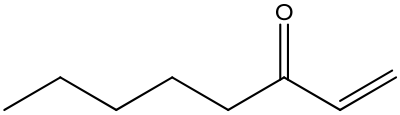
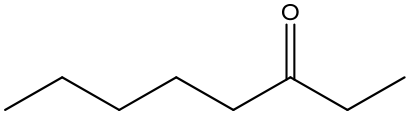
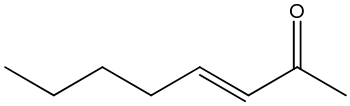
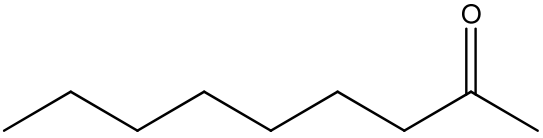
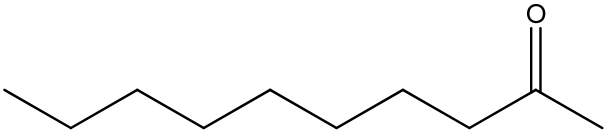
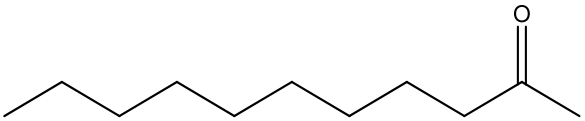
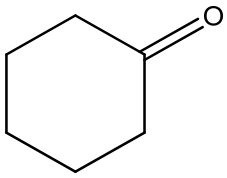
 Acetone	 2-Butanone
 2-Butanone,3-methyl	
 2-Pentanone	 2-Heptanone
 2-Octanone	
 1-Octen-3-one	 3-Octanone
 3-Octen-2-one	
 2-Nonanone	 2-Decanone

 2-Undecanone	 Cyclohexanone	
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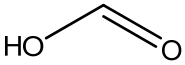
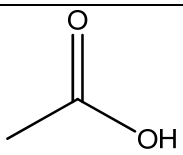
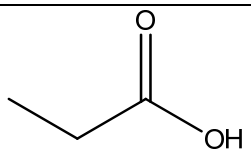
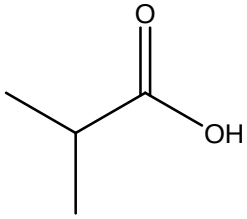
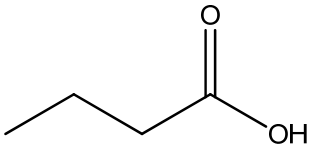
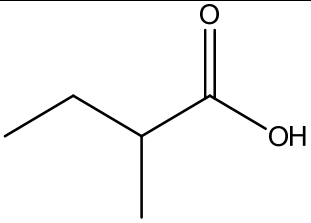
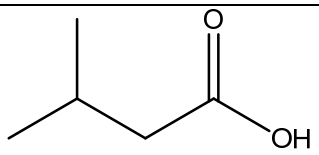
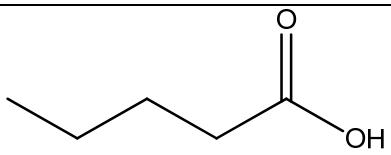
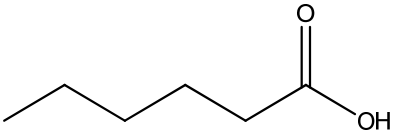
 Formic acid	 Acetic acid	 Propanoic acid
 Propanoic acid, 2-methyl	 Butanoic acid	 Butanoic acid,2-methyl
 Butanoic acid,3-methyl	 Pentanoic acid	 Hexanoic acid
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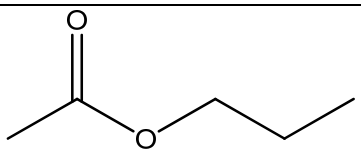
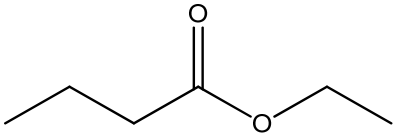
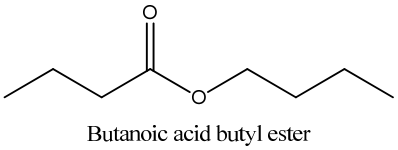
 Acetic acid propyl ester	 Butanoic acid ethyl ester	 Butanoic acid butyl ester
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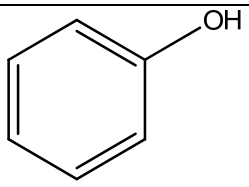
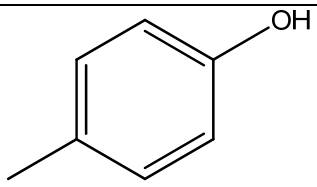
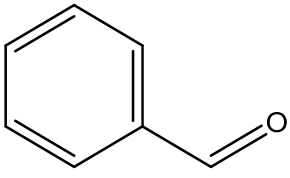
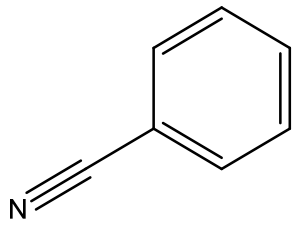
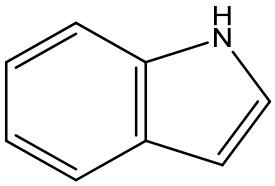
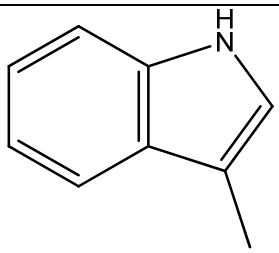
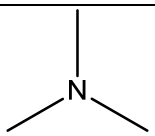
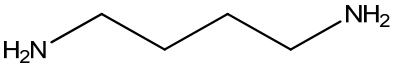
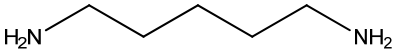
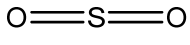
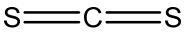
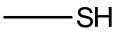
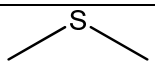
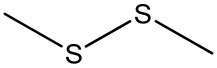
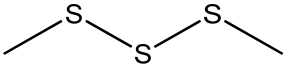
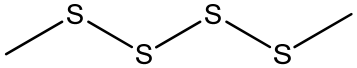
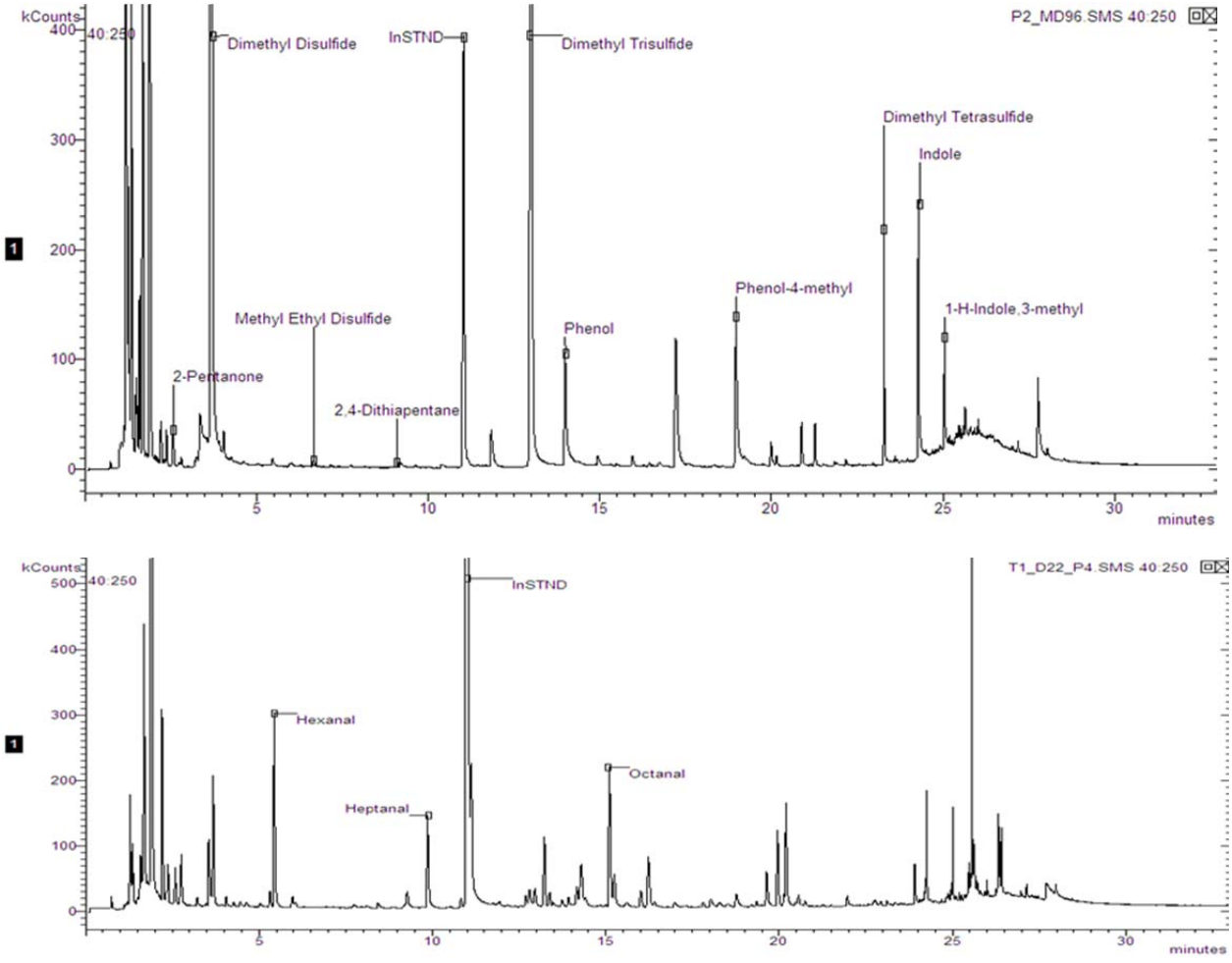
 Phenol	 4-Methylphenol	 Benzaldehyde

 Benzonitrile	 Indole	 3-methyl-1*H*-indole
Nitrogen		
 Trimethylamine	 Putrescine	 Cadaverine
Sulfides		
 Sulfur dioxide	 Carbon disulfide	 Methanethiol	 Dimethyl sulfide
 Dimethyl disulfide	 Dimethyl trisulfide	 Dimethyl tetrasulfide

Appendix B: The experimental day and accumulated degree days (ADD) for the onset and endpoint of the decomposition stages for the microcosm trial and outdoor Trials 1, 2 and 4.

		Fresh		Bloat		Active Decay		Advanced Decay		Dry Remains	
		Onset	End Point	Onset	End Point	Onset	End Point	Onset	End Point	Onset	End Point
Microcosm	Experimental Day	0	3	3	26	26	Study End	-	-	-	-
	Accumulated Degree Days (ADD)	0	59.10	59.10	500.02	500.02	Study End	-	-	-	-
Trial 1	Experimental Day	0	2	2	6	6	11	11	22	22	Study End
	Accumulated Degree Days (ADD)	0	35.93	35.93	98.07	98.07	179.52	179.52	395.05	395.05	Study End
Trial 2	Experimental Day	0	2	2	6	6	8	8	34	34	Study End
	Accumulated Degree Days (ADD)	0	29.02	29.02	101.31	101.31	145.83	145.83	658.10	658.10	Study End
Trial 4	Experimental Day	0	2	2	10	10	14	14	17	17	Study End
	Accumulated Degree Days (ADD)	0	29.35	29.35	157.35	157.35	237.70	237.70	297.50	297.50	Study End

Appendix C: Sample chromatogram of compounds identified during the microcosm trial and outdoor Trials 1, 2 and 4 using TD-GC-MS.



Appendix D: Percent composition of each chemical class for each experimental day in Trial 1, 2 and 4

Trial 1						
Experimental Day	ADD	Decomposition Stage	% Polysufdies	% Aromatics	% Aldehydes	% Ketone
0	0.00	Fresh	0.46	75.18	23.20	1.16
2	35.94	Bloat	90.08	5.59	0.84	3.49
4	69.46	Bloat	97.66	1.82	0.28	0.24
6	98.07	Active	93.92	5.58	0.15	0.36
8	126.26	Active	36.56	61.38	0.07	1.99
11	179.52	Advanced	40.83	52.42	0.57	6.18
14	233.83	Advanced	29.15	53.20	7.37	10.28
22	395.06	Dry	33.88	32.85	16.96	16.31
26	471.37	Dry	89.07	0.00	9.02	1.91
34	626.27	Dry	88.29	0.00	11.02	0.70
41	787.61	Dry	82.41	0.00	15.28	2.31
48	947.49	Dry	45.46	0.00	39.87	14.67
55	1099.99	Dry	65.48	0.00	23.99	10.53

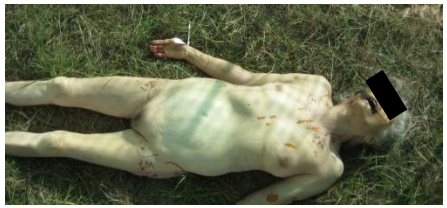
Trial 2						
Experimental Day	ADD	Decomposition Stage	% Polysufdies	% Aromatics	% Aldehydes	% Ketone
0	0.00	Fresh	0.00	99.55	0.45	0.00
2	29.02	Bloat	39.26	53.77	2.63	4.33
4	59.42	Bloat	94.18	5.49	0.00	0.33
6	101.31	Active	85.66	12.59	0.03	1.72
8	145.84	Advanced	34.52	63.42	0.07	1.98
11	195.58	Advanced	85.31	12.17	0.88	1.64
14	248.55	Advanced	78.36	21.64	0.00	0.00
17	312.26	Advanced	58.30	15.09	21.34	5.26
20	371.42	Advanced	50.31	1.04	43.40	5.25
27	513.83	Advanced	11.58	58.19	30.10	0.13
34	658.10	Dry	32.34	0.00	58.98	8.69
41	796.21	Dry	6.68	1.59	86.73	5.00
48	991.68	Dry	3.57	0.00	96.43	0.00
69	1474.56	Dry	67.24	0.00	20.65	12.11

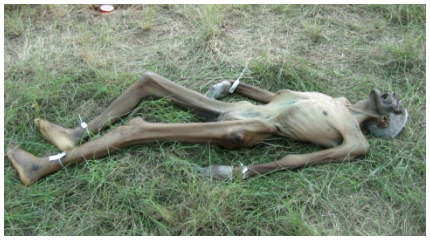
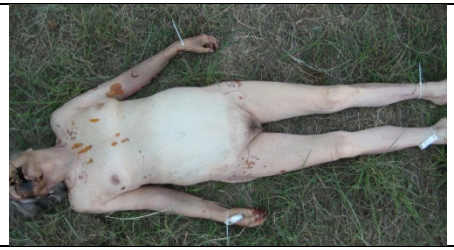
Trial 4						
Experimental Day	ADD	Decomposition Stage	% Polysufdies	% Aromatics	% Aldehydes	% Ketone
0	0.00	Fresh	0.00	0.00	0.00	0.00
2	29.35	Early Bloat	51.13	0.00	0.00	48.87
4	55.61	Bloat	99.14	0.36	0.50	0.00
6	83.87	Full Bloat	99.40	0.30	0.11	0.19
8	118.34	Post Bloat	98.80	0.48	0.00	0.72
10	157.35	Active	55.70	43.29	0.06	0.95
12	202.27	Active	64.73	34.18	0.06	1.03
14	237.70	Advanced	70.74	21.47	5.83	1.96
17	297.50	Dry	68.04	5.45	16.71	9.80
20	364.75	Dry	88.15	0.57	9.97	1.31
27	508.17	Dry	93.97	0.00	6.03	0.00
34	657.56	Dry	44.35	0.00	55.65	0.00
41	812.83	Dry	27.28	0.00	72.72	0.00
48	979.05	Dry	28.26	0.00	71.74	0.00
55	1136.35	Dry	9.32	0.00	88.60	2.08

Appendix E: The experimental day and accumulated degree days (ADD) for the onset and endpoint of the decomposition stages for outdoor Trial 3.

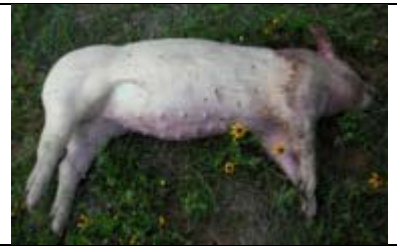
		Fresh		Bloat		Active Decay		Advanced Decay		Dry Remains	
		Onset	End Point	Onset	End Point	Onset	End Point	Onset	End Point	Onset	End Point
Trial 3	Experimental Day	0	2	2	4	4	11	11	40	40	Study End
	Accumulated Degree Days (ADD)	0	54.96	54.96	107.22	107.22	260.98	260.98	845.71	845.71	Study End

Appendix F: Photographs of decomposition observed during human remains trial 1 (November 2011) at the Forensic Anthropology Research Facility in San Marcos, Texas, United States.

Experimental Day / ADD	H1 (Insect Inclusion)		H2 (Insect Exclusion)		P1
D0			No Photo Available		
D1					No Photo Available
D2					

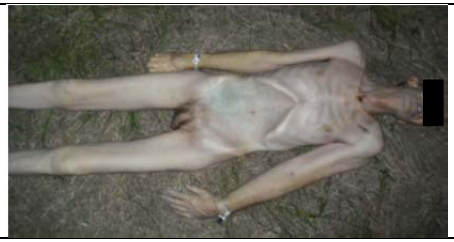
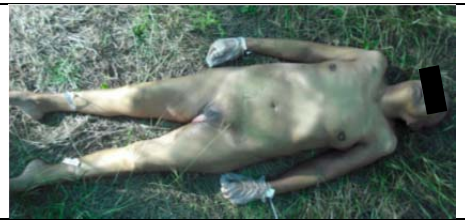
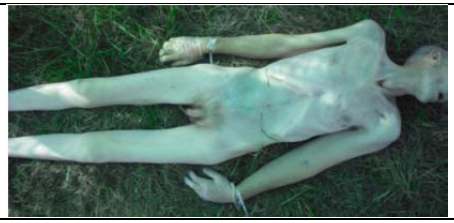
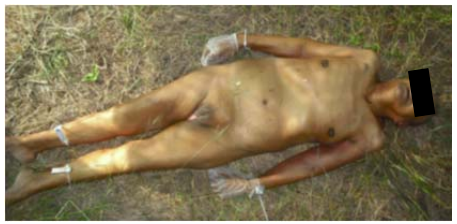
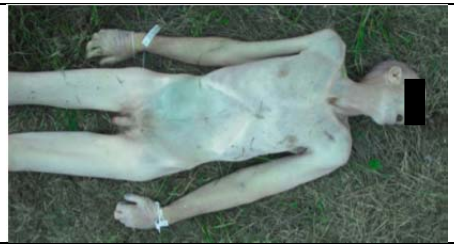
D3						

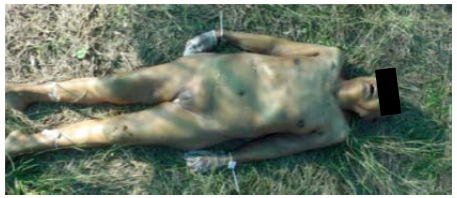
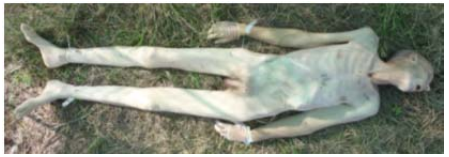
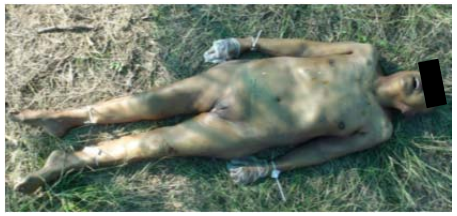
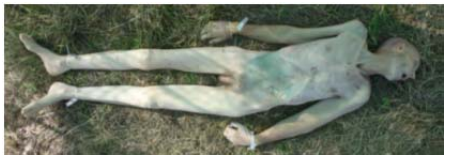
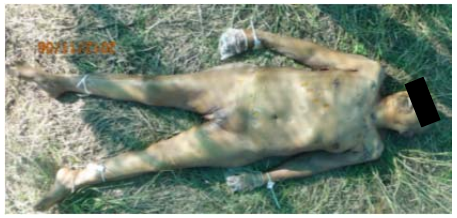
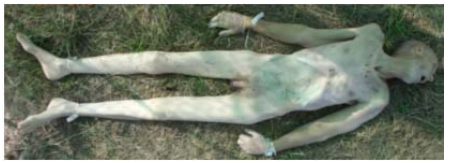
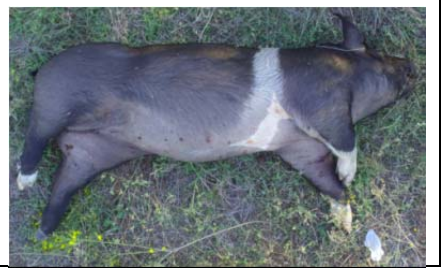
Appendix G: Photographs of decomposition observed during human remains trial 2 (May 2012) at the Forensic Anthropology Research Facility in San Marcos, Texas, United States

Experimental Day / ADD	H3 (Insect Inclusion)		H4 (Insect Exclusion)		P2
D0					
D1					
D2					

D3					
D4					Missing Photo
D5					

Appendix H: Photographs of decomposition observed during human remains trial 3 (November 2012) at the Forensic Anthropology Research Facility in San Marcos, Texas, United States. (*) photo not taken at the time of sampling, but on the night of that sampling day

Experimental Day / ADD	H5 (Insect Inclusion)		H6 (Insect Exclusion)		P3
D0					
D1					
D2					

D3					
D4					* 
D5					* 

Appendix I: Principal Component Loadings (coefficients) from the PCAs conducted on data sets from the outdoor Trial 3 and human remains trials 1, 2 and 3. The values indicate the relationship between the new principal component and the original variable.

	Trial 3: Pig 1		Trial 3: Pig 2		Human remains		Human remains		Human remains trial	
	Axis 1	Axis 2	Axis 1	Axis 2	Axis 1	Axis 2	Axis 1	Axis 2	Axis 1	Axis 2
Alcohol	-0.0587	-0.5082	0.0220	0.4647	0.1662	-0.1351	-0.2006	-0.3050	-0.0960	-0.5704
Aldehyde	0.0060	0.0571	0.0243	-0.0867	0.1325	0.0044	-0.0077	-0.0021	-0.0133	0.0391
Aromatic	0.1180	0.3974	0.4728	-0.1348	0.6589	-0.1763	-0.0907	0.4621	0.0400	0.7828
Carboxylic Acid	0.5148	0.6015	0.8719	-0.0461	N/A	N/A	0.8986	-0.2649	0.8571	-0.1216
Ester	-0.0012	-0.0097	-0.0003	0.0054	-0.2418	-0.3634	-0.0463	0.1442	-0.0052	-0.0127
Halogen	0.0004	0.0067	0.0026	-0.0184	0.1744	0.1347	0.0114	0.0533	-0.0628	-0.1105
HC	0.0048	-0.0030	0.0072	0.0186	0.0462	0.8088	-0.0252	0.1867	-0.1340	0.1556
Ketone	0.0010	0.0933	0.0308	-0.1358	0.1258	0.0527	-0.0434	0.2268	-0.0490	-0.0700
Nitrogen	0.0205	-0.0430	0.0567	0.2489	0.3874	0.1475	-0.1034	-0.0292	-0.0294	-0.0254
Sulfide	0.8469	-0.4557	0.1046	0.8213	0.2812	0.1789	-0.3567	-0.6838	-0.0295	-0.0081
Other	0.0012	0.0174	0.0035	-0.0248	0.4273	-0.2965	-0.0360	0.2119	-0.4778	-0.0588