Development of Standard Operating Procedures for the detection of Volatile Organic Compounds associated with the decomposition process

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Abstract

Decomposing meat produces a characteristic profile of Volatile Organic Compounds (VOCs) that changes as a function of time. This has been considered by the forensic community as a possible means of estimating Post Mortem Interval (PMI) and thus could be of importance in criminal investigations.

Previous work has focussed on obtaining VOC profiles for the decomposition stage and the key chemicals identified include alcohols, aldehydes, acids and sulphur compounds including Dimethyl disulphide (DMDS) and Dimethyl trisulphide (DMTS). The type and levels of these VOCs change throughout the decomposition of mammalian cadavers and are of considerable interest in the forensic community in a range of areas including 'time profiling' as a means of estimating PMI, locating clandestine graves and to assist in the training of Victim Recovery (VR) dogs. VOCs can accumulate in the air surrounding a cadaver (the 'headspace') and the current research focussed on methods to collect, detect and identify these.

This research was carried out using Thermal Desorption Gas Chromatography Mass Spectrometry (TD-GC-MS) and Headspace Gas Chromatography Mass Spectrometry (HS-GC-MS). TD-GC-MS uses an adsorbent tube and pump system to concentrate a sample and HS-GC-MS uses a sealed vial system to introduce and analyse solid or liquid without the requirement for extensive sample preparation. GC-MS was used for this study because of its ability to combine separation and identification, which is particularly appropriate for complex matrices, such as those seen in the decomposition processes.

A combination of pure chemicals and pork were used to develop methodology for TD-GC-MS and HS-GC-MS where the sampling parameters (such as mass of meat, size on container, presence of insects, type of substrate) were investigated in detail. No Certified Reference Material (CRM) was available so a standard mix of chemicals was devised based on a literature review and the results of preliminary experiments. For pork studies, it was found that optimum results were obtained using 500 g pork samples placed on sand as a substrate and contained within clip lock plastic boxes. The results confirmed those of other workers that the accessibility of insects to the meat plays an essential role for a realistic decomposition profile.

A key aim of this project was the development of Standard Operating Procedures (SOPs) for the detection of VOCs produced in the headspace above a cadaver during its decomposition. SOPs are a way of reproducing methodology of an, often complex, but routine activity. The SOPs were developed as an iterative process throughout the project and then further tested and validated using repeatability studies and intra-laboratory evaluation. Validation studies involved repeatability studies by the author and two competent analysts. It was shown that the SOPs fulfilled their basic requirement in terms of ease of use and suitability for detecting VOCs associated with decomposition.

The robustness of these SOPs was successfully demonstrated using chemically similar systems in the phyto-kingdom. The robustness was evaluated by applying them, after some adaptation to the sample collection procedure, to plants that mimic decomposition by producing chemicals that attract insects. The presence of DMDS and DMTS was detected in plants known to attract blowflies which are of key entomological importance in decomposition events. The research was extended to carnivorous plants particularly the lifecycle of *Sarracenia flava*, which has not been previously studied in this way. In addition to the work on plants, a short study on a commercial odour reduction system was undertaken which further confirmed the scope of the SOPs. The SOPs were found to be applicable to these systems and the robustness was shown by being able to withstand minor modifications while still being fit for purpose.

Overall, limitations were seen in terms of repeatability for the technique and due to the complexity of the decomposition matrix. However, the SOPs showed great promise to be used as a starting point for forensic practitioners to test their own methodology and instrumentation for decomposition studies.

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1. INTRODUCTION

1.1 Forensic taphonomy and project context

Taphonomy is the study of how organisms decay. Forensic taphonomy specifically focusses on the post-mortem fate of human remains including the biological and chemical changes that are linked to decomposition.[1, 2 3] It impacts on a variety of fields ranging from anthropology to entomology [4, 5] and the training of victim recovery (VR) dogs.[6-8 9] However, a particular application is the estimation of the post-mortem interval (PMI) which is often crucial in investigations of suspicious deaths.[10, 11] Although some taphonomic research is observational, e.g. monitoring the changes in skin colour during decomposition, there is increased use of analytical techniques to monitor chemical changes.[12-20]

Of growing interest is *thanatochemistry* or the chemistry of death, [21] specifically the detection, identification and quantification of volatile organic chemicals (VOCs) whose nature and concentration are known to alter throughout the various stages of decomposition. [12, 14, 22 23-25 18, 26-28 19, 29] This 'time profiling' of decomposition VOCs offers not only a means of estimating PMI but also a possible method for locating clandestine graves that would not involve the complexity of dog training. [30]

Gas chromatography - mass spectrometry (GC-MS) with its ability to separate and identify components in complex mixtures is used for decomposition VOC analysis, especially when coupled with a vapour capture technique such as thermal desorption (TD) or headspace (HS). [12-15, 18, 22, 23, 26, 27, 29, 31, 32 6, 19, 20, 25, 33-35] However, there seems to be little in the literature on a more fundamental (rather than case based) study of the reliability of these techniques for this type of work, or a standardised methodology. It is this gap that this project seeks to address.

1.2 Stages of decomposition

Human decomposition begins almost immediately after death with characteristic changes.[36 37] Decomposition can be divided into various stages with different categories used by different researchers according to their particular area of interest. [36, 38-41] For the purposes of this study, the decomposition process can be simply divided into four main stages: *Early, Active, Advanced* and *Skeletonisation*.

Early decomposition starts as soon as cells become deprived of oxygen when the heart stops beating. During this stage the main post-mortem indicators are observed: *livor mortis* (where blood pools under gravity to the lowest part of the body), *rigor mortis* (where muscles become stiff) and *algor mortis* (the general reduction in body temperature).[41]

Active decay is associated with the production of most putrefactive odour and includes bloating and subsequent purging of gases from the body tissues which leads onto the stage of *advanced decomposition.*[42] It is these 2 stages that are mainly of interest for the study of decomposition VOCs.

Finally, during *skeletonisation* all remaining soft tissue is removed from bone

Most early research in this area focussed on *early* and *active decay*. However, more recently there has been interest in studying the chemicals evolved throughout the whole decomposition process, with a greater focus on later stages. [12-15, 17-19, 22, 26-28, 30, 33, 39, 41, 43-46] It has even been reported that it is possible to identify the chemicals given off during *skeletonisation* even in the absence of soft tissue. [10, 20]

The goal of this work was to be able to link the abundance profile of certain key VOCs to their time profiling. Their associated abundance profile over time has great potential to link to the decomposition stage and ultimately allow some estimation of time since death.

1.3 Decomposition and the forensic importance of PMI

Estimation of the post-mortem interval (PMI) is of forensic importance as time since death can allow the link to a suspect using a certain timeline. The estimation of time since death in the early stages of decomposition (i.e. 24-48 hours) is relatively well established but it is much more difficult to estimate PMI if a body is in later stages of decay.[11]

Forensic investigators have used many different approaches in an attempt to more accurately determine PMI during the later stages of decomposition. These have included the study of the body itself, the environment surrounding the cadaver (e.g. soil) [43-45, 47]and the entomological approach [48], where the lifecycle of the fly is an established means of PMI determination. [49] However, a less well established but interesting area of investigation is the study of VOCs surrounding the cadaver as decomposition occurs.

During decomposition the breakdown of cells in the body leads to tissue disintegration. In the early stages this is aided by bacteria and fungi inside the body while in later stages the action of bacteria, fungi and insects exterior to the body become more important.[11, 41, 43, 47, 50-52] Biological macromolecules in the body (e.g. carbohydrates, proteins, lipids and nucleic acids) breakdown into simpler molecules, which give off the distinctive foul odour of rotting flesh.[44]

Different tissues at different stages of decay produce distinctive chemical compounds which can also be affected by environmental, soil, bacterial and entomological factors. [12, 36, 38-40, 44, 46, 53, 54] Some of these compounds have relatively low boiling points and have a measurable volatility under ambient conditions. Previous work suggests that the identification of these VOCs evolved by human remains (or pigs as human models) could aid forensic science in understanding decomposition for applications such as victim recovery (VR) canines, etiology of death and potentially PMI estimation. [12-14, 17, 19, 22, 23, 26-28, 44] Although there is considerable interest in investigating chemical and biological variables and techniques based on monitoring the decomposition process itself under different physical conditions,[11-13, 22, 36, 39-41, 55] for the purposes of PMI determination, there is little established, standardised methodology which this project hopes to try and develop.

1.4 The biochemistry of decomposition and the origin of VOCs

Following death, the body undergoes autolysis and cell death. The general order in which the body decomposes has been classified by Gill-King [2] as follows: -

- **Digestive organs and heart and circulatory muscle.** Due to the digestive enzymes already present these organs show rapid deterioration.
- **Air passages and lungs.**
- **Kidneys and bladder.**
- **Brain and nervous tissue.** Due to their high metabolic activity, neurons are one of the first to undergo cell breakdown (autolyse) following death
- **Skeletal muscles.**
- **Connective tissue and skin.** Protein collagen is difficult to hydrolyse and thus these tend to be the last to decompose

As the body reaches the end of autolysis an anaerobic environment is created which favours growth of both intestinal and soil bacteria. Those that act on the body break down carbohydrate, protein and lipids into their constituent parts as well as gases, acids leading to colour changes, bloating and intense odour in a process known as *putrefaction.* [2] Different gases and VOCs are given off depending on the putrefactive bacteria present. [44]

1.4.1 Decomposition products of carbohydrates

Bacterial fermentation of carbohydrates, which occurs primarily during early decomposition, gives rise mainly to gases (methane, hydrogen, carbon dioxide and hydrogen sulphide).[10, 30] This involves the breakdown of complex polysaccharides into sugars which sometimes involves complete oxidation into CO_2 and water. If incompletely decomposed by enteric flora e.g. by *Clostridium spp.* in anaerobic conditions it gives rise to organic acids and alcohols, resulting in an acid environment. [10, 30] There are different bacterial processes involved depending on the depth of soil and therefore different levels of oxygen [44]. Ethanol is produced by microorganisms during this decomposition process. [12, 31]

1.4.2 Decomposition products of proteins

Bacteria are capable of the breakdown of proteins into their constituent amino acids with the release of exoenzymes. [2] This proteolysis is very dependent on environmental conditions particularly temperature and moisture as well as this bacterial action. [44]

Protein breakdown by bacteria is responsible for the predominance of the offensive odour associated with decomposition [28] when they are converted into VOCs via their amino acid constituents. Ammonia and amines can both be products of amino acid breakdown where ammonia is liberated from the nitrogen in amino acids. [44] Amines of particular note are cadaverine and putrescine which are produced by the decarboxylation of lysine and orthinine respectively. [2, 28, 56] Other amino acid reactions include: - deamination of tyrosine by anaerobic bacteria to yield phenyl acetic and propionic

acids; phenylalanine gives phenyl acetic acid (oxidation), phenylpropionic acid (reduction), benzoic acid and phenyl pyruvic acids; leucine also breaks down into VOCs of interest. [2, 15]

Volatile sulphur compounds are also associated with putrefaction and early/active decay. [27] The anaerobic conditions of body decomposition favours sulphide production due to the action of bacteria e.g. desulfhydralation on the sulphide containing amino acids (cysteine, cysteine and methionine). [44] Of particular note is DMDS which is produced abundantly in early decomposition from the breakdown of methionine. [28, 44] Its low odour threshold means it is associated with offensive odour of decomposition.

Other offensive odour molecules associated with protein breakdown are thiols, mercaptens and phenolic compounds such as indole and skatole [28] [44] but what VOCs are actually evolved depends very heavily on the state of the body, environmental conditions (particularly the levels of available oxygen) and what bacteria are present. [1, 4, 38, 44, 46, 54, 55, 57]

1.4.3 Decomposition products of fat

Fat is contained in adipose tissue which is made up of lipids, triglycerides and some proteins. Triglycerides are molecules of glycerol with 3 fatty acids attached and thus their hydrolysis yields fatty acids which can undergo subsequent oxidation or hydrogenation. [44] Following the ethanol production of fermentation, these fatty acids can react with sodium or potassium giving rise to adipocere ('grave wax') through a process called *saponification* ('soap production').[2, 17, 23, 33, 44, 45, 57-60] Adiopocere mainly comprises saturated fatty acids (myristic, palmitic and stearic acids) with varying levels of unsaturated fatty acids (oleic, palmitoleic) plus other minor components. These can result in Fatty Acid Methyl Esters (FAMEs). [17, 30]

Adipocere is particularly affected by environmental conditions including pH and soil type and tends to occur more in anaerobic conditions. Adipocere can take a long time (sometimes up to years) to fully break down.[17]

High concentrations of unsaturated fatty acids allow fat hydrolysis to produce aldehydes and ketones. [44] Volatile fatty acids (VFAs), which are carboxylic acids with low molecular weight (C2-C7) come from aerobic degradation in early decomposition. Below are relevant examples from Swann *et al* (2010) of VFAs that also have amino acid sources: -

- Acetic acid from alanine, glycine and serine
- Propionic acid from lysine and threonine
- Butanoic acid from alanine, glutamic acid and aspartic acid [15]

Fat breakdown is a complex process with multiple pathways involved depending on conditions, again, particularly the level of oxygen.

1.4.4 Decomposition products of bones

This stage does not yield many VOCs although some researchers have investigated chemical detection from dry remains.[12]

1.5 VOCs produced during decomposition

In the transition from early to active decay, *bloating* is usually seen and this sub-stage has been linked mainly to the formation of alcohols (butan-1-ol), sulphur compounds (including DMDS), and nitrogen compounds (including trimethylamine).[19]

Active decay gives the strongest olfactory signature, and many chemicals reach levels where they can be detected using analytical instrumentation.[33] The literature has a wide range suggested which includes cyclic compounds (indole, phenol, and 4-methyl phenol), sulphur compounds (DMDS and dimethyl trisulphide-DMTS) and organic acids (butanoic, 2- and 3- methylbutanoic acid). During advanced decay the relative proportion of aldehydes increases. [12, 19]

One VOC of particular note is DMDS (mentioned earlier) which is produced from the breakdown of proteins, specifically the amino acids cysteine, cystine and methionine, which are sulphur containing and undergo microbial desulfhydralation and degradation to produce DMDS and other sulphur containing species.[12, 18, 19, 25, 26, 28, 44, 61, 62]

The number and types of VOCs evolved at different stages are very complex and depend on a great number of variables including the tissue broken down at that stage and the microorganisms present both internally and externally.[33] As well as decomposition stage, tissue type e.g. meat, skin, fat etc., can have an impact on VOCs evolved and therefore detected as this will determine which body component is present for breakdown.[13]

1.5.1 VOC detection and odour threshold

Odour threshold is defined as the lowest concentration of a certain odour compound that is perceivable by the human sense of smell. The more odourous a compound the lower the threshold and thus the lower the concentration at which it can be smelt. Often more offensive smells can have a much lower odour threshold because this is a useful protective mechanism for danger detection in animals. [63] As a VOC may be smelt by the human (or canine) nose but not be detected by instrumentation there is a need to consider the important differences between olfaction in animals and the Limit of Detection (LOD) of instrumentation. Very early decay can often yield insufficient VOCs to be detectable by current instrumentation [19] even when smell of decay would be apparent to most people. For example, DMDS has an odour threshold of as low as 0.0012 ppm [63] which is below the LOD estimated for the instrumentation in this study.

1.8 Standard Operating Procedures (SOPs)

Standard Operating Procedures (SOPs) are used as a way to allow reproduction of a method of analysis. They are used to complete an often complex, but routine, activity. In essence they are step-by-step instructions, somewhat like a recipe, that allow analysts in different laboratory settings to produce consistent and comparable data. This is particularly important for auditing, accreditation bodies (such as UKAS) and for the production of and adherence to standards e.g. ISO 9001. As such, SOPs often form part of a Quality Management system, particularly for use within the context of an accredited laboratory setting. Within a SOP you may also find a Standard Operating Instruction (SOI) for the use of analytical instrumentation and laboratory equipment.[64-66]

Many early papers investigating decomposition are presented in the style of a case study based on an actual forensic investigation (e.g. collecting the VOCs evolved from bodies washed up on a beach [28]) and as such have very little context. Being 'one-offs', the repeatability and reproducibility of the measurements are unknown. There have been reviews that have compared and contrasted between authors' methodologies and more recently there has been a move towards attempts at repeats. However, these tend to still be in the style of that particular author with little consistency between papers.[23]

All experimental scientific papers (and research theses) should allow the reader to be able to repeat the experiments they describe with minimal referencing elsewhere. As such they can, at least to a certain extent, themselves fulfil some of the functions of an SOP. However, the aim of the current work was to develop more formal SOPs than that usual to a thesis, with the broader aim of enabling the scientific and forensic community to have consistent analysis of VOCs given off during the decomposition process.

1.7 Previous work

Previous work in the literature includes making attempts at VOCs databases, most notably from Vass and co-workers, and their use for different comparisons of conditions. It is the combination of the literature and preliminary experiments that has helped the rationale of choice of decomposition chemical standards and the instrumentation used in the current study.

Dent *et al.* (2004) reviewed previous research on human decomposition processes in soil providing detail of different stages of decay and the associated chemicals of breakdown. It was noted that it was important to take into account soil and environmental factors in addition to those of bacterial and physicochemistry as well as the body itself when investigating in-soil (i.e. in-grave) decomposition processes.[44]

Hoffman *et al.* (2008) attempted to characterise the VOCs present in the headspace of decomposing human remains. Using Solid Phase Micro Extraction (SPME) and GC-MS they identified 33 VOCs associated with the decomposition process. They also suggested that, although there is much shared

between them, there are some VOCs specifically associated with different body tissue types. This may be useful for determining decay stages as it is known that different tissues breakdown at different stages (i.e. Dent *et al.,* 2004).[33]

Researchers had previously relied on VR canines for chemical detection strategies. However, Hoffman *et al.* (2008) and others have shown there is potential that these VOCs could be more scientifically identified and profiled for such work.

Vass *et al.* (2004) conducted a detailed study of the VOCs and semi-volatiles that migrate upwards from buried human remains using triple sorbent traps (TSTs). This was in an attempt to identify and profile these compounds over time in order to create a decompositional odour analysis (DOA) database. The work was undertaken over a relatively long time period and identified many compounds associated with decomposition. The authors suggest that fingerprinting the chemicals around the human body during decay was of great potential forensic importance.[14]

Statheropoulos *et al.* (2005) used TD-GC-MS to study the VOCs evolved from a decaying human body. They were able to identify 80 compounds and indeed go some way towards their quantification. However, this was done at a single time point of the decay process and as decay is a dynamic process they identified that the study would be better as a function of time.[32] The current study aims to address these limitations.

Statheropoulos *et al.* (2007) continued their previous work using TD-GC-MS but at different time intervals during the early stages of decay where they measured the VOCs accumulated from human decomposition over time in order to get a profile. They reported a common core of compounds that potentially could be used as chemical detection signals in the search for human remains.[26]

Dekeirsschieter *et al.* (2009) studied cadaveric VOCs released by decaying pig carcasses in 3 different environments over time using passive diffusion into a sorbent tube followed by thermal desorption into a GC. Despite differences between the three environments, they found a common core of 35 VOCs with similarities to the smell signature of human decomposition in terms of chemicals released. They surmised that the VOCs released during decomposition attract insects as well as potentially VR canines and that a better understanding of the olfactive profile of human decomposition would be useful for various forensic applications.[19] This and other work strongly suggested the use of TD-GC-MS for this current work.

Vass (2012) The most recent VOCs database work from Vass has much relevant information on chemicals evolved during decomposition. This key paper includes information on: -

- Chemicals specific to decomposition stages. It was found that during early decomposition there were fluorinated halogen compounds, substituted benzene compounds, and some specific sulphur compounds (e.g SO_2) and then as time progresses substituted benzenes become simpler. More complex sulphur compounds as well as aldehydes and ketones are detected. As the late phase approaches there is a noticeable increase in aldehydes (butanal, decanal, heptanal, nonanal, octanal), which supports the work mentioned earlier by Dekeirsschieter *et*

al.[19] Straight chain hydrocarbons (alkanes) also become more prevalent (hexane, decane, nonane, octane) as well as furans. Xylenes were only seen in this study in the dry stages of decomposition.

- Chemicals that could possibly be used as markers of decomposition events. Xylenes, 2-butanone, acetone, DMDS, hexane, pentane, toluene, chloroform, carbon disulphide were seen to be present at most decomposition events.
- Chemicals that are human specific (carbon tetrachloride, pentane and when skeletonised decane and undecane). Chemicals were also seen in different ratios when comparing human to animal and thus could be used as markers (e.g. in an animal carcass 2-methyl butanal was seen to always be in higher levels than 3-methyl butanal).
- Chemicals derived from microorganisms (e.g. ethanol is not linked to human decomposition *per se* but is important as it is produced by the action of microorganisms during decomposition).[12]

Forbes and Perrault (2014) A more recent paper studied the decomposition odour profile in the air and soil surrounding vertebrate carrion. This focussed on the comparison of VOC profiles from both air and soil for different decomposition stages and in reference to environmental parameters including microorganisms present. It was found to be not only an extremely complex profile, with 249 compounds detected across the areas of study, but also to be specific to stage and sampling location. This supports findings in the current study of the complexity of decomposition processes and the associated need for variable control.[25]

More recent work continues to build on these principles studying the VOCs evolved as well as attempting to find human specific markers in the VOC profile[35] and focussing on soil chemistry and cadaver dog training. [50, 67 68]

Rosier *et al* (2016) studied the VOC profile over time in the laboratory setting in an attempt to identify a marker specific for human remains which was the focus as well as cadaver dog training aids [67, 69] However, very little work has been done on the study of the actual fundamental parameters and repeat study in controlled conditions from an analytical point of view.

1.8 Context of previous work with respect to current project

The literature as a whole suggests that pigs are suitable as human models for decomposition studies although this still requires further investigation. [12, 18, 19, 23, 24, 30, 53, 70] There is a need for profiling of chemicals and their relative ratio and signature over time as a tool to estimate PMI and it is hoped that this work can apply the chemistry of decomposition to the area of PMI determination. [5, 7, 11-13, 15, 18, 19, 23, 25, 28, 36, 40, 46, 48-50, 53, 57, 70-72]

Although many different methodologies were used by previous workers the potential of both Thermal Desorption-Gas Chromatography-Mass Spectrometry (TD-GC-MS) and Headspace-Gas Chromatography-Mass Spectrometry (HS-GC-MS) for the analysis of VOCs is commonly emphasised and both of these techniques were used for comparison in this study. [13, 23, 44 73] Much of the previous

work reported in the literature has focussed on one aspect of decomposition, perhaps with a few whole organisms. However, to determine their use and any limitations of PMI estimation through VOC time profiling, it is of vital importance to investigate the sampling and analysis parameters from a fundamental point of view. Thus the work presented here is in the format of an analytical project with a forensic application rather than solely from a forensic perspective.

1.9 Aims and objectives

The aim of this project was to investigate the VOCs produced during the decomposition process from a fundamental analytical point of view for the purposes of developing SOPs. Five key objectives have been identified: -

1. To develop a methodology capable of studying the relevant chemicals evolved during the decomposition process and compare the validity of such in terms of repeatability and reproducibility.
2. To collect, identify and profile as a function of time the VOCs produced during decomposition using (primarily) Headspace Gas Chromatography Mass Spectrometry (HS-GC-MS) and Thermal Desorption Gas Chromatography-Mass Spectrometry (TD-GC-MS) and compare and contrast these techniques.
3. To investigate the effect of experimental parameters such as sample size, headspace volume and other factors, on the VOC profiles.
4. To develop Standard Operating Procedures (SOPs) for the analyses of the VOCs evolved during decomposition
5. To test the robustness of SOPs using the phyto-kingdom as a related system with similar chemicals as decomposition.

By controlling as many variables as possible it was hoped that using the chemicals characteristic of decomposition over time (e.g. alcohols, aldehydes, acids and sulphur compounds) will enable the development of SOPs and use of analytical instrumentation with a view subsequently to link the olfactory signature of decomposition VOCs to PMI. Development of SOPs was an iterative process where results from method development and use of preliminary SOPs were used to feed-back to previous chapters to allow their further development. It was hoped to have SOPs by the end that were capable of being applied to this and other related fields including the chemically similar area of decomposition mimicry in the phyto-kingdom.

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2. MATERIALS AND METHODS

2.1 Introduction

Chromatography is a family of techniques where separation of components in a mixture is obtained by their relative affinity to a stationary phase through which they are transported by a mobile phase. The project utilised Gas Chromatography (GC) which is particularly suited to the separation of mixtures containing low boiling point components such as VOCs. GC utilises an inert gas as a mobile phase and a solid coated capillary column, typically several metres in length, comprising the stationary phase and situated in a temperature controlled oven. The time that each component takes to pass through the column due to differential adsorption is known as the *retention time* (RT) and is characteristic to that particular substance for a given set of conditions. Different forms of column coatings are available depending on the nature (usually polarity) of the mixture being analysed. Other conditions, e.g. the oven temperature and gas flow rate, can be varied to optimise separation of components of interest.[1-5]

2.2 GC Detectors

2.2.1 Flame Ionisation Detector (FID)

A Flame Ionisation Detector (FID) uses an intense hydrogen and air flame to pyrolyse and ionise components as they exit from the GC column. The signal produced by the detector is the extent to which these ions and electrons conduct electricity. Different chemicals produce different signal intensities based on the quantity present and how readily they are ionised. FIDs are easy to use, robust with low sensitivity to interferences and are quantitative if calibrated with known amounts of the analytes of interest. [2, 6]

2.2.2 Mass Spectrometer (MS)

Mass spectrometry (MS) is based on ionisation of a compound to produce ions which are then separated based on their mass-to-charge ratio (m/z). The mass and relative abundance of these ions is unique for that compound as well as their resulting fragmentation pattern. Coupled with GC it forms a powerful analytical technique as it allows for identification of the separated components when used in conjunction with spectral database.[1, 7, 8]

2.3 Headspace-GC-MS

Headspace (HS) is used to describe the vapours that equilibrate above a heated sample (solid or liquid) when in a sealed container. It can be used as a sample introduction system for GC where, typically glass, vials (20 ml) are used with septum-sealed lids with a GC syringe being used to collect a quantity of the headspace for injection into the GC inlet. It is used for analysis of VOCs to allow direct introduction of solids and liquids into the GC without the need for sample preparation as compared to simple liquid injection.[9]

The chemicals in the HS vial reach equilibrium between that of the sample matrix and the headspace above it. The exchange rate of the chemicals across this interface is correlated via the principle of Henry's law constant. [9]

2.3.1 HS-GC-MS

Initial experiments were carried out using a HS-GC-FID system due to its availability in the laboratory. Once work justified and funds allowed, a HS-GC-MS system was obtained and used for subsequent experiments (see Tables 2.1 and 2.2 for HS-GC-FID and HS-GC-MS conditions). See Chapter 3 for GC oven programme rationale.

Headspace sampler	Agilent technologies 7697A with 111 place autosampler
GC-FID	Agilent technologies 6890N with ChemStation software
Column	Zebron ZB-5MS Dimensions - 30 m x 0.25 mm x 0.25 um Phase chemistry – 95 % dimethylpolysiloxane, 5 % phenyl Temperature range – isothermal - -60 ˚C to 360 ˚ C, TPGC - - 60 ˚C to 370 ˚C
Carrier gas	Helium
Vials	Agilent technologies 20 ml septum-lidded crimp sealed specifically designed for use with the system.

Table 2.1 HS-GC-FID conditions

Figure 2.1 Capped 20 ml Headspace vial

Headspace sampler	Agilent technologies 7697A with 111 place autosampler
GC-MS	Agilent technologies 7890B GC with 5977 A Mass Selective Detector with MassHunter and ChemStation software
Column	Zebron ZB-5MS Dimensions - 30 m x 0.25 mm x 0.25 um Phase chemistry – 95 % dimethylpolysiloxane, 5 % phenyl Temperature range – isothermal - -60 ˚C to 360 ˚ C, TPGC - - 60 ˚C to 370 ˚C
Carrier gas	Helium
Vials	Agilent technologies 20 ml septum-lidded crimp sealed specifically designed for use with the system.
MS	Single quadropole

Table 2.2 HS-GC-MS conditions

2.4 Thermal Desorption (TD)-GC-MS

Thermal Desorption (TD) is a sample introduction technique which utilises a metal tube containing an adsorbent (discussed below) through which air containing VOCs is drawn by suction. During analysis the TD tube is heated while a flow of inert gas passes through it. Volatiles and semi-volatiles are desorbed and collected on a cold trap. Rapid heating of the cold trap then introduces the VOCs into the inlet of the GC column (see Figure 2.2). TD combines ease of use, minimal sample preparation and concentration enhancement (see Table 2.3 for TD-GC-MS conditions).[10]

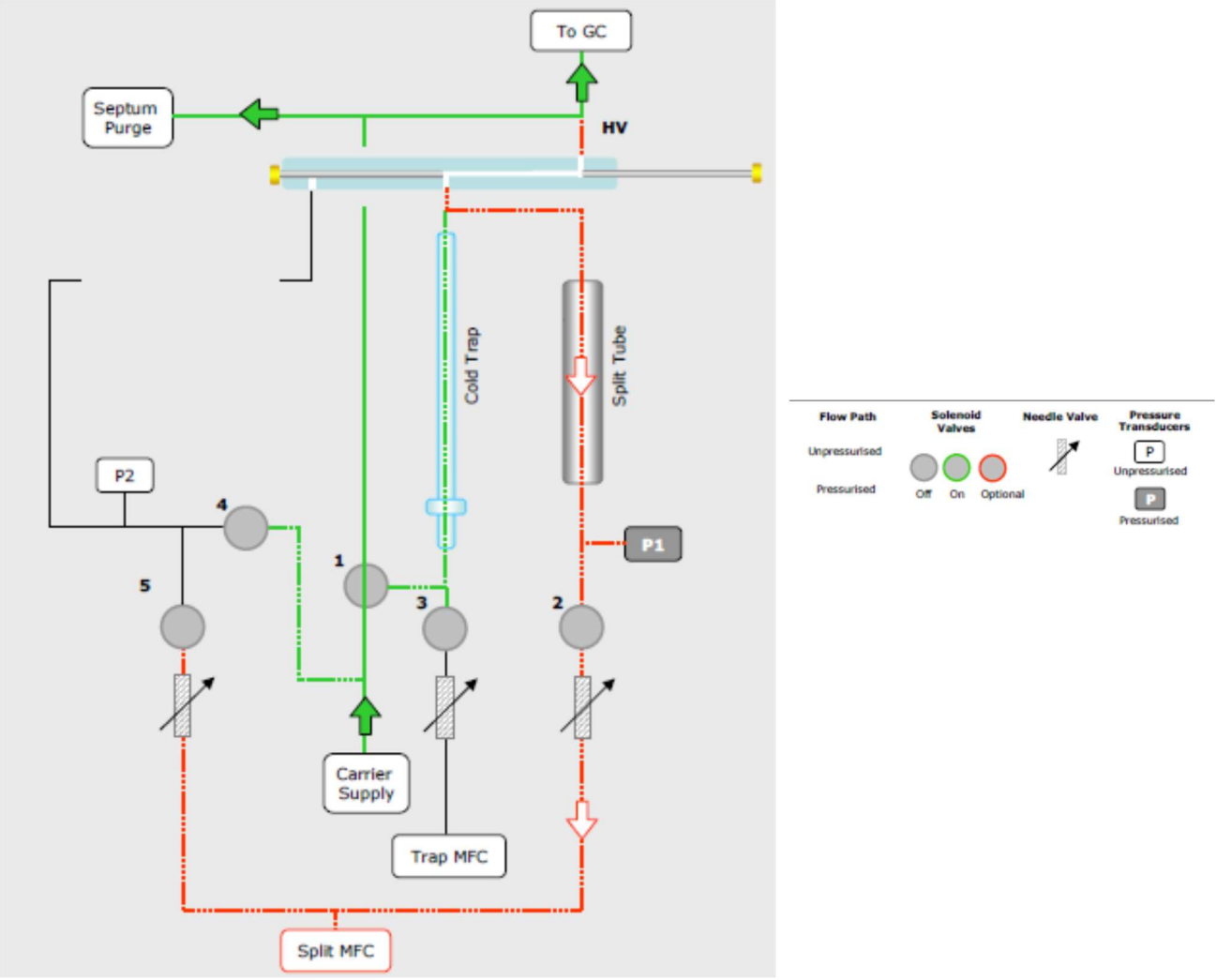

Figure 2.2 Schematic of the Thermal Desorption flow path (no recollection) (*figure supplied by Markes International*). This shows the flow paths in a snapshot of time. The valves are opened and closed as each area pressurises during the programme. MFC is the Mass Flow Controller which measures and controls the gas flow to ensure a specific flow rate

Thermal desorption	Markes International TD-100 Thermal Desorber with 100 place autosampler
GC-MS	Agilent Technologies 6890N GC with 5975 inert XL Mass Selective Detector. ChemStation software and NIST library.
Column	Zebron ZB-5MSi Dimensions - 30 m x 0.25 mm x 0.25 um Phase chemistry – 95 % dimethylpolysiloxane, 5 % phenyl Temperature range – isothermal - -60 ˚C to 360 ˚C, TPGC - - 60 ˚C to 370 ˚C
Carrier gas	Helium
MS	Single Quadropole

Table 2.3 TD-GC-MS conditions

2.4.1 TD tubes

These are metal tubes filled with adsorbent (see Figure 2.3) in various ratios and combinations according to the experiment and sample type. For this work *Tenax TA/Carbograph 1A* tubes were used. Tenax TA is a porous polymer resin and Carbograph is carbon based.[11, 12]

This type of adsorbent is sold by Markes international (Markes International, CF72 8XL) as covering a wide mass range. Tenax TA is a porous polymer adsorbent with a maximum temperature limit of 350 C. Carbograph 1A is graphitised carbon black used as a general-purpose sorbent, often in two- or three-bed mixed sorbent tubes.[11, 12]

The tubes are uni-directional and the end exposed to the target vapour has a notch to identify it as the *sampling end*. TD tubes can be used passively, i.e. VOCs enter the tube via diffusion, but in this work *active sampling* (suction pumping) was used to maximise the VOCs collected in a given time period (see section 2.4.3).

Brass storage ends (Swagelok) are used to seal the tubes and they can be stored for up to a week in the fridge (5 °C) or up to a month the freezer (-18 °C). This manufacturer's claimed storage times were tested and found to be acceptable by the author in preliminary studies. Prior to analysis these need to be replaced with Difflok caps which restrict static diffusion into the tube but allow desorption when the external gas flow is applied.

TD tubes, in theory, should be clean following desorption (which can be checked by a GC run). They are typically regenerated by additional heating if they are still contaminated in a process called *conditioning* (heated for 30 minutes at 230 °C).

Figure 2.3 TD tube with brass storage ends attached

2.4.2 TD tube sampling

Sampling using TD tubes allows concentration of volatile analytes and is usually carried out as a discrete sample, which tends to be a one-off collection of headspace at one time, or over a period of time but not accumulation of the headspace over the length of an experiment. The aim is to collect the headspace of interest and was a balance between collecting enough for analysis and to avoid overloading the tube, which has a finite adsorption capacity.

2.4.3 TD sampling pumps

TD tubes can be used for passive sampling but *active sampling,* where a hand or electric pump draws vapour through the tube, is the most convenient means of collecting sufficient VOCs for analysis in the shortest time. Initial experiments were done using a Drager bellows hand pump (already available in the laboratory) and then two pumps, both manufactured by Markes International (the manufacturer of the TD unit) were trialled.

2.4.3.1 EasyVOC hand pump

This hand-held bellow-style pump was attached to the non-notched end of the TD tube and squeezed for operation. Each squeeze corresponds to 100 ml of air sample.

2.4.3.2 ActiVOC electric pump

This electronic automatic pump was attached to the non-notched end of the TD tube and then timed for the required sample volume. Using a flow meter and the manufacturer's instructions the pump was calibrated to sample 100 ml/min.

2.4.3.3 Pump testing

Once the pumps were calibrated as above, they were tested in terms of their resistance and for carry over. Resistance testing was done by pulling a sample through the tube to ensure the desired flow rate was still maintained. This was checked periodically. Carry over was tested by running two tubes in series and sampling one tube with another. Even with levels classed as overload there was no carry over into the next tube.

2.4.4 Recollection

It is possible to 'recollect' samples back onto the TD tube after analysis (see Figure 2.4). Following heating of the cold trap a split allows some of the volatiles to go to the GC oven while the rest is passed back onto the sample tube and re-adsorbed. Generally, this option was not used in this project as it added another complexity to the analysis.

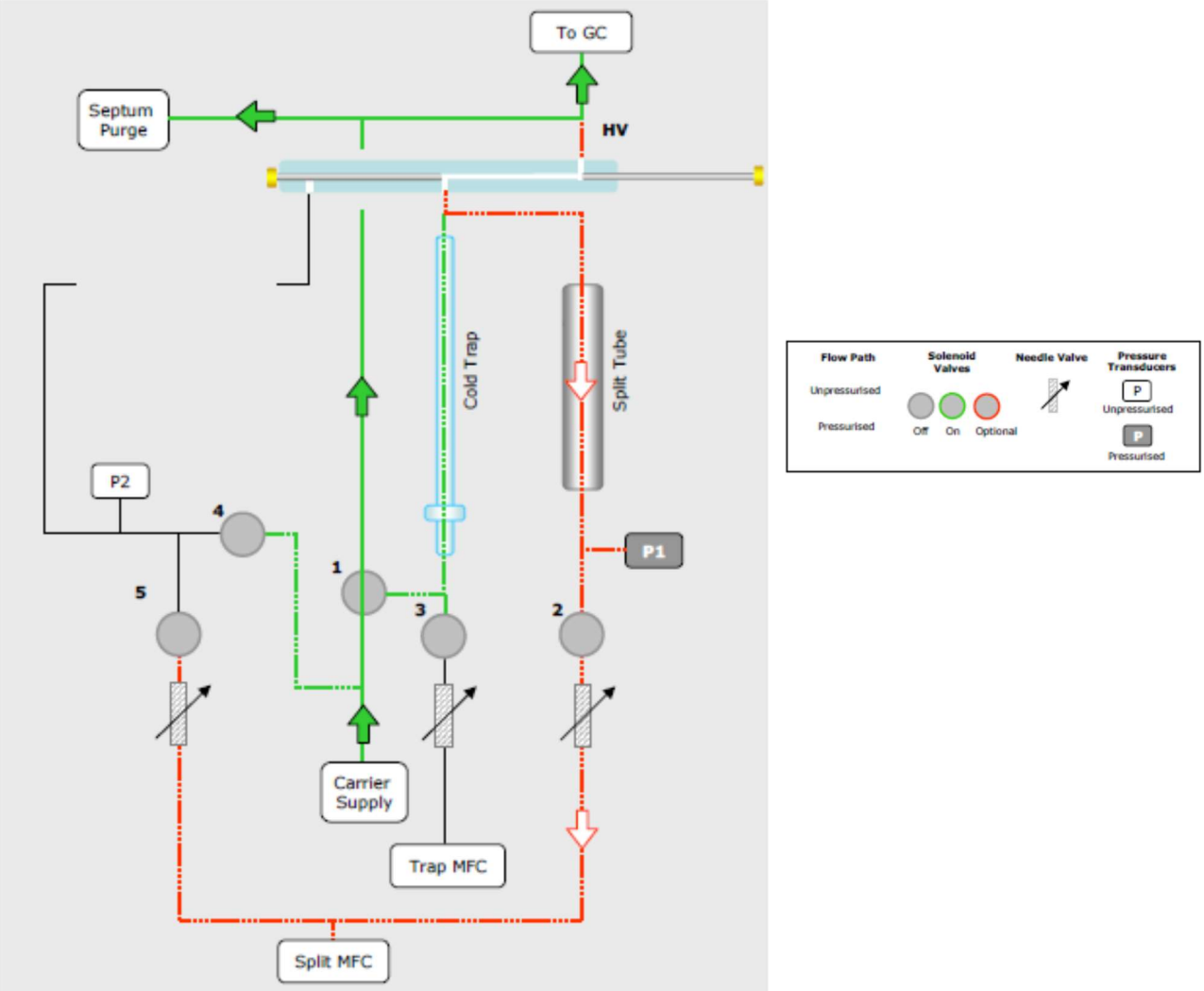

Figure 2.4 Schematic of Thermal Desorption flow path (with recollection) (*figure supplied by Markes International*). This shows the flow paths in a snapshot of time. The valves are opened and closed as each area pressurises during the programme. Recollection allows the flow of sample to return to tube rather than to the split tube. MFC is the Mass Flow Controller which measures and controls the gas flow to ensure a specific flow rate

2.5 Data analysis

Data from the HS-GC-MS and TD-GC-MS were processed in a similar way with the exception that data from the former was first converted from MassHunter to ChemStation to be compatible with the latter.

All chromatograms were first examined to determine if the peaks were well resolved, of significant level and clearly differentiated from the background (see below and section 3.4.5 for adequate tube cleaning criteria). Peaks fulfilling these criteria were then identified using the NIST library.

Quantitation was not carried out in the scope of this study so a *significant peak* was defined as a value of >3 x baseline and if peaks were clipped or above 10^7 it was classed as peak overload i.e.

between approximately 3000 and 1,000,000 counts was taken as a *significant peak*. For data presentation, peak height was chosen even though not all peaks were Gaussian in shape as some peaks were not well resolved e.g. carboxylic acids (see Figure 2.5) and it made the use of peak area difficult due to the integration parameters in the software. This fronting of a peak usually indicates column overload or inadequate resolution between two peaks.[4-6] In this case the chromatography conditions are not ideal for the analysis of butanoic acid. This is far from ideal but was an accepted compromise to ensure that analysis of all peaks was carried out under the same conditions to allow consistency. This was possible because the peak did not affect or overlap with any other peaks of interest.

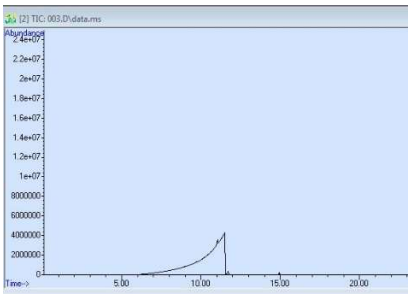

Figure 2.5 showing the chromatographic spread peak for butanoic acid

2.6 Decomposition containers

During method development and initial studies many different sample containers of various sizes and materials were trialled. Eventually polypropylene boxes with clip-on lids (Really Useful Products Ltd) were adopted (see Figure 2.7) as they were UV and weather resistant and themselves emitted no VOCs (e.g. phthalates) that could interfere with the VOC profile.

Preliminary decomposition experiments used boxes of various sizes (see Figure 2.6) with a hole drilled at either end. One hole (6 mm) allowed for the insertion of a TD tube while the other (2 mm) allowed air flow when sampling. Rubber bungs were used to seal the holes to maximise the build-up of VOCs in the headspace. These also had an outer box containing a 2 cm hole to allow insect access but to protect from scavengers. If an experiment was to have insect exclusion the hole was meshed with a fine mesh to allow airflow but to prevent insect access.

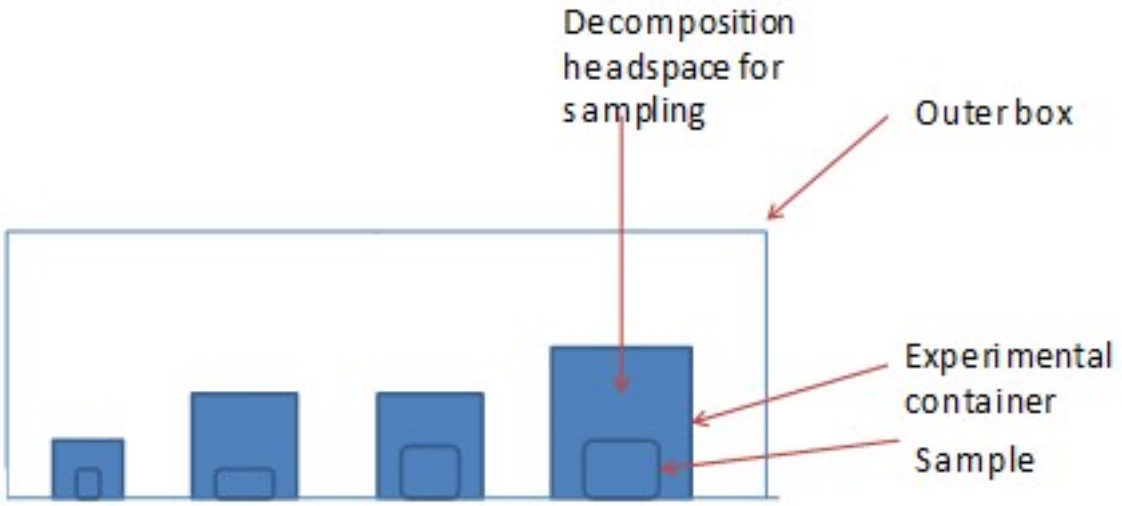

Figure 2.6 Diagram showing the decomposition headspace for sample collection

Following these various trials and preliminary experiments, for later experiments and chemical studies a standard box was used with the diameter of 22 x 17 x 15 cm being chosen as the optimum VOC sampling chambers. These boxes were robust enough to provide scavenger protection without the need for outer box (see Figure 2.7). Unless otherwise stated boxes were unmeshed to allow insect access and un-bunged for realistic airflow conditions. Boxes were disposed of at the end of an experiment and not reused as this would be considered a contamination risk.

Figure 2.7 VOC sampling chambers chosen as optimum from the trials and preliminary experiments

2.7 Sand for standard substrate use

Several of the decomposition experiments required the use of an inert substrate. Sand (British Play Sand-Sinclair Pro, Lincoln, LN6 7AH) was selected because it was quoted to be pre-washed, had a low iron oxide content and used sub-rounded grains to reduce abrasion. The quoted particle size was 63 microns to 1 mm with the majority of grains being in the 125 – 355-micron range (these were not tested by the author). All sand was dried in an oven at 105 °C for 5 hours before use and placed to a depth of approximately 1-2 cm in the bottom of the sample container.

2.8 Location of decomposition experiments

Experiments were set up in the laboratory and then sampling boxes were located on an external roof space on the Science Building, University of Huddersfield (OS grid ref. 414674 –latitude, 416346-longitude), exposed to the natural variation in weather and entomological conditions. Insects present included flies, blowflies, beetles and wasps. Boxes were weighted down with bricks to prevent their movement in the wind.

The outdoor conditions have not been controlled but all the samples for comparison in one experiment are subjected to the same conditions by their location. Control of all variables would be impossible and would not give a realistic decomposition event.

A weather station was present within a few metres of the site and this was used to confirm any observations made during sampling events. Temperature, humidity and pressure were considered but not controlled.

2.9 Chemicals for decomposition mix

Chemicals were analytical grade reagents from Fisher Scientific, Loughborough, LE11 5RG

- Trimethylamine (in ethanol) (TMA) – 33 wt % solution in ethanol
- Dimethyl disulphide (DMDS) – 99 %
- Butan-1-ol – 99 %
- Pentan-1-ol – 99 %
- Propanoic acid – 99 %
- Butanoic acid- 99 %
- Hexane (initially mixed fraction of all 5 isomers) – extra pure fraction, low in aromatic hydrocarbons SLR
- 2-methyl butanal – 99 %

2.10 Meat samples used for decomposition studies

- Fresh food grade pork loin (Sainsbury's) without additives or a nitrogen flushed packaging (to avoid any effect of preservation), comprising flesh, fat and skin was cut to size as required. Unless otherwise stated meat was placed fat/skin side upwards in the sample container.
- Chicken in jelly cat food (Felix, Nestle Purina Pet Care (UK) ltd, Liverpool, L9 0EW) was used as received.

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3. METHOD DEVELOPMENT

3.1 Introduction

Any analytical instrumentation requires a great deal of initial method development if there is not already an established or validated method available for the analyte or sample of interest. [1] This is particularly true of difficult or complex matrices (such as decomposition) [2] and thus the vast majority of the initial experimental work was instrumentation familiarisation and method development for the HS-GC-FID and the TD-GC-MS systems.

To allow their direct comparison a lot of the work was done in tandem but the fundamental differences between the two systems did necessitate individual method development. Although both systems are based upon GC, allowing the majority of chromatographic parameters to be investigated simultaneously, differences in sample introduction technique and detector required their own study.

The main longer-term aim was to lay out the basis of the planned SOPs for decomposition studies. However, the initial aim was to get adequate resolution of peaks for different chemical types with a reasonable analysis time. Analysis time was considered an important factor as it was planned to progress to experiments requiring multiple analyses using autosamplers and the use of TD and HS sample introduction techniques introduces substantial additional time on top of that for the chromatography itself.

Most early work was carried out using the TD-GC-MS system as the MS detector provided the ability to correlate individual peaks with chemicals. By using identical GC conditions, including column type, this allowed any identification of peaks using the TD-GC-MS to be applied to peaks observed using the HS-GC-FID, based on retention time.

3.2 GC parameters

Due to the complexity of the area of study, initially parameters such as flow rate, inlet temperatures and the split ratio were kept at their default values to allow the influence of alterations in other important parameters (column, oven temperature programme and detector) to be studied. This was with a view that once chosen and established, it would be possible to alter these subtler parameters later if necessary.

3.2.1 Column choice

Initial trials were carried out using amines as the literature suggested their importance as decomposition VOCs. [3-12] These used both polar (BP20) and non-polar (BP5) columns but it was quickly realised the need to standardise on the latter for both systems as analytes can be quite column specific so the two cannot be used to fairly compare two systems. BP5 was chosen as decomposition chemicals span a wide range of chemical groups and it was felt that a more specialist column could limit detection of certain chemicals. [7, 10, 13, 14] However, if one class of VOCs was considered more important for study in the future then a more specific one could be used without significant changes to overall methodology.

3.2.2 GC oven programme

This is generally considered the single most important parameter for the purposes of chromatographic separation. [15] The GC programme used as default (fast ramp to high temp) was tried first but it was quickly noted that most of the analytes of interest were very volatile so it would be necessary to slow the initial programme to allow better separation in the first few minutes.

Different GC programmes were found in the literature (these papers show some examples [6, 8, 11, 12, 14, 16, 17 8, 13, 14, 16-30]) and some of these were evaluated (those in these papers [6, 8, 11, 12, 14, 16, 17]) to determine which gave best separation in the least analysis time. This work was done using the HS-GC-FID system to avoid the additional complexities of the sample introduction stage of TD (to be discussed later in section 3.4). Several GC programmes selected from the literature were tested on the HS-GC-FID using the decomposition mix (see section 3.7). The most appropriate all round for decomposition studies was found to be that of Dekeirsscheiter *et al.* [6] as it extends the near isothermal portion and covers a wide range of VOCs. After confirmatory studies on the TD-GC-MS, this was adopted for all subsequent method development including for SOP development (see Table 3.1).

	Temp (°C)	**Hold (min)**	**Ramp (°C/min)**
Initial temp	35	5	3
Second temp	50	0	6
Third temp	110	0	10
Final temp	245	5	

Table 3.1 GC oven temperature programme taken from Dekeirsscheiter *(2009)* [10]

3.2.3 Detector choice

Detector type is a major consideration when establishing a new methodology for chromatographic study. The detectors here were first chosen simply on their availability in the department where the work was taking place. The MS detector attached to the TD-GC allows for identification capability and by keeping other conditions as closely linked as possible then data from this could be used for identification on the HS-GC-FID as well.

During this period of work an additional GC-MS was purchased and it was decided to link this with the HS. This meant that both systems were identical, apart from the sample introduction technique, and the ability to directly identify peaks on the HS was very beneficial. These systems were used for the remainder of the project and were used in subsequent stages of method development.

3.3 HS-GC-MS

Method development was largely done using the HS-GC-FID initially and then applied to the HS-GC-MS once in place.

3.3.1 Headspace (HS) sampler temperature

In addition to the GC parameter development discussed above, it was also necessary to undertake familiarisation and method development for the Headspace (HS) sampler itself.

3.3.2 Headspace Temperature setting

It was necessary to set the oven temperature for the HS sampler in addition to the GC oven. To prevent unwanted condensation of sample, the HS oven, loop and transfer line need to have an increasing temperature gradient. Early studies illustrated the importance of the need to consider the boiling point and vapour pressure of all components present. It needs to be a balance between volatilising analytes of interest with higher boiling points without creating too much of a build-up of pressure for the more volatile ones. [31] The oven in the HS sample introduction system doesn't have sophisticated cooling systems so time for cooling is a big consideration when using runs of different oven temperature methods.

3.3.3 Headspace Sample volume and LOD

Preliminary studies were carried out to determine the optimum sample volume to be used in terms of limit of detection (LOD) and for avoiding overload. This work was continued in Chapter 4.

Additional experiments were undertaken using various granular activated carbons as adsorbents using the standard chemicals, alone and as the decomposition mix. The aim was to determine the optimum quantity of chemical, type and mass of carbon used with a view to use carbon as a potential alternative method of VOC collection. After initial experiments, it was found that the optimum was 100 µl of pure chemical and approximately 0.4 g of carbon (sufficient to cover the bottom of the vial).

This carbon was also used in the VOC sampling boxes in conjunction with TD. The presence of water was always a major issue when using decomposition samples. Getting the balance between collecting the volatiles of interest in the presence of water and then desorbing them without the water vapour raising the pressure in the headspace vial too high during analysis was difficult. Carbon work for HS-GC-MS was trialled (see section 3.5) but not continued in the scope of this project so would be of interest for future work.

3.3.4 Reproducibility and repeatability of Headspace analysis

Another major consideration when using HS is the length of time a sample will be stable in the HS vial and the tolerance for multiple injections in the same vial. Work was done on repeatability of experiments by using standard chemicals and carrying out repeat injections over time. It was found that GC peak heights did not significantly change if injected at least five times from the same vial even with a week between injections. Based on this result, it was decided to accept up to five repeat injections if required. However, further work would be needed especially with regard to storage time and if using decomposition samples rather than pure chemical which was not in the scope of this project.

3.3.5 MS detector

As described in section 3.2.3, the HS-GC-FID system was upgraded to HS-GC-MS. Evaluation of this system was done in conjunction with a study on decomposition chemicals and is discussed in Chapter 4.

3.4. TD-GC-MS

3.4.1 Thermal desorber (TD) autosampler

In addition to the optimisation of the GC parameters, it was also necessary to develop the methodology in terms of the Thermal Desorption (TD) aspect of the system. For the majority of experiments, the manufacturer's (Markes International, CF72 8XL) recommended desorption conditions for volatiles of 30 mins at 90 °C tube desorb with trap flow of 42 ml/min were used.[32]

3.4.2 TD tubes and sampling

The adsorbent used in a TD tube can be chosen to allow for selective adsorption of analyte and therefore has an impact on the extent of collection of certain compounds. The adsorbent used in the standard TD tube is designed to be suitable for a wide range of volatiles (see section 2.4.1). However, selective adsorption is inevitably a factor when a complex mixture of VOCs is being sampled and can lead to undesirable concentration of certain compounds and underrepresentation of others.[32, 33]

3.4.3 TD sample pumps

Initial experiments utilised a manual Drager bellows pump which could be set for either 50 ml or 100 ml of air pulled through the TD tube per action. This is squeezed the according number of times for the desired volume. These initial experiments used decomposition material available from another project and, encouragingly, subsequent TD-GC-MS analysis revealed the presence of the expected chemicals, most notably DMDS and TMA. Additional work was carried out using cat food as an easily available odourous material known to produce decomposition VOCs and also the chemical mix in order to obtain a working method for the pump and instrumentation.

Different sampling volumes were necessary depending on approximate concentration of chemicals present (as pure or in decomposition experiment), the odour threshold in terms of perception of analyst versus instrumental LOD, and experiment type in terms of container headspace volume (see Chapter 5). It was found to be necessary to select different sampling volumes for each type of experiment based on experience. For pure chemicals, 100 ml was usually sufficient but for collection from decomposition experiments standard practice was to attempt to 'exhaust' the headspace by collecting a volume equating to the container volume i.e. if for a 1000 ml container then a 1000 ml volume of headspace was collected.

With larger sampling volumes, manual pumps were impractical in terms of collection time and effort – as well as exposure of the collector to offensive foul odour. Electric pumps that could be left unattended

for the duration of the sampling were used for the majority of the later experiments but, again, care was taken to find a balance between collecting sufficient VOCs for reliable analysis and not overloading the system.

3.4.4 Collecting decomposition headspace using TD

In addition to the sampling volume which is pumped through the TD tube, the sample headspace volume i.e. the amount of space above and around the sample that allows accumulation of VOCs, is another important consideration (see Chapter 2, Figure 2.6 and section 5.2). A larger volume allowed for a potential greater quantity of VOCs to accumulate. It should be noted however, that although the boxes were lidded they were not airtight. VOCs were contained enough to be captured in a pseudo-equilibrium at higher concentrations than if the boxes were open but not to levels that might actually affect the decomposition process.

The holes could be bunged to allow accumulation of headspace or left un-bunged for a more realistic airflow scenario. If bunged, both holes are opened when pumping the headspace.

3.4.4.1 Generation and regeneration

Generation is defined as the time taken to accumulate headspace in the sampling container with components of interest having sufficient vapour pressure for TD detection.

Regeneration is the same as above but is the re-accumulation following a sampling event.

3.4.5 TD tube and system cleaning

The manufacturer (Markes International, CF72 8XL) claimed the analysis process run itself would be sufficient to clean the TD tubes ready for another sampling. However initial experiments showed that not all the VOCs were removed under the analytical conditions used and the development of a reliable cleaning cycle was critical. This was particularly true because not only could the adsorbent in the tube become overloaded but the subsequent high levels of VOCs desorbed could then contaminate the TD-GC-MS system itself. Indeed, even levels present from previous experiments that may be acceptable in the study for other systems could cause sensitivity problems when studying the low level of certain volatiles in this project. Ensuring adequate cleaning ability and to distinguish peaks from background was essential to allow accurate library searching and for any work based around limit of detection (LOD).

During the initial phase of the project, it was difficult to determine what was an acceptable background on the chromatograms because, at the time, experiments were performed without knowing the concentration of chemicals in the headspace and in the absence of an internal standard (IS). Deciding what was experimentally relevant, what was contamination and indeed if tubes were clean was difficult. It was not possible to simply state that below a certain abundance was acceptable as clean so a procedure was developed based on a set of decision criteria which were all applied for each tube: -

- Produce no peaks at all (fully clean, the ideal situation)

- Produce no peaks above approximately 3000 abundance units (in accordance with suggestions from Agilent for LOD[31]). Although actual quantification of analytes was not part of the project plan it was still considered desirable to get the best possible LOD when working with low level VOCs.
- Produce no peaks that were identifiable using the MS spectral database and considered relevant in the experiment being performed.
- Produce no peak corresponding to a chemical known to be collected on the tube previously when performing experiments with pure chemicals (as this could indicate carry over).

An additional factor is that higher temperatures and longer times are generally more effective at cleaning the tubes but risk degrading the adsorbent. It was determined that conditions of 320 °C and a hold time of 30 minutes with a 50 ml/min ramp with any desorbed material being sent to waste rather than to the GC was the best compromise for cleaning.

The whole process was time-consuming as checking if a tube was clean itself required an additional full analysis on the TD-GC-MS on top of that checking the GC system and column. A balance had to be made between being confident that the whole system was clean and the ability to run sufficient tubes to be useful experimentally in the time available.

As it was thought that some of the contamination could have accumulated during the normal low temperature GC runs, a specific GC temperature programme for tube cleaning experiments was developed which had a longer hold at higher temperature (see Table 3.2).

	Temp (°C)	**Hold (min)**	**Ramp (°C/min)**
Initial temp	50	0	20
Second temp	280	10	

Table 3.2 GC programme used for testing state of TD tube

In most cases, it was accepted as adequate for cleaning for most analyses of a series of TD tubes to start and end with an empty tube (i.e. no adsorbent) with the GC cleaning run (see above). In addition, other cleaning procedures were adopted: -

- Periodically all tubes were *conditioned* (as in section 2.4.1)
- Random tubes were checked for contamination after an experiment.
- If the system was left for any period of time, then blank TD trap fire (where the trap is heated in isolation) and blank GC runs were carried out to check the system.
- In addition to tube cleaning, if any TD contamination was suspected then the entire TD autosampler was heated and held at a high temperature in an attempt to remove any contamination (‘*baking out’*).

Tube performance also declined over time. This was monitored using the individual serial numbers provided by the manufacturer on the tube. If artefacts were seen that are known to be associated with

tube breakdown (lots of benzenes, siloxanes etc.) [33] or levels of collected sample declined then a tube was removed from use.

3.4.6 Recollection

The TD system supports a process called *recollection* (see section 2.4.4, Figure 2.4) when a proportion of the material adsorbed onto the cold trap is returned to the sample tube while the rest is passed to the GC-MS for analysis (by default excess adsorbates are sent to waste). This would be potentially beneficial because it would provide a 'second chance' for analysis of a sample that would be difficult to obtain again (for instance in the middle of a series of experiments profiling VOCs over time). However, the procedure does add further complications in terms of time and implications for tube cleaning and this was not routinely used.

3.4.7 Initial testing of method evaluation using decomposition chemicals for TD-GC-MS analysis

Initial method evaluation was carried out using the pure chemicals chosen for the decomposition mix (see section 2.9). For comparison, these chemicals were both sampled onto the tubes (100 ml suck) and injected directly (1 µl) onto the tubes and then put into the TD-GC-MS.

The results indicated that, as expected, direct injection allowed better repeatability but the results showed that it was not appropriate for use with headspace analysis above decomposing material. Therefore, it was decided to do any standard chemical work as air/headspace samples as well to allow truer comparison.

The complexity of the samples and the challenges of using TD suggested that little quantification would be possible. However, the ratios of the size of peaks from the compounds of interest could be useful. These initial trials also suggested it may be necessary to add a known amount of internal standard (IS) by direct injection to allow the monitoring of peaks and background for ratio analysis. Standard doping onto the meat was also eventually carried out (see Chapter 6).

3.4.8 Preliminary experiments using pork for TD-GC-MS analysis

These were undertaken to get some idea of the decomposition process both from a simple observation point of view and to aid in the development of sampling protocol, choice of meat and container used and for development of instrumental parameters.

Pigs are considered an accepted human model for decomposition studies [6, 10, 19] [6, 10, 18, 19, 24, 26] and thus the meat selected for use was pork. To allow the study of variables the size of meat and container used needed to be able to be controlled from one experiment to the next.

During these very preliminary experiments, different masses of pork were used both as a means of identifying optimum decomposition conditions and also to initially evaluate vapour collection and analysis methods. The effect of presence of insects, different meat tissues, preservation (with sodium nitrite and salt) and substrate presence in the containers was also studied (some of these are studied in more detail in Chapter 5).

Although not presented in this thesis, these studies included observational and photographic recording of the decomposition process, evaluation of any insect activity and its effect and recordings of mass loss undergone by the pork by the end of the experiment. The main finding from these initial studies in context of this project was that there is a minimum amount of pork (approximately 100 g) that can be used before total desiccation occurs, effectively terminating the decomposition process and that the volume of headspace above the meat is important for sampling purposes. The chemicals seen related to those in the literature suggested the TD-GC-MS as promising and appropriate for VOC profiling for decomposition studies.

Due to the nature of decomposition experiments, there is not always material available at the stage required so it was decided that having an odourous substance would be necessary and so for the purposes of quick testing of sampling volume and instrumentation, cat food was used if decomposition experiments were not available.

On a very basic level these studies also allowed the development of health and safety and disposal protocols and determined the need for consistent protocol.

3.5 Preliminary experiments using carbon for HS analysis

Alongside the preliminary experiments for TD-GC-MS, granular carbon was placed in the sampling boxes in an attempt to simultaneously study decomposition by TD and HS. This was exposed to the decomposition headspace throughout the process and removed for testing by HS at different stages. This did show promise but was not pursued as it involved disturbing the headspace for its removal and may have affected the TD data. Thus it was decided to only sample for HS at the beginning and end of TD experiments or to carry HS out separately. A MSc project under the supervision of the author and supervisor was carried out investigating HS parameters and carbon analysis which showed promise but would require further study. [34]

3.6 Development of a paper' sniffer'

It was hoped that in addition to the instrumental techniques used, that a paper test based 'sniffer' could also be developed as a quick presumptive test for decomposition at a scene if a VR canine was unavailable for scenting. The author guided undergraduate projects in this attempt, using chemicals that reacted specifically to amines, aldehydes, alcohols and sulphur groups. In combination with pH it was hoped these would give an indication of a decomposition event to justify the need for more detailed GC-MS analysis. This showed promise but would require further investigation and was not under the scope of this project.

3.7 Selection of chemicals for decomposition standard chemical mix

Following the initial studies above, to provide a comparison to the decomposition experiments, it was decided that having a standardised mixture of chemicals to emulate, at least partially, those produced during decomposition was necessary. A standard mixture would assist method development because sampling of the vapour produced by the mixture would be generally more consistent than could be achieved with actual decomposition, where the number of variables is far greater. Rationale for the selection was based on an extensive literature search, [6, 8, 10, 14, 16-18, 26, 27, 29, 35-37] the results of initial experiments and the desire to cover the range and types of chemicals produced throughout the decomposition process (see Table 3.3).

As no quantitative measurements were planned for the project, the molar ratio or concentration of the constituent chemicals (all liquids) was not important and a simple equi-volume mixture was prepared. It was realised that the vapour pressure of the constituents would depend on the chemical, its interaction with other chemicals in the liquid phase and the temperature. However, provided these factors did not change significantly this was not considered important.

Trimethylamine (in ethanol) (TMA)	Propanoic acid
Dimethyl disulphide (DMDS)	Butanoic acid
Butanol	Hexane (initially mixed fraction of all 5 isomers)
Pentanol	2-methyl butanal

Table 3.3 Chemicals used in the standard decomposition mix.

3.8 Comparison of TD-GC-MD and HS-GC-MS

During the development of methodologies, it was necessary to investigate the advantages and limitations of both the TD-GC-MS and the HS-GC-MS techniques and their comparison to each other. To facilitate their comparison, the systems were developed in conjunction with each other and run under the same conditions wherever possible e.g. GC oven temperatures, flow rates etc.

TD-GC-MS has the benefit of concentrating VOCs through sampling of larger volumes of headspaces. The ability to cap the ends of the tube means that collected samples could be stored for some time prior to analysis thus facilitating field sampling. However, the sampling of headspace itself adds another stage in the method development process and additional uncertainty in terms of preferential selectivity of certain analytes with different adsorbents. The latter could be advantageous if selectivity is necessary but less so when a broad coverage of varied analytes, such as those produced by decomposition, is required. There was also the issue of reliable and consistent cleaning and the decline in the performance of the TD tubes with time (monitored according to protocol in section 3.4.5).

HS-GC-MS, although providing more precise data compared to TD-GC-MS, has the limitation of not providing sample concentration and the volume of headspace sampled is restricted to the volume that can be sealed into the vial. In addition, material and vapours left in the vial may continue to react unlike TD tubes which provide a fixed snapshot of the VOCs at the time of collection. However, the HS-GC-

MS does allow better consistency of results when analysing standard chemicals as once the sample is sealed in the vial and reaches equilibrium it can simply be injected into the GC without the complexity of an adsorbent to consider. HS vials allow multiple injections assisting method development as there is a higher degree of certainty that identical concentrations of VOCs are being injected each time. Although recollection in the TD-GC-MS theoretically allows similar repeatability, the extra stages this process involves adds further uncertainty.

Both the TD-GC-MS and HS-GC-MS systems in tandem, with as much conservation of conditions across the two, gives a powerful combination to tackle the complex area of study that is decomposition.

3.9 Chapter conclusion

The complex sample matrix and sampling techniques employed required extensive method development. In addition, HS-GC-FID and particularly TD-GC-MS are complex analytical techniques with their own advantages and disadvantages but they do complement each other well. It was justified to purchase the GC-MS to replace the GC-FID for the HS system to allow much accurate comparison with TD-GC-MS system.

Once a working method was established, initial experiments with decomposition chemicals and meat showed promise for further study as relevant VOCs for decomposition were profiled. Investigation of various parameters and the method scope allowed subsequent development of a Standard Operating Procedure (SOP) for studying VOCs evolved during the decomposition process.

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3.10 References

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4. USE OF CHEMICAL STANDARDS TO INVESTIGATE FUNDAMENTAL SAMPLING PARAMETERS

4.1 Introduction

Much of the literature relating to cadaver decomposition focuses on the link between the chemicals and biochemical reactions observed and the environmental conditions [1-8] [9-17]or the stage of decomposition [1, 2, 4, 6, 10, 14, 16-22] where the experimental variables are numerous and complex.

However, there has been little previous work at the level of the collection and analysis of the VOCs just as chemicals. If reactions and interactions are significant even from a simple mix of chemicals, under laboratory conditions, then this would inevitably impact on the reliability of conclusions drawn from the far more complex matrix of decomposed remains in a natural environment.

4.1.1 Aims

This chapter describes work undertaken using a relatively simple mixture of chemicals to provide an insight into the fundamental parameters that affect the collection and analysis of VOCs under controlled laboratory conditions. The aim was to provide information that could be used to:

A) Further guide the development of the SOPs

B) Develop a 'baseline' measure of the variability of the sampling and analysis

The experiments performed were:

a) Investigation of the time to generate and regenerate headspace VOCs
b) Repeatability of headspace sampling using TD
c) Effect of quantity of chemicals
d) Direct injection of chemicals onto TD tubes
e) Direct analysis of the chemical standards using GC-MS
f) Chemical standards with HS-GC-MS

4.1.2 Materials and methods

4.1.2.1 Chemicals

A standard chemical mix, based on chemicals commonly observed during decomposition, had been used previously (see section 3.7, Table 3.3). However, it was thought that the full mix would be too complex for the type of study planned and so four chemicals from the original mix were used (see Table 4.1). These were selected so as to represent some of the main categories of chemical observed during the decomposition process (alcohols, aldehydes, sulphur groups and acids as observed in the literature [4-6, 10, 15, 19, 22, 23]) and also to cover a range of boiling points and volatilities. A 1:1:1:1 equi-volume mixture was prepared, which was only mixed at the beginning of experiment. The approximate pH of all liquids was determined using universal indicator paper. This was done by paper and not meter due to the low volumes present.

Chemical	Category	Mw (g/mol)	B.P. (°C)	pH
2-methyl butanal	Aldehyde/oxygenated compound	86.134	91	4
DMDS	Sulphur-containing compound	94.19	110	5
Pentanol	Alcohol	88.15	137-139	5
Butanoic acid	Volatile fatty acid	88.11	163.5	3
1:1:1:1: v/v mixture				4

Table 4.1 Chemicals used in the study of fundamental sampling parameters

4.1.2.2 VOC headspace chambers

Several of the experiments required the monitoring of the vapours formed above the chemicals. To facilitate this, a series of lidded boxes was used (see section 2.6). The holes were left unsealed for the duration of the experiment. The aim was to provide a controlled environment where VOC levels could rise but also escape in a semi-consistent manner

For the experiments using the VOC sampling chambers, the chemicals were placed individually in four separate open 5 ml glass vials or in a single open 5 ml vial for the mixture. Typically, 50 μl of each individual chemical was used and 200 μl for the mixture (see Figure 4.1).

Figure 4.1 Chambers showing the 'individual' and 'mix' set-ups (lids removed for clarity)

4.1.3 Procedure for TD-GC-MS

For all experiments using the TD-GC-MS, 500 ml from the VOC headspace chamber was collected on a clean TD tube using the Markes EasyVoc hand sampling pump. The tubes were analysed using the standard methodology in section 2.4 and GC programme outlined in section 3.2.2, Table 3.1

4.1.4 Data processing and significant peaks

All data processing was carried out instrumentally as described in section 2.5 and using the cleaning criteria in section 3.4.5. Peaks below an abundance of 3000 (approximately 3 x baseline) were considered as 'not detected' (N/D). Peaks between 3000 and 1,000, 000 were considered '*significant*' and above 1,000,000 as 'high'.

4.2 Investigation of the time to generate and regenerate headspace VOCs

4.2.1 Aims

a) To determine the time required for a measurable quantity of vapour to generate / regenerate in the VOC chambers.

b) To compare the TD-GC-MS profiles for the individual chemicals and the mixture

4.2.2 Experimental

Experiments were performed in triplicate i.e. three VOC headspace chambers containing the individual chemicals ('individual') and three containing the mixture ('mix') (see section 4.1.2.2).

Samples were left for an initial equilibration (*generation*) time followed by a *regeneration* time (see section 3.4.4.1). 1 hour and 2 hour equilibration time and then after a further 30 mins and 1 hour were tested.

4.2.3 Results and Observations

Time after	% RSD for chemical detection as 'individual'				% RSD for chemical detection as 'mix'			
	1	**2**	**3**	**4**	**1**	**2**	**3**	**4**
A-2 hours	50.13	3.53	98.34	N/D	30.53	16.44	N/D	N/D
B-Further 1 hour	N/D	7.10	35.59	N/D	27.55	12.92	N/D	N/D

Table 4.2 showing the % RSD for chemical detection for 'individual' and 'mix' for generation times where A- generation time 2 hours B- regeneration time 1 hour and where 1-:2 methyl butanal, 2-: DMDS, 3-: pentanol, 4-: butanoic acid.

The following observations were made from the data obtained:

- Data was questionable (and thus not presented) for 1-hour equilibration and 30-minute regeneration suggesting insufficient VOC generation in these times. Therefore, further experiments used 2 hour equilibration and 1 hour regeneration were shown (see Table 4.2).
- The initial two-hour period prior to the first sampling allowed sufficient time for generation of vapour for significant peaks to be observed. However, the repeatability in peak areas between the headspace chambers was poor (>50 % RSD).
- There was considerable variation in the behaviour of the different chemicals.

In terms of each individual chemical, it was noted that:

- Butanoic acid was not consistently detected and even in the instances in which it was the peak size was very small (lower than the accepted 3000 abundance) suggesting very low concentrations. This was true when butanoic acid was as an individual chemical or part of a

mixture. The fact that butanoic acid had been detected in other experiments when smaller quantities were used may be attributed to changes in the temperature of the laboratory affecting its volatility. It may also have been due to its relatively low vapour pressure.

- DMDS was observed at high levels from all samples. This may partly be the result of the tenax/carbograph used as the adsorbent in the TD tubes as this is known to have a high affinity for sulphur compounds [24, 25]
- Pentanol was detected when the chemical was present in isolation but not when part of the mixture.
- 2-methyl butanal was detected at significant levels both when present as an individual chemical and as part of the mix for the initial sampling. However, in the subsequent sample no 2-methyl butanal was detected possibly because the chemical may have completely evaporated during the experiment or it may have been more reactive.

4.2.4 Discussion

Although of the same magnitude, repeatability was noted to be not very good between boxes. As a result, it was decided that both repeatability and reproducibility needed further investigation as well as some monitoring of the chemicals over time (see later in this section).

After the initial 2 hours equilibrating time significant peaks were seen.

- Butanoic acid appeared to be less volatile and was found to require more time to achieve a vapour pressure capable of being detected in the headspace or indeed may require a greater sample volume especially at lower lab temperatures. Further experiments with higher volume were carried out in order to see if enough was volatilised for detection if the volume is 'non-limiting' (see section 4.4). Also direct injection onto the TD tube was undertaken in an attempt to show if the butanoic acid is simply not getting into the headspace or if another factor is influencing its detection (see section 4.5).
- Pentanol was seen in significant levels in all 'individual' boxes but not seen in any of the 'mix' boxes indicating a suppression effect of the mix on pentanol and suggesting some reaction or interaction is occurring. This would require further investigation. It was thought that direct TD injection may show if it is just not being detected in the headspace or liquid injection on the GC-MS may show if a true reaction in the mix is taking place so these were investigated later in this chapter.
- DMDS was seen in consistently high levels on all boxes. This indicates a sufficient level of DMDS has volatilised from all samples including the 'mix', even following a regeneration time. Although the DMDS is consistently present there may also be a factor from the adsorbent in the TD tube as the tenax/carbograph is known to be effective at adsorbing sulphur compounds (Markes international[24, 25]) and may be showing proportionally higher levels than are truly present. Again this would require further investigation

- 2-methyl butanal is detected in significant levels for both the 'individual' and 'mix' for the initial sampling. However, after the 1-hour regeneration time it is not detected on the 'individual'. The relatively low volume has probably entirely evaporated due to high volatility and low boiling point over even this short time. Monitoring weight loss over time could confirm this (see section 4.4.2.1). The higher total volume of the 'mix' may be enough to stop this or there may be some interaction in the mix that allows its prolonged detection.

4.3 Repeatability of headspace sampling using TD

4.3.1 Aims

a) To further investigate interaction between the standard chemicals.

b) To determine the repeatability of TD air sampling for pure chemicals in chambers

4.3.2 Experimental

Experiments were performed in triplicate with three VOC headspace chambers containing the individual chemicals and three containing the mixture as detailed in section 4.1.2.2.

Repeatability was not acceptable (>50 % RSD) for low volume sampling so non-limiting volume (500 µl and 2000 µl) was used for these experiments

Samples were left for 3 hours to equilibrate and a further 2 hours to regenerate to ensure sufficient vapour pressure.

4.3.3 Results and Observations

Time after	% RSD for chemical detection as 'individual'				% RSD for chemical detection as 'mix'			
	1	2	3	4	1	2	3	4
A-3 hours	21.58	28.70	48.60	N/D	4.75	1.19	20.30	N/D
B-Further 2 hour	25.23	11.99	18.69	N/D	15.52	15.31	72.80	N/D

Table 4.3 showing the % RSD for chemical detection for 'individual' and 'mix' for generation times where A- generation time 3 hours B- regeneration time 2 hour and where 1-: 2 methyl butanal, 2-: DMDS, 3-: pentanol, 4-: butanoic acid

The following observations were made from the data obtained: -

- Repeatability was generally better for the 'mix' than for 'individual' (between 4 and 20 % RSD as compared to 21-48 respectively).
- Repeatability varied between the different chemicals with DMDS giving the best result for both 'individual' and 'mix' (with just over 1 % RSD in the mix).

- % RSD was overall higher for the regenerated headspace than for the original equilibrated headspace. However, there was less variation in % RSD between the 'individual' and 'mix' and also between each chemical after regeneration.

4.3.4 Discussion

Repeatability was very inconsistent both within an experiment and between experiments especially as compared to chromatograms from the direct TD injection (discussed in section 4.5)

Inter-chamber repeatability was generally poorer after regeneration time for the 'mix'. The chemicals themselves showed range between 'mix' and 'individual' with some chemicals, interestingly, actually showing better repeatability after regeneration. This indicates that the chemicals that present in lower headspace concentrations may require time to equilibrate within the headspace mix before consistency and repeatability is seen. The 'mix' overall produced better repeatability than 'individual' initially except for pentanol which worsened after regeneration. Indeed, pentanol does seem to produce inconsistent results.

The better repeatability seen for DMDS may have been due to higher levels volatilised in the headspace or the TD tube more consistently capturing it due to the aforementioned selectivity of the carbon for sulphur-compounds. It is interesting to note that 2-methyl butanal has the lower B.P but was worse in terms of repeatability after regeneration especially for the 'mix'. This may be due to evaporation giving rise to problems with detection.

As for other discussion points it may be that the chemicals need to be considered more individually than as a collective which is difficult in the lab environment and near impossible in the field.

More work on repeatability and indeed reproducibility needs to be done, especially in context with pork as a human model as this will aid in the validation of the SOP under development here (see Chapter 6).

4.4 Effect of quantity of chemicals

4.4.1 Aims

a) To investigate the effect of an excess volume of the chemicals
b) To monitor the chemical evaporation over time in conjunction with a mass loss experiment to determine if there is a relationship between detection of compound and mass loss
c) To determine the link between volatility and mass loss.
d) To investigate if the relationship between volatility and mass loss is affected by being in a mix in terms of increased volume or interaction.

4.4.2 Experimental

Experiments were performed in triplicate with three VOC headspace chambers containing the individual chemicals and three containing the mixture as detailed in section 4.1.2.1.

However, to ensure the standard chemical volume was non-limiting (500 µl and 2000 µl respectively) for the 'individual' and 'mix' were used (ten times larger than standard volumes). Also, to ensure the headspace volume was fully saturated and therefore non-limiting, 3 hours' equilibration and 2 hours' regeneration times were used.

4.4.2.1 Mass loss experiment

An initial mass of each vial plus sample was taken, using an Ohaus Pioneer 4 figure balance. A weight of vial plus sample was taken at each sampling event.

4.4.3 Results and Observations

Chemical	Boiling point (Bpt) (°C)	Approximate mass loss (%) (mean of 3 values)	Last detection day ('individual')	Last detection day ('mix')
2-methyl butanal	91	50	Day 1 (24 hours)	Day 5 (120 hours)
DMDS	110	near 100	Day 0 (5 hours)	Day 5 (120 hours)
Pentanol	137-139	10	Day 5 (120 hours)	Day 1 (24 hours)
Butanoic acid	163.5	12	Day 1 (24 hours)	Not detected
Mix		40		

Table 4.4 showing approximate percentage mass loss from vials at the end of the experiment (5 days)

The following observations with regard to non-limiting chemical volume and associated mass loss were made: -

- Peaks were of significant levels for analysis on day 0 and dramatically dropped after 24 hours thus only day 0 is discussed for non-limiting volume. However, for the mass loss aspect of the experiment, any level of TD detection was noted in conjunction with mass loss for subsequent days.
- Non-limiting volume - Butanoic acid was only seen on one 'individual' chamber indicating its detection is related to something other than volume (as discussed). However, the other peaks including pentanol were seen on both the 'individual' and the 'mix' chambers. Formic acid 1-methylpropyl ester was also detected initially and was also detected on 2-methyl butanal alone for HS analysis (see section 4.7) so was attributed to the 2-methyl butanal. Use of butanoic acid and 2-methyl butanal as standards meant it was possible to differentiate between these and the ester during experimentation.
- Mass loss **-** Individual components lose weight faster and thus lose TD peak detection before the 'mix'.

- The ‘mix’ loses less than the total loss of the other components and thus DMDS and 2-methyl butanal are detected much later in experiment in ‘mix’ than in ‘individual’. DMDS is detected on the TD even when its weight loss is near total**.** Pentanol was detected throughout on all and this has the lowest mass loss.

4.4.4 Discussion

It appears that the suppression effect seen for pentanol in low volumes when in the mix, is not seen when there is a non-limiting volume.

Formic acid 1-methylpropyl ester, which may be a reaction product, was initially observed but was not seen on subsequent sampling events. This may have been due to its low level so had entirely evaporated later or had not gained sufficient vapour pressure for detection.

Mass loss is lower in the ‘mix’ than ‘individual’. Mass loss also appears to not just be dependent on boiling point as DMDS is nearly all lost but has a higher B.P than 2-methyl butanal (see Table 4.1). Butanoic acid is not always detected in ‘individual’ and never in ‘mix’. It has a relatively high B.P and the mass loss observed was small. Indeed, it was only detected on days when the laboratory temperatures were observed to rise above 20 °C. This may have an effect on its vapour pressure.

Volatility and TD detection seem based on additional physical factors such as polarity, pH and interactions between chemicals in the higher volume liquid mix. The ‘mix’ loses less than the total loss of the other components and thus DMDS and 2-methyl butanal are detected much later in the experiment in ‘mix’ than in ‘individual’. DMDS is seen on the TD even when its mass loss is near total which may support the idea that it more effectively adsorbs onto the TD tube. Miscibility may have an effect as there may be layering or incomplete mixing and there could be differences in solubility between the chemicals in the mix.

Interestingly pentanol was not seen in ‘mix’ in the earlier low volume experiments but was seen here so perhaps there is a critical volume which is required to allow its detection.

When the original standard chemical volumes were non-limiting and the headspace generation time was sufficient, data was more consistent with significant peaks observed for ‘mix’ and ‘individual’. This confirms that time, volume and vapour pressure have an impact on the headspace generation and therefore detection.[26]

4.5 Direct injection of chemicals onto TD tubes

4.5.1 Aims

a) To see if all chemicals can be detected when directly injected onto the TD tube as compared to air sampling.
b) To determine optimum dilution for the chemical mix when injecting on TD tube

c) To obtain an approximate indication of LOD

4.5.2 Experimental

The four chemicals were diluted in HPLC grade methanol to make a mix with each chemical having 1 in 10 dilution (concentration was not calculated as quantification was not carried out). This was then used to make the following dilutions: -

- 1 in 100
- 1 in 250
- 1 in 500
- 1 in 1000

Previous experiments had suggested 1 µl to be an appropriate volume for direct injection onto to the TD tube so 4 ul of each of the above dilutions was used due to there being 4 components in the mix. These were mixed and then immediately injected onto the sampling (notched) end of a TD tube using a SGE Analytical Science 10 µl GC syringe.

Using the 'tube to split tube' adapter (made in-house-see Figure 4.2) the split flow on the front of the instrument was used to 'blow' the samples onto the TD tube for 10 seconds.

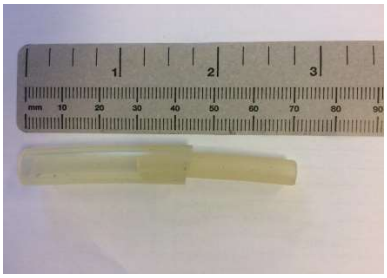

Figure 4.2 In-house adaptor for 'tube to split tube'

All tubes were run in duplicate on the TD-GC-MS as section 3.4 and with GC oven temperature programme in 3.2.2.

4.5.3 Results and Observations

For direct TD injection the following observations were made: -

- 1 in 100 produced an overload for all peaks and so was not considered. Butanoic acid was not seen for the 1 in 1000. Therefore, only the 1 in 250 and 1 in 500 were processed in more detail.
- All four chemicals were observed although the chromatographic peak for butanoic acid was very broad and distorted, making retention time determination problematic (see Figure 2.5). Unfortunately, the standard integration parameters used in the ChemStation software processed these as a series rather than an individual peak. Manual integration was not carried out to ensure experimental consistency of parameters across all analytes studied.

- Peaks are at high levels for both 1 in 500 and 1 in 250 with some overload for both. As all peaks are seen for both it was decided that 1 in 500 was the most appropriate choice as there was slightly less overload for the higher peak levels and the lower ones were still observable.
- Only the four chemicals injected were seen and no additional potential reaction products were detected

4.5.4 Discussion

The problem with integration of the butanoic acid peak meant that peak heights were chosen when processing this compound at high levels. This is not ideal but considered the only way to ensure consistency. However, at lower levels integration proved more reliable. It appears the levels for butanoic acid were higher for the more diluted sample but this is probably due to the uncertainty in data processing rather than actually lower levels of vapour in the headspace. This would need to be considered especially for any quantification work.

Overload was observed but was worse for some components than others. This suggests that some chemicals may require more dilution than others, calculated using their weights and densities, not on volume ratio alone. This would be interesting for future work

No detailed peak ratio work was carried out during this series of experiments. However, it was observed when comparing duplicates of chromatograms, that the peaks were generally more consistent in their levels between runs and as a ratio in reference to each other when directly injected onto the TD tube than when using TD air sampling. This would need to be considered as the majority of sampling will always be from air.

Only the four components injected were detected which indicates no reaction had occurred that produced a volatile product or the relatively high levels of these chemicals may have masked any low level reaction or storage products. Injection immediately following the mix means that the chemicals would be distributed onto the adsorbent inhibiting any further reaction or indeed interaction.

It was decided to directly inject into the GC-MS to see if any effects observed were due to interactions with the adsorbent of the TD tubes.

4.6 Direct analysis of the chemical standards using GC-MS

4.6.1 Aims

a) To ensure that any effects seen when using TD were not due to any variation in interaction with the adsorbent
b) To see if there had been any chemical reactions giving rise to distinct products.

4.6.2 Experimental

The four chemicals and mix were each diluted 1 in 10 in HPLC grade methanol and then serial diluted in methanol to 1 in 100 and 1 in 1000.

All the 1 in 100 and 1 in 1000 dilutions were run in duplicate using the S/SL liquid autosampler on the GC-MS portion of the TD-GC-MS system using the same conditions as section 3.2.2, Table 3.1

4.6.3 Results and Observation

For liquid injection on the GC-MS the following observations were made: -

- I in 100 showed complete overload and thus only 1 in 1000 was processed.
- All peaks were at significantly high levels and gave some overload for the individual chemicals.
- Pentanol had a particularly large peak area when injected individually but when injected in the mix was not observed at all.
- DMTS (associated with DMDS) and 2 methyl butanal were observed as well as some esters thought to be associated with butanoic acid and 2-methyl butanal storage, perhaps through their oxidation or hydrolysis, but no expected pentanol products (discussed in section 4.7).

4.6.4 Discussion

The same peak resolution issues were experienced for the butanoic acid peak as for the TD direct injection (see section 4.5).

The apparent lack of pentanol in the mix could indicate there has been a true chemical reaction and not simply an interaction that has suppressed its detection, as was suggested in the earlier experiments in this series (TD air sampling).

The experiments used an auto-sampler with the mixture at the end of a sequence of approximately 10 hours. This meant that the mix had a considerably longer period for reaction compared to the direct TD injection experiments. The unavoidable longer potential reaction time for the mix (last tubes in the run) before injection could give enough time for any chemical reactions to occur which are slowed or likely stopped early by adsorbent as with TD injection (see section 4.5.4). It was expected that polarity of the solvent (methanol) would retain the pentanol in detectable levels ready for the injection but perhaps the levels were too low (1 in 1000). It may be possible to show this by reversing the run order.

The esters seen were associated with butanoic acid and 2-methyl butanal storage and not pentanol but there was nothing in significant enough levels to account for the apparent loss of pentanol i.e. nothing that could be attributed to breakdown, oxidation or reaction products of pentanol. This may be a suppression effect due to the overall pH, polarity etc. of the mix. This would require further investigation in the future.

4.7 Chemical standards with HS-GC-MS

4.7.1 Aims

a) To determine if there is any reaction/interaction in the chemical mix observable in the vapour phase using HS-GC-MS
b) To test method development for the HS-GC-MS system
c) To compare chemical mix results to those from the TD-GC-MS system
d) To get an optimum volume for chemical use and an estimate for LOD for the HS-GC-MS

4.7.2 Experimental

Pure standard chemicals were tested individually or as a mix in capped 20 ml headspace vials. Various volumes were tested which yielded peak overload. Split ratios were changed to try and avoid overload but no conditions gave optimum peaks for all components involved so 0.5 µl was chosen. Although this still gave some overload, it was the lowest practicable volume for pipetting without dilution. For the mix, 0.5 µl of each were put in the vial but mixing was not really possible due to the small volumes.

The vials were run in duplicate on the HS-GC-MS system (see section 3.3) using a 37 °C HS oven. Different splits were attempted (20:1, 50:1 and 100:1) but to ensure butanoic acid was detected in acceptable levels the standard 20:1 split was used despite some overload on the other components.

4.7.3 Results and Observations

For the analysis of chemicals using HS-GC-MS the following observations were made: -

- All individual chemicals could be seen in significant levels when using 0.5 µl. There was still some peak overload but the lowest peak (butanoic acid) was always seen in acceptable levels.
- The 'mix' showed all four chemical peaks in significant levels including pentanol (which was absent in the 'mix' when using liquid GC-MS 'mix').
- In addition, there were also several other peaks seen that were not detected in the other experiments in this chapter. This included esters that could have been reaction products but most were probably attributed to storage (see below)
- DMTS – this had been noted occasionally using the other techniques including when using DMDS alone so could possibly be a storage product of DMDS.
- Formic acid methylpropyl ester and 2-methyl butanoic acid were seen in the mix but were also seen on HS with 2-methyl butanal individually. 2-methyl butanoic acid is a possible oxidation product of 2-methyl butanal and the ester may also be attributed to storage [24].
- Butanoic acid esters (propyl, butyl, pentyl). Work from an MSc student in the department in conjunction with the author and supervisor [27] suggests these are present from butanoic acid storage but this would require further analysis. The pentyl ester could have resulted from reaction with the pentanol.

4.7.4 Discussion

For HS analysis 'mix', the four chemicals were put into the vial together and not specifically mixed prior to reaction due to the low sample volume (i.e. the majority of the mixing would likely have occurred in

the vapour phase). All the 'mix' chemicals were detected, plus additional ones, suggesting that when the vial reached equilibrium there would be sufficient vapour pressure to allow any reactions and/or interactions to occur.

Possible explanations for the detection of pentanol here but not with GC-MS are:

- less liquid mixing so minimal liquid reaction time
- any reactions would occur in the headspace
- there was no solvent to have an effect.

Once the sample is sealed into the headspace vial there can be little influence from external factors and evaporation is prevented. Although using an autosampler, it is expected that the chemicals would all have reached equilibrium between the liquid and the gas phase before being analysed so there should be less effect from sequence order time than for liquid GC-MS, where the contents of the vials could have been subject to evaporation, further reactions etc.

It was interesting that the chemical components detected in addition to those put into the vials (discussed above) were only significantly noted when using HS analysis. The difference with TD is the adsorbent, which may not have adsorbed some of these, may have preferentially adsorbed others (thus masking those of lower levels) or it may be due to the increased reaction time in the HS where the TD effectively stopped reactions once volatiles are dispersed on the adsorbent. [28] However, the most likely explanation is the heating step in the HS oven as this could allow volatilisation to sufficient vapour pressure of some compounds that would not volatilise in the lab situation alone. In this way HS may show trace levels of lower volatility compounds while the other techniques do not.

4.8 Chapter conclusion

a) TD air sampling

This allowed the mixing of chemicals both as liquid and in the headspace. Not all components were seen in the 'mix' at low volume and suppression effects for pentanol were observed in low volume experiments which were absent when sample was in 'non-limiting' volumes. Low B.P 2 –methyl butanal evaporates quickly when in low volume individually but not in the 'mix'.

TD air sampling concentrates the sample but may mask lower level components by the preferential adsorption on the adsorbent of other components e.g. sulphur containing compounds like DMDS.

Volatilisation over time was monitored in conjunction with mass loss. Mass loss experiments showed evaporation and volatilisation to be a complex process involving factors other than just boiling point and that the 'mix' showed less evaporation overall compared with the individual chemicals in isolation either simply due to increased volume of the mix or intra-liquid interactions.

The sampling of headspace using TD is shown to be a complex process with many factors as compared to the other methodologies.

b) TD direct injection

The 'mix' only had minimal reaction time before injection onto the adsorbent when interaction between components would effectively cease. All components were seen in the 'mix'. This indicates that any not seen in the TD air sampling was due to insufficient vapour pressure of components in the headspace and not an issue with the adsorbent.

c) GC-MS direct liquid injection

All peaks were seen in significant levels but pentanol was not seen in the 'mix'. This may be due to reaction or interaction of the liquids in the vial or loss through evaporation during the long sequence times.

d) HS analysis

All peaks were seen in significant levels and some additional trace components were also detected. HS may aid in trace level detection due to the heating step. Although TD allows sample concentration, preferential adsorption effects by the tube may limit what is detected. Theoretically, HS has the potential to detect all compounds present as vapours in the vial.

All experiments described in this section used fixed quantities of chemicals. However, it is realised that in actual decomposition, these chemicals are continuously generated (and possibly consumed) by metabolic processes.

The interactions and possible reactions of chemicals even in their pure form and under laboratory conditions in a simple mix is complicated. Therefore, the decomposition matrix in field conditions, with very little control of the numerous experimental variables would suggest an even higher level of complexity difficult to account for. However, the combination of TD-GC-MS and HS-GC-MS with as much variable control as possible still shows good potential in this area of study.

Although it is an area of analysis with many variables, profiling of the chemicals associated with decomposition is still a promising area of forensic research in terms of decay studies, victim recovery and PMI estimation. Much of the previous work in this area focusses on full organisms in a natural environment [11, 18, 19, 23, 29]. In contrast this work investigates the analysis at a more fundamental level with controlled experimental conditions to hopefully provide a firmer basis for consistency of methodologies and procedures as they are moved from a laboratory setting to the field.

In this chapter, fundamental parameters with an emphasis on sampling using standard chemicals were investigated in detail for the main instrumentation techniques as the aim of this work was to establish experimental protocol that would form a basis to develop the SOPs for TD-GC-MS and HS-GC-MS. These methods, once developed, would need to be tested and attempt to be validated as well as their evaluation through their application to pork studies (see Chapter 6)

For the development and validation of SOPs for the analysis of decomposition VOCs using both TD-GC-MS and HS-GC-MS, the main instrumentation techniques have thus far been compared and contrasted in terms of their relative advantages and disadvantages for decomposition applications

throughout this thesis. The fundamental parameters work that investigated chemical standards in this chapter was used as a basis for SOP development (see Chapter 6).

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5. INVESTIGATION OF THE SCOPE OF METHODOLOGY USING PORK

5.1 Introduction

Decomposing meat is a complex matrix with many variables affecting the process.[1-9] The presence and type of substrate the meat is on was hypothesised to have an effect on rate and profile of decomposition.[9-16]

The overall aim here was to establish the scope of methodology for the development of the SOPs using the findings of this chapter in conjunction with earlier method development and the work on sampling parameters using standard chemicals.

This chapter describes three sets of related experiments utilising pork as a source of decomposition VOCs. The first set involved 'time-profiling' the decomposition VOCs using periodic sampling to follow the presence of different chemicals over 4 months during the summer.

The second set of experiments focussed on comparing larger pieces of meat and were aimed at evaluating the optimum period between VOC collection and the impact on the decomposition of the substrate (soil or sand) the meat rested upon.

The third set of experiments extended the investigation of the effect of the substrate.

5.2 Initial evaluation on time-profiling decomposition VOCs

In order to further evaluate the TD-GC-MS method discussed in the previous chapter, a series of experiments were set up with containers of different material and size and using different amounts of meat. The purpose was to investigate the effect of varying the size of container and meat i.e. the meat to volume ratio to see if their relative ratios had an impact on the VOCs produced during decomposition over time. In addition, the inclusion or not of insects was also studied.

5.2.1 Materials and methods

Experiments were set up to include different containers, different quantities of meat and with and without insect access as shown in Table 5.1. These were set up in duplicate so in total there were four experimental sets

Set	Sample number	Container type	Code	Weight of meat (g)
A-insect access	001	plastic box, 12 x 18 x 8 cm	**LBLM**	288.09
	002	plastic box, 9 x 12 x 6 cm	**SBMM**	133.35
	003	plastic box, 9 x 12 x 6 cm	**SBSM**	20.78
	004	glass jar, 125 ml	**SJSM**	22.54
B – insect access	005	plastic box, 12 x 18 x 8 cm	**LBLM**	321.04
	006	plastic box, 9 x 12 x 6 cm	**SBMM**	133.87
	007	plastic box, 9 x 12 x 6 cm	**SBSM**	23.69

	008	glass jar, 125 ml	**SJSM**	23.95
C – insect exclusion	009	plastic box, 12 x 18 x 8 cm	**LBLM**	269.34
	010	plastic box, 9 x 12 x 6 cm	**SBMM**	145.41
	011	plastic box, 9 x 12 x 6 cm	**SBSM**	31.63
	012	glass jar, 125 ml	**SJSM**	34.83
D – insect exclusion	013	plastic box, 12 x 18 x 8 cm	**LBLM**	274.68
	014	plastic box, 9 x 12 x 6 cm	**SBMM**	145.11
	015	plastic box, 9 x 12 x 6 cm	**SBSM**	28.05
	016	glass jar, 125 ml	**SJSM**	27.75

Table 5.1 Experimental set-up for meat to volume ratio experiments with and without insect access

Key: LBLM – Large box, large meat, SBMM – Small box, medium meat, SBSM – Small box, small meat, SJSM – Small jar, small meat.

Two pieces of pork (as section 2.10) were used. One piece was used for experiments A and B and the other for C and D. The air from each container was sampled using a TD tube prior to the experiment to act as a 'blank'. Meat was cut, weighed and added to the appropriate container meat down/fat-skin up and the total mass recorded. The containers for each set were placed in a tray with an over box to protect from scavengers (see section 2.6, Figure 2.6). This outer box had a 2.5 cm hole in the side which was unmeshed for A and B to allow insect access and meshed for C and D for insect exclusion. These complete sets were then placed in the decomposition location (see section 2.8) between May 2013 and August 2013. The weather and temperature were recorded by the nearby weather station.

An experimental procedure was developed as outlined below: -

i) Blanks

These were done initially by sampling each container before any meat was added and also periodically in the surrounding area.

ii) Sampling events

During each sampling event the temperature was taken on the roof using a standard laboratory thermometer (range 0-110 °C) and also periodically inside the sampling containers (to be compared with weather station data). General observations of the condition of the meat and insect activity, where relevant, were recorded. A TD sample was taken for each of the containers and periodically a blank roof sample. TD sampling was carried out using a Markes EasyVOC hand sampler and had a sampling volume of 500 ml (5 x 100 ml) was used in all cases. This was achieved by setting the pump to 100 ml and sampling 5 times. Sampling events were carried out periodically but typically twice per week.

iii) **TD Tubes**

TD tubes were sealed following a sampling event (as above) using the storage brass ends and run on the TD-GC-MS as soon as possible. In cases where the instrument was not

immediately available then tubes were stored with sealed ends in the fridge (5 °C) and run within a maximum of one week. The TD-GC-MS was operated as detailed in section 2.4 and 3.2.2.

5.2.2 General observations for the completed experiment

- **Container type 1**
  Rapid onset of decomposition (within a day). Indicated by the presence of insects where applicable, the change in appearance of the meat and the smell (simply by nose) that was observed. Moisture present.
- **Container type 2**
  Once decomposition occurred, the decomposition products couldn't drain/move away from meat sample so covered it.
- **Container type 3**
  Very quickly dried out (within 3 days) and this desiccation meant no meaningful decomposition VOCs observed throughout.
- **Exclusion of insects**
  set C and D underwent a different decomposition process in terms of smell and visual observation and the onset of decomposition was initially slower and less rapid throughout.

5.2.3 Data presentation

It was quickly realised from the general observations above that the only appropriate meat to volume ratio was the container type 1 with the larger quantity of meat (**LBLM**). All the others either dried out or the container didn't allow sufficient drainage. The results presented below are therefore only from those experiments

5.2.3.1 TD-GC-MS chromatograms

Figures 5.1-5.8 show a selection of chromatograms LBLM experiments where the containers allowed insect access (Figures 5.1, 5.3, 5.5 and 5.7) and where the containers excluded insects (Figures 5.2, 5.4, 5.6 and 5.8). These show resolution to be adequate and are representative of other experimental chromatograms.

It is worth noting that this part of the study occurred chronologically earlier into the thesis and peak area was used. Subsequent experiments showed peak height to be more appropriate (due to the reasons discussed in section 2.5) and it is thus used.

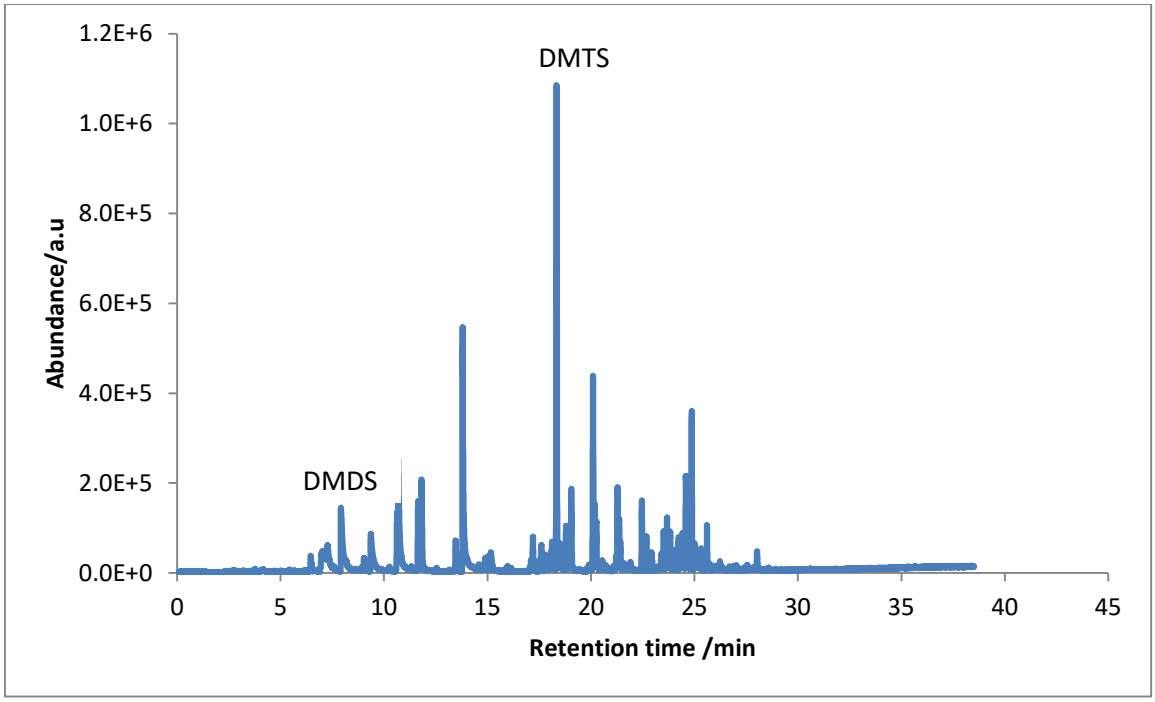

Figure 5.1 Chromatogram from TD-GC-MS showing retention time against relative abundance for day 4 of the experiment from box 001 (LBLM with insect access)

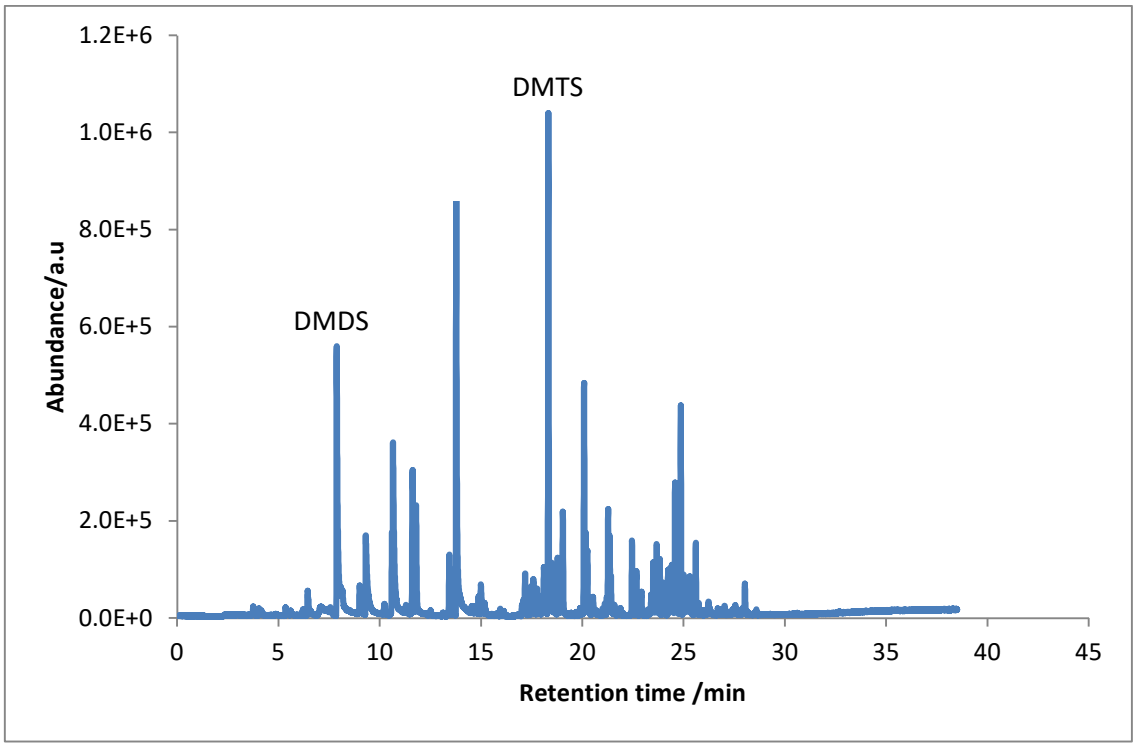

Figure 5.2 Chromatogram from TD-GC-MS showing retention time against relative abundance for day 4 of the experiment from box 013 (LBLM with insect exclusion)

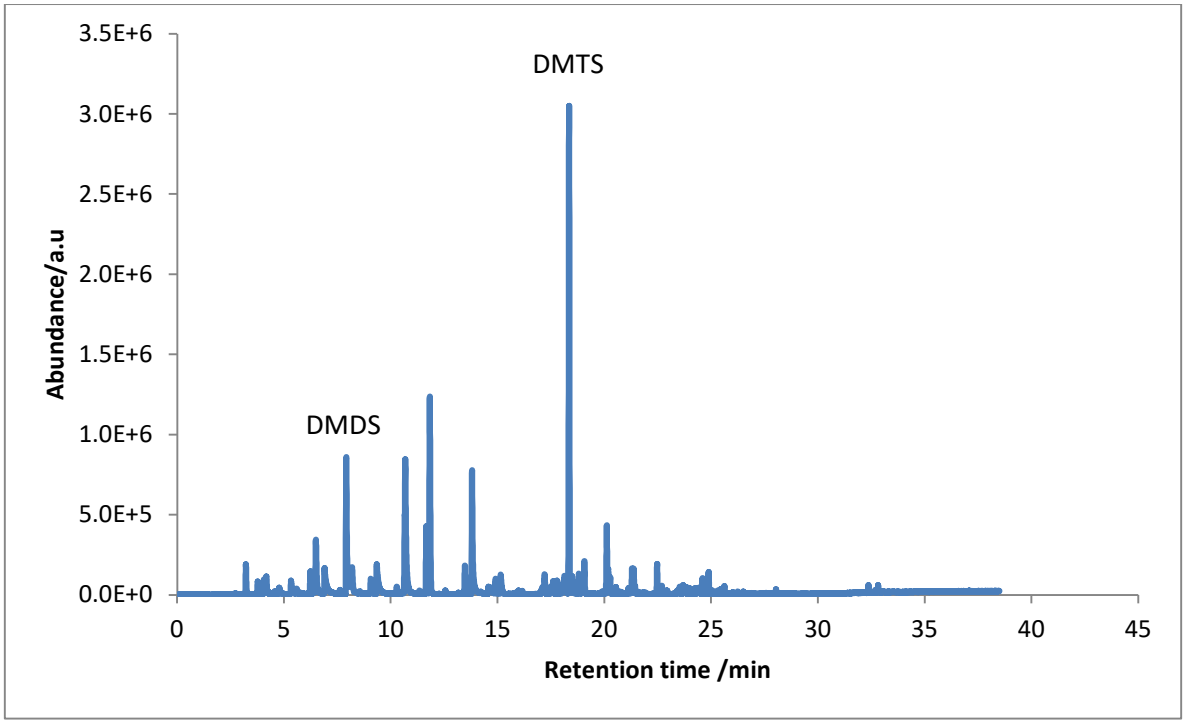

Figure 5.3 Chromatogram from TD-GC-MS showing retention time against relative abundance for day 7 of the experiment from box 001 (LBLM with insect access)

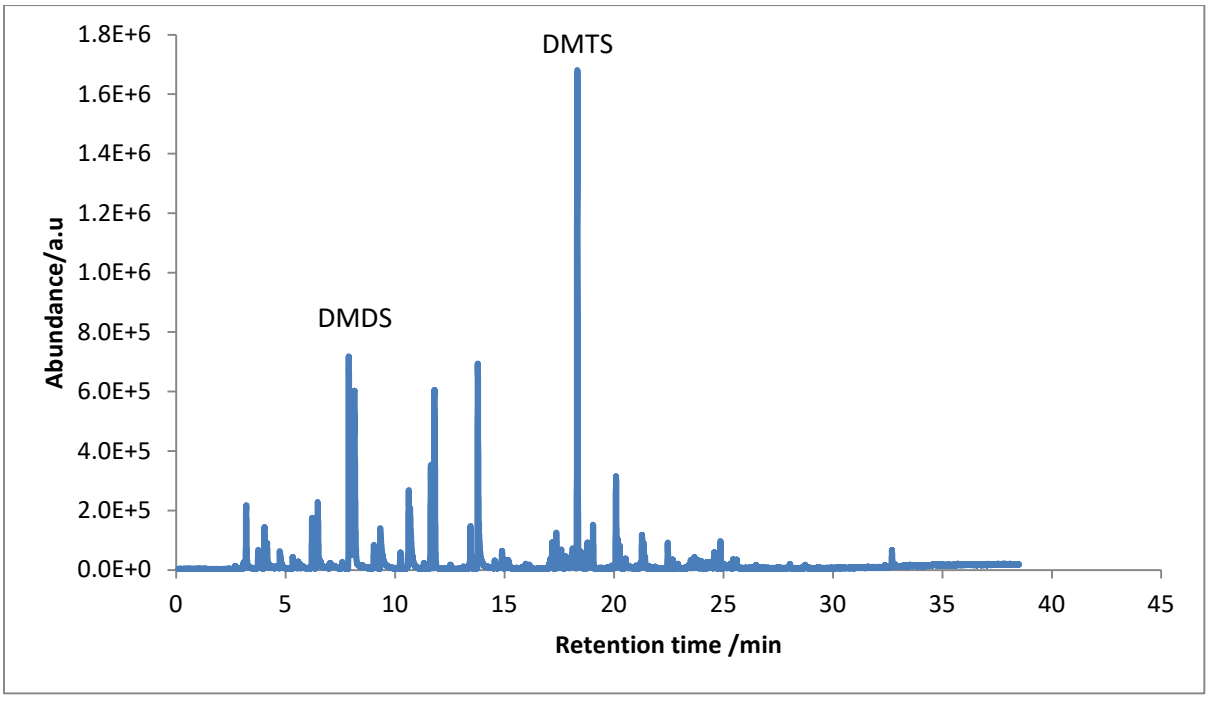

Figure 5.4 Chromatogram from TD-GC-MS showing retention time against relative abundance for day 7 of the experiment from box 013 (LBLM with insect exclusion)

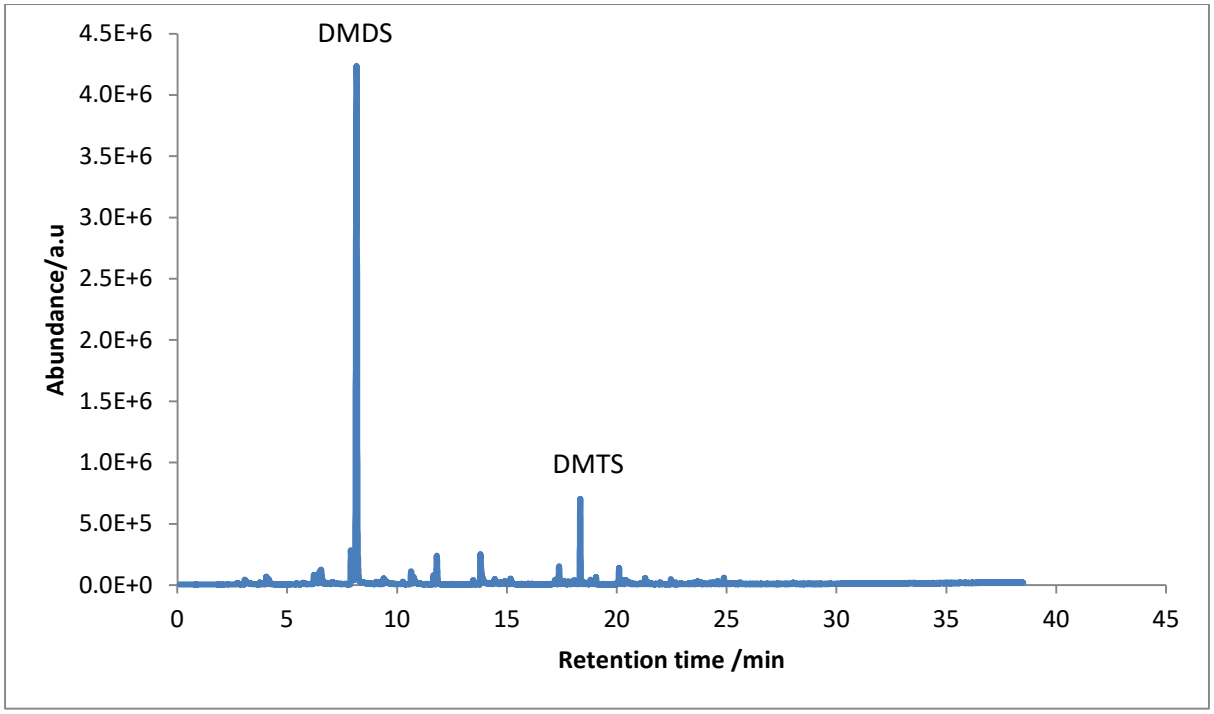

Figure 5.5 Chromatogram from TD-GC-MS showing retention time against relative abundance for day 14 of the experiment from box 001 (LBLM with insect access)

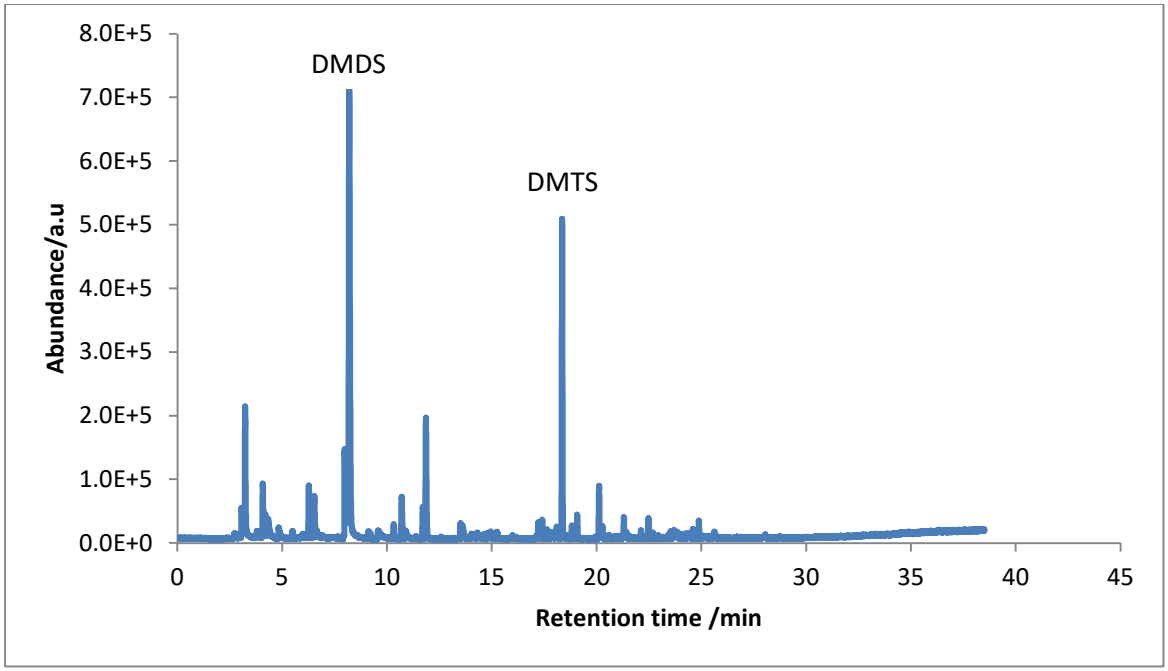

Figure 5.6 Chromatogram from TD-GC-MS showing retention time against relative abundance for day 14 of the experiment for box 013 (LBLM with insect exclusion)

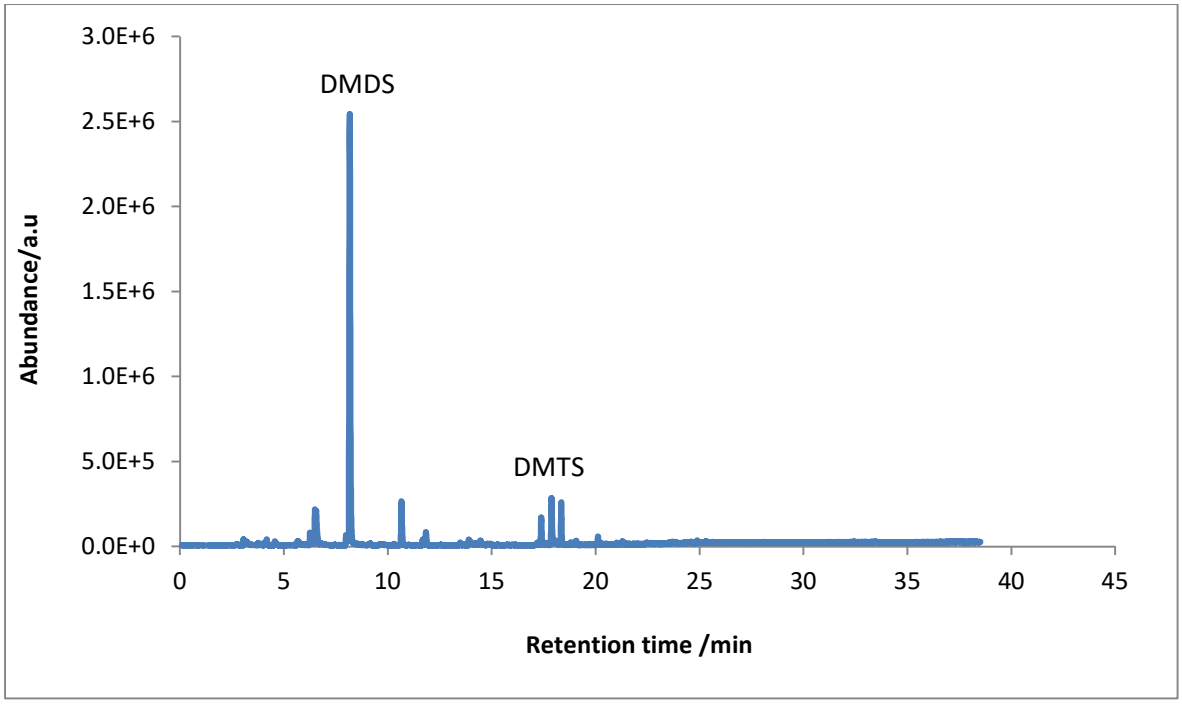

Figure 5.7 Chromatogram from TD-GC-MS showing retention time against relative abundance for day 23 of the experiment from box 001 (LBLM with insect access)

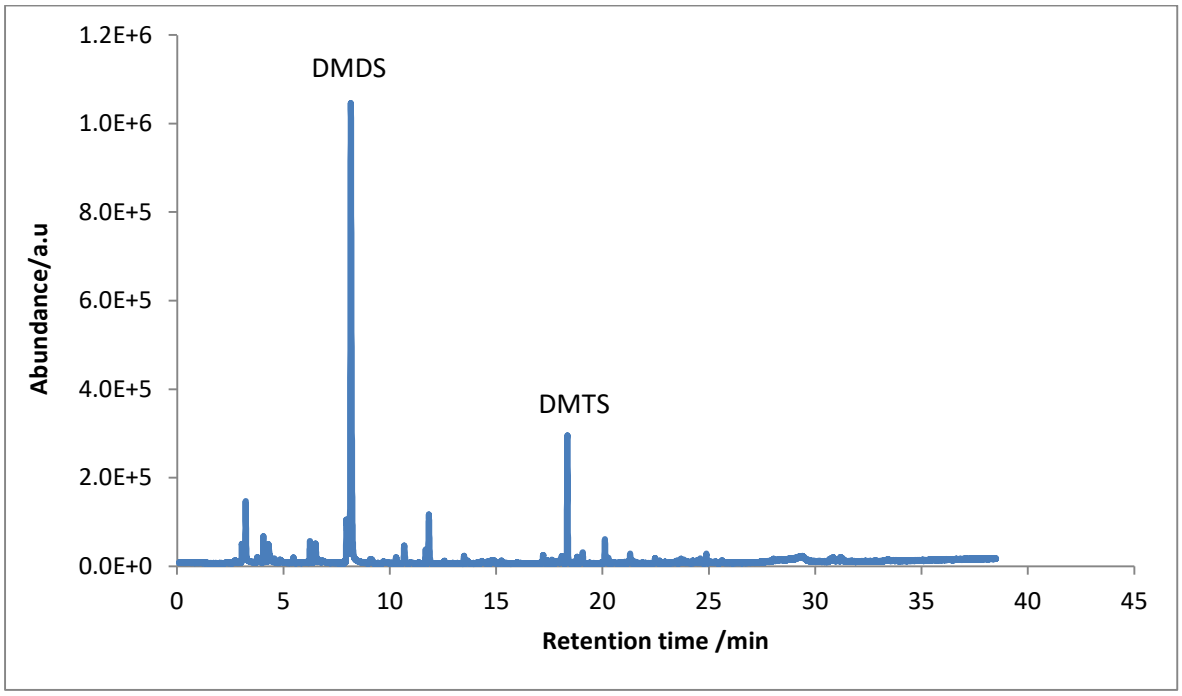

Figure 5.8 Chromatogram from TD-GC-MS showing retention time against relative abundance for day 23 of the experiment from box 013 (LBLM with insect exclusion)

Chromatographs largely indicate decomposition to be a slower in the absence of insects. The decomposition profile is different between insect access and exclusion groups. The VOCs characteristic to decomposition are evolved later and often with lower levels with insect exclusion and

the profile shows complexity of chemicals but not with those known to be characteristic for normal decomposition.

5.2.3.2 Mass spectral data

Mass spectral data analysis was done as in section 2.5. on any peaks on the chromatogram which were well resolved from background and of significant level (as discussed in section 3.4.5). Table 5.2 shows example data obtained from 001 (LBLM with insect access) experiment on day 14. This was selected because decomposition was well advanced by this time and was considered to representative of the '*active decay'* stage.

Retention time / min	Area / %	Library/ID	NIST Ref	CAS	Quality
8.15	60.14	Disulfide, dimethyl (DMDS)	2474	000624-92-0	96
18.07	0.40	1-Decene	17321	000872-05-9	96
6.21	0.75	1-Heptene	3229	000592-76-7	95
17.40	1.54	Dimethyl trisulfide (DMTS)	10606	003658-80-8	95
11.80	2.72	Cyclotrisiloxane, hexamethyl-	73123	000541-05-9	91
14.44	0.32	2,4-Dithiapentane	5103	001618-26-4	91
18.33	5.87	Cyclotetrasiloxane, octamethyl-	122481	000556-67-2	91
6.47	1.10	Heptane	3886	000142-82-5	90
13.79	3.70	1-Hexanol	4314	000111-27-3	90
10.64	1.23	Octane	7421	000111-65-9	87
11.65	0.90	Heptane, 2,4-dimethyl-	12300	002213-23-2	87
13.46	0.40	Octane, 4-methyl-	12272	002216-34-4	87
7.89	4.56	1-Butanol, 3-methyl-	2084	000123-51-3	83
20.40	0.56	S-Methyl methanethiosulphonate	10601	002949-92-0	83
4.04	0.65	1-Hexene	1427	000592-41-6	81
3.05	0.52	Trimethylamine	248	000075-50-3	80

Table 5.2 Example of the library searching results from the mass spectrum TIC for Day 14 box 001 (LBLM with insect access)-see Figure 5.5

The table shows GC retention time, peak area and the percentage quality of fit to a compound from the NIST library. Only compounds with a quality of fit above 80 % were presented. Below this are only

considered for further analysis if relevant to decomposition and even above this are only possible identifications to be examined further. Only confirmation using GC retention time for known chemical standards would categorically confirm an identification.

From the chromatographic and mass spectral results a main peak of interest was identified. It was present in high levels from day 7 onwards and throughout, in both insect access and exclusion experiments and was identified as DMDS using the NIST library. From this it was decided to monitor DMDS and DMTS (related to DMDS) over time (as separate profiles). DMDS and DMTS are both associated with decomposition as well as TMA, 1-hexanol and 1-butanol, 3-methyl .[1, 3, 13, 17, 18] The other compounds would make up a profile unique to a decomposition event and although not the main compounds, may add to the subtle profile. Siloxane compounds are known to be associated with column bleed which is expected (and accepted to an extent).

Figure 5.9 shows an example of how VOCs can be time-profiled. The graph shows two sets of results (with and without insects) for DMDS which was very abundant on the chromatograph from day 7 in both insect access and exclusion experiments for 001 and 013.

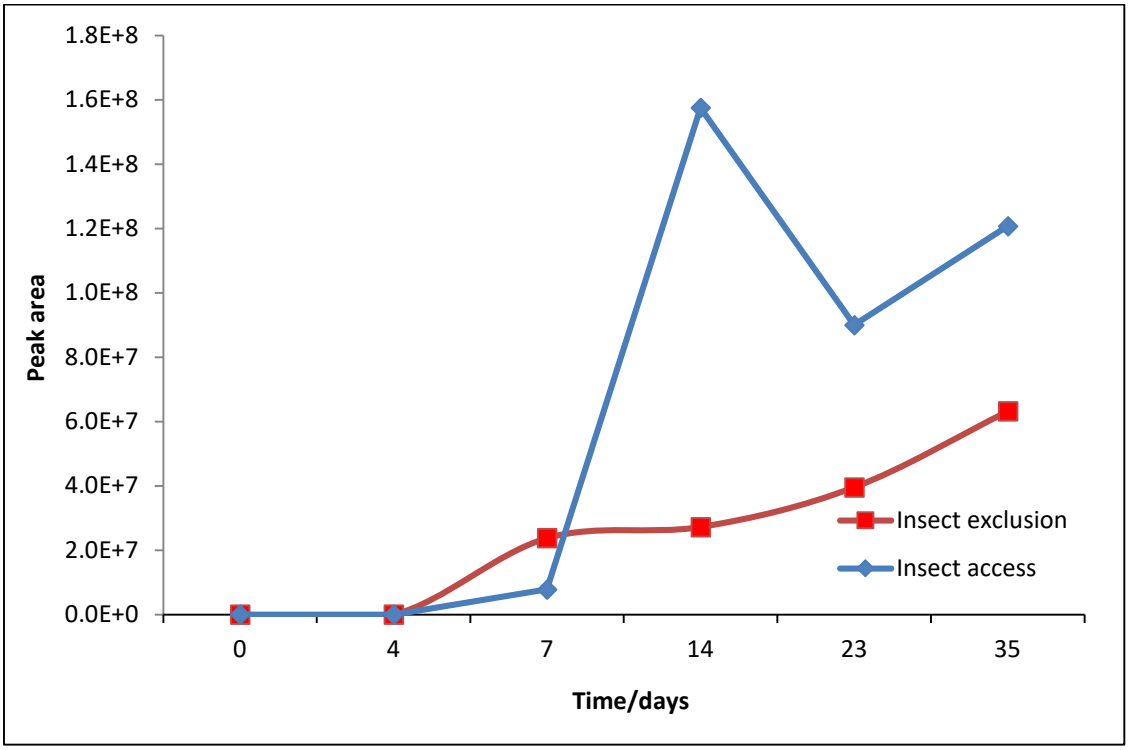

Figure 5.9 Levels of DMDS in terms of peak area from the TD-GC-MS chromatograms over time for box type LBLM (insect access and insect exclusion experiments)

It can be seen that the onset of decomposition, as revealed by the presence of DMDS, occurs after a similar amount of time (5 days) and continues to rise for both experiments. However, the importance of insect activity in accelerating the decomposition process is clearly apparent. This effect can be attributed to a combination of the direct action of maggots digesting the meat or indirectly through the changes in microbial activity [12, 13]

Figure 5.10 shows another example of VOC time profiling. The graph shows two sets of results (with and without insects) for DMTS which was seen in both groups from day 5, generally decreasing with time, following an initial rise, with insect access and increasing with time in the absence of insects (box 001 and 013)

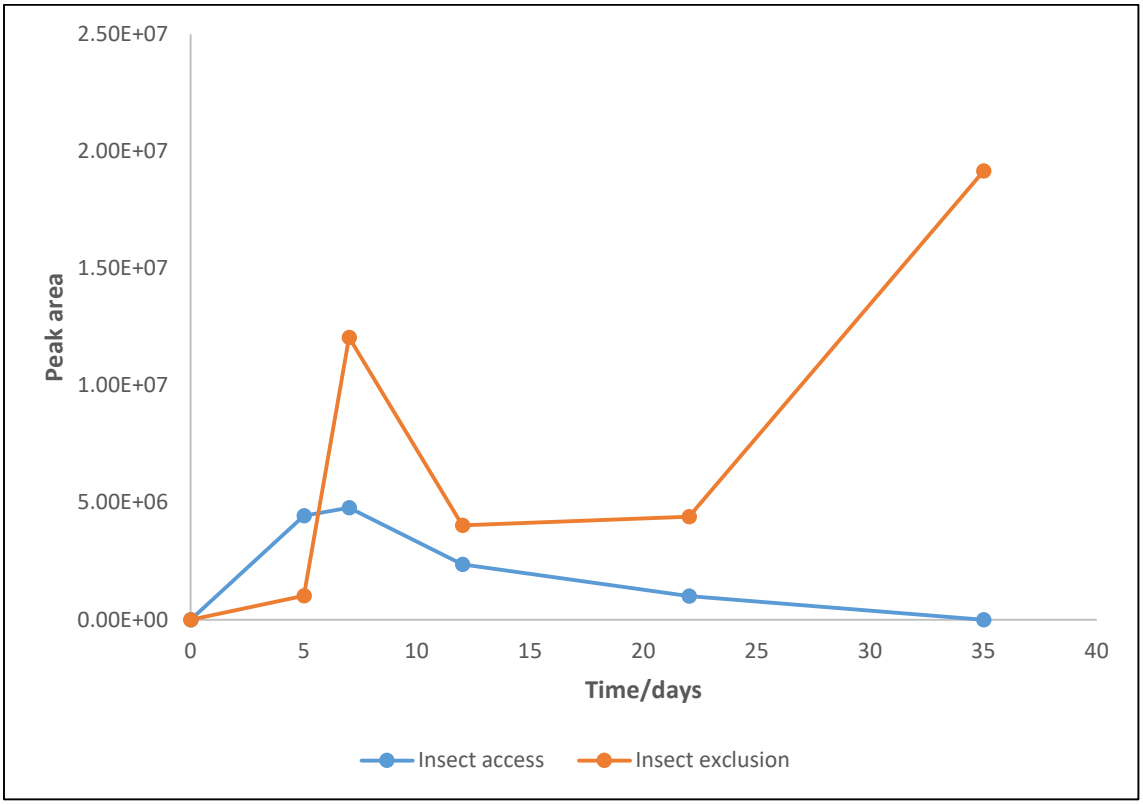

Figure 5.10 Levels of DMTS in terms of peak area from the TD-GC-MS chromatograms over time for box type LBLM (insect access and insect exclusion experiments)

5.2.4 Discussion

The experiments were performed over the summer period where maximum temperatures often exceeded 20 °C. Thus the onset of decomposition was quite rapid (within a day) for both set-up boxes but the overall decomposition process was slower in the absence of insects and different in terms of chemicals given off, shown with time profiling of the sulphur-containing VOCs, DMDS and DMTS. These were chosen as the component for time profiling (see Figure 5.9 and 5.10) as they were present in significant levels and have been repeatedly reported in the literature associated with decomposition. [12, 15, 19-24 25]

Insect activity has a big impact on decomposition both in terms of rate and the type of decomposition.[1, 3, 13, 17, 18] and this could explain the slower rate seen in the production of DMDS in the insect exclusion group as compared to that of insect access. DMTS generally decreased with insect access but

increased in their absence, illustrating the difference between profiles of the two groups. This preliminary study suggests the importance of the presence of insects for a realistic decomposition profile which supports the findings of other workers [1, 3, 9, 13, 16-18, 23, 26-32]

As a preliminary study primarily designed to evaluate the method with both relatively infrequent sampling and lacking internal standards for quantitation, only limited interpretation of the results is possible. However, they do confirm that time profiling of decomposition VOCs is feasible although ideally this would require the use of multiple chemicals either in terms of their individual concentrations or expressed as ratios with each other.

5.3. Establishing effect of size, substrate and sampling interval

The preliminary studies described above indicated that the clearest results were obtained when using larger pieces of meat and the larger plastic boxes. There was clear balance between there being sufficient meat to decompose in a realistic manner and produce the concentration of VOCs in the headspace of the container for reliable analysis and allowing sufficient to drain away to resemble the more realistic conditions found in nature.

If the large meat was contained in too enclosed a box and the decomposition products are allowed to build up too much then the VOCs could be in such a high concentration that they would no longer represent those in the natural conditions which could give rise to extra reactions not normally seen.

Using this as starting point, a similar set of experiments was then undertaken to further investigate the role of sample size, the effect of substrate and the sampling interval.

Spectra and data analysis are not presented as this trial suggested the need to investigate the effect of substrate in more detail.

5.3.1 Materials and methods

All the experiments used the plastic boxes as described in section 2.6 (see Figure 2.7). Two experimental parameters were investigated, mass: 500 g or 1000 g and substrate: sand or nothing. Each of these four categories was set up in triplicate and a further empty box was used as a blank. All VOC sampling followed the protocol given above but using the ActiVOC pump to collect 2000 ml. Insects were allowed access to ensure realistic decomposition.

In addition, the impact of 3 sampling intervals: 1, 2 and 3 hours, on the regeneration of VOCs in the headspace was investigated. A sample was taken initially and then another from the same box after an interval as specified above.

5.3.2 General observations

- **Size**

  The results of the experiments indicated that there was no significant difference between using 500 g and 1000 g of pork. Therefore, the smaller 500 g quantity was used for all subsequent experimentation.

- **Substrate**

  The experiments confirmed the importance that substrate had on the decomposition process both in terms of how fast it occurred and the complexity of the VOC profiles produced. This was considered significant enough to justify further investigation in a dedicated experiment.

- **Sampling interval**

  There were some differences between levels seen after both 1 hour and 2 hours compared to initial but no significant difference between 3 hour and initial (see Table 5.3).

5.3.3. Data presentation

- **Size**

The stacked chromatographs below (Figure 5.11) show the 500 g (black) and 1000 g (blue) pork with no substrate. The difference in levels between the two masses can be seen to not be significant in terms of VOCs present and their abundance.

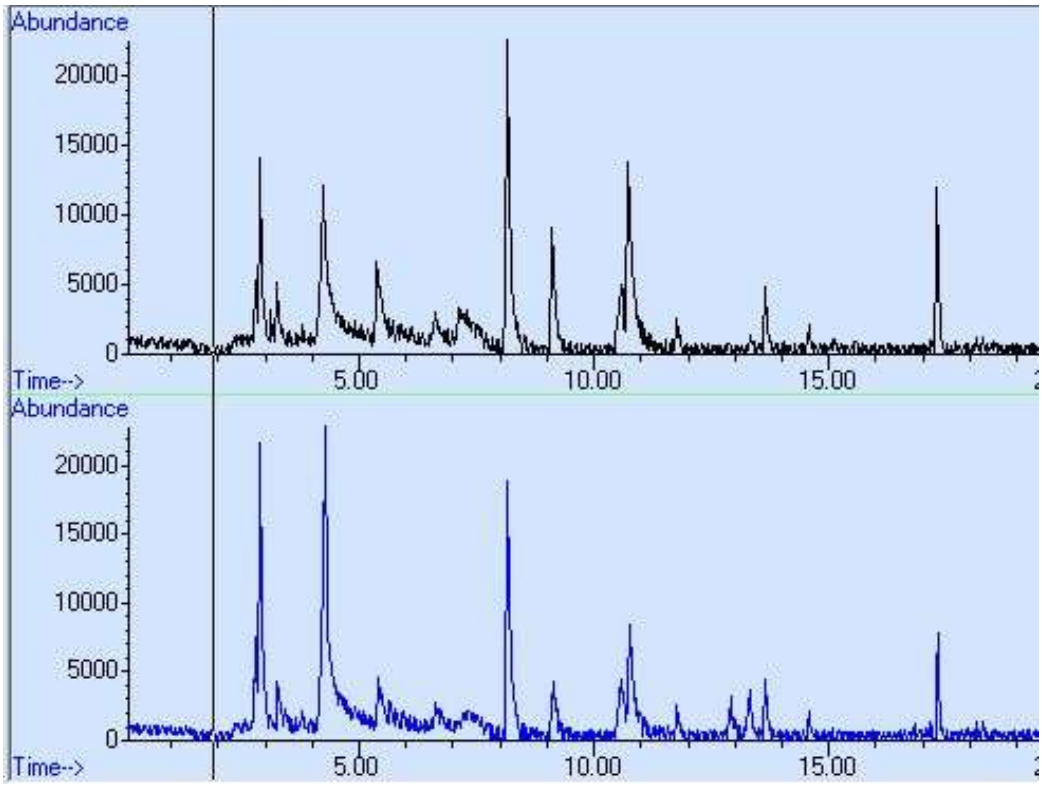

Figure 5.11 showing the difference between 500 g (black) and 1000 g (blue) pork with no substrate. This is a screen shot from the ChemStation software

- **Substrate**

Data is not presented as it was clearly observed to warrant further investigation (see section 5.4).

- **Sampling interval**

The table below (Table 5.3) shows levels of three key chemicals for initial sampling and subsequent sampling (from the same box) after three separate sampling intervals (*regeneration* times). Differences can be seen between the subtle (lower level chemicals) although not in the major components (high level) for both 1 and 2 hours but not for 3 hours. This shows the need for a minimum of 3 hour sampling interval to allow headspace regeneration.

Sampling interval	**Chemical**		
	TMA	**DMDS**	**DMTS**
Initial	28508	2937086	91453
After 1 hour	19343	2465713	0
After 2 hours	19633	2851940	18627
After 3 hours	23424	2953523	92664

Table 5.3 showing the effect of sampling interval on chemical levels

5.3.4 Discussion

Size of carcass has been shown to have an effect on decomposition by previous workers [1, 9-13, 15, 18, 22, 23, 25, 26, 31-46] so it was important to investigate this variable. Although the mass used in this study was relatively low its importance has been shown throughout. Below a certain critical mass (100 g) of meat it dries out and the volume of headspace surrounding the mass has an effect, with the relative ratio of the two affecting volatile collection.

An additional factor governing the mass of pork selected for subsequent experiments was the overall 'olfactory impact' in the relatively limited space of the roof area used for decomposition studies. A taphonomy project was using the space at the same time but utilising whole organisms and the overall decomposition VOC production was sufficient to unrealistically distort the entomological profile in the area. In addition, the 'smell overload' also caused offence to inhabitants of the building and so of course needed to be avoided in the future. Using the minimum quantity of meat consistent with obtaining usable results was considered sensible and thus 500 g was used for subsequent experiments.

Presence of substrate was clearly shown to have an effect on profile and rate of decomposition so that was further investigated in the next part of this chapter (section 5.4).

From the results based on sampling interval, differences can be seen between the subtle (lower level chemicals) for both 1 and 2 hours but not for 3 hours but not for DMDS that is at high levels. There was no difference between major levels as it probably allowed sufficient vapour pressure and thus detection anyway. Therefore, it was concluded that for multiple samplings of the same box a minimum 3 hours regeneration time was required. In terms of method development and evaluation,

variations in VOCs arising from partially exhausted headspaces was not overly important as each container was treated as a replicate. However, it was noted that any potential SOP may need to consider the implications of sampling interval.

5.4 Further investigation of the effect of substrate on decomposition

In the previous experiments, the effect of substrate on the decomposition was observed to be sufficiently significant to merit further investigation in isolation with other parameters being fixed.

5.4.1 Materials and methods

500 g of pork was used in the standard containers as described in section 2.6 (See Figure 2.7). Three substrate conditions were investigated: none, sand and soil. Each substrate experiment was performed in triplicate with a further empty container used as a blank. The containers were filled such that the bottom was covered to a uniform depth of 1 - 2 cm with either sand or soil. The soil was all-purpose compost dried overnight at 105 °C. The sand was as described in section 2.7. Insects were allowed access for a realistic decomposition scenario.

5.4.2 General observations

It was noted that the decomposition was faster for meat in the absence of substrate than when it was on either sand or soil. This was probably due to the substrates either adsorbing the ‘decomposition fluid’ or at least preventing the remaining meat from standing in liquid as occurred in the absence of substrate.

5.4.3 Data presentation

Figures 5.12 to 5.14 show the VOCs detected presented as average peak height (for the 3 boxes used), over a 28-day period in the absence of substrate and with sand and soil as substrate. Error bars were obtained using a mean average of 3 boxes and presented as ± 1SD. All peaks associated with decomposition and of significant level were presented.

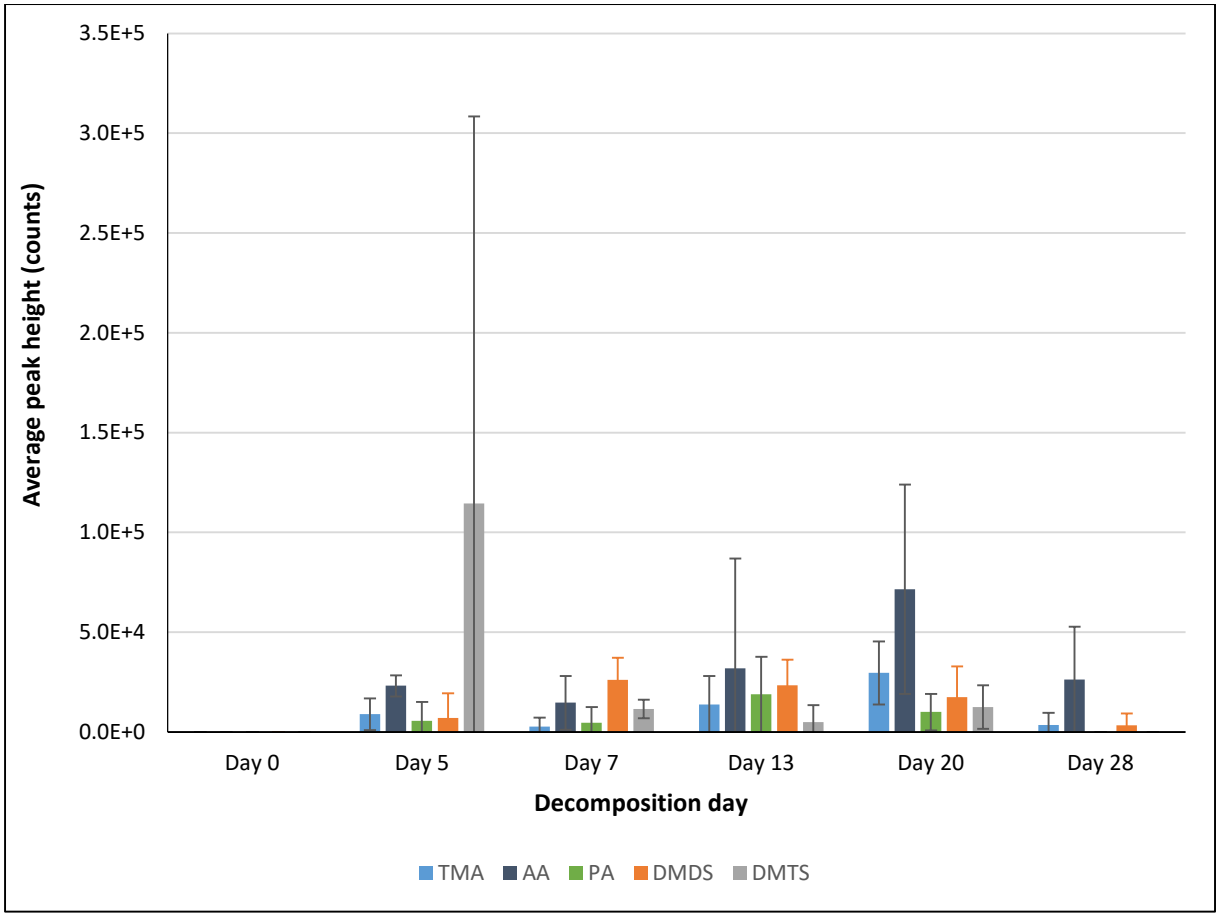

Figure 5.12 Levels of decomposition chemicals seen over 28 days with no substrate

Figure 5.12 shows the levels of various VOCs associated with decomposition in the absence of a substrate. Day 0 had no peaks detected. Decomposition is well underway by day 5. The VOC profile observed as compared to those with substrate is complex and remains so throughout. DMDS changes in its levels throughout the process.

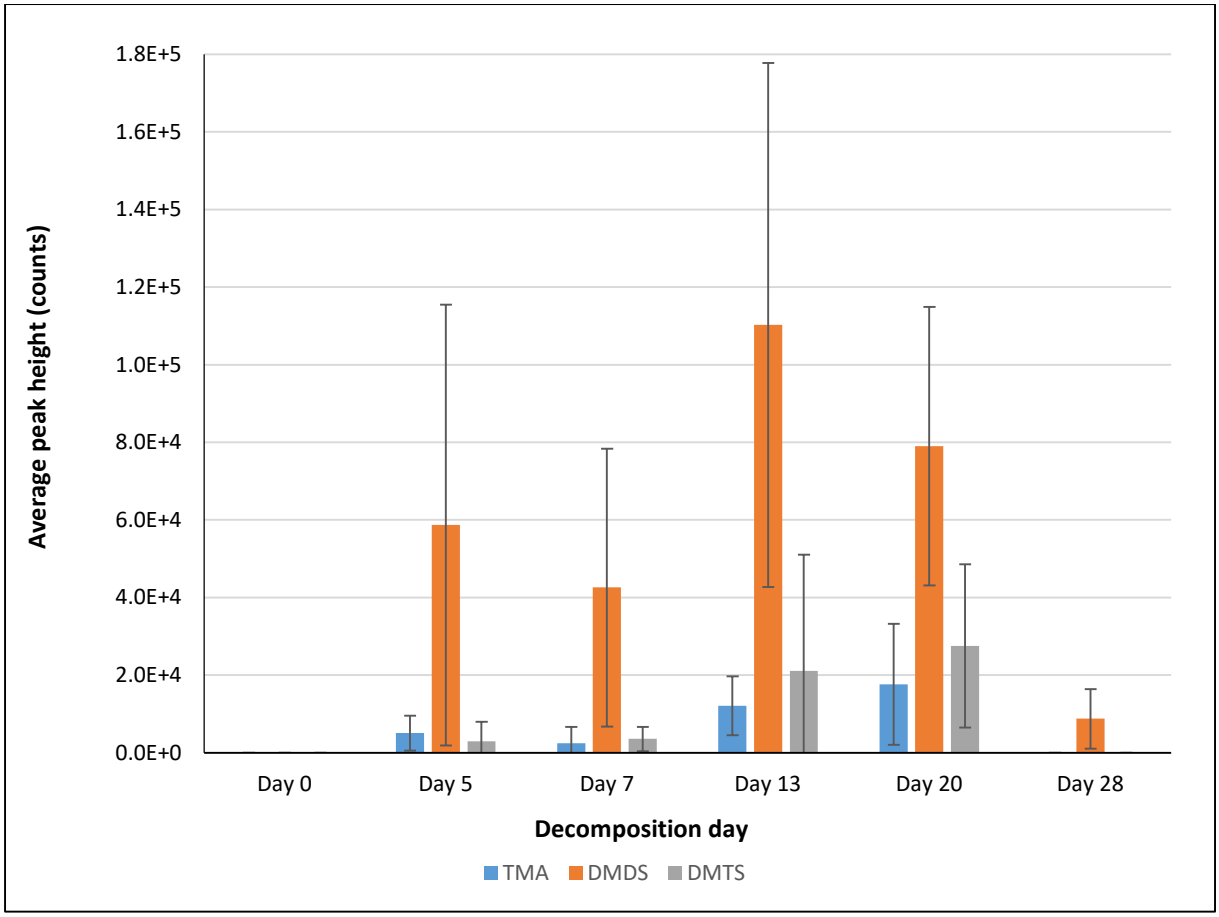

Figure 5.13 Levels of decomposition chemicals seen over 28 days with sand as a substrate

Figure 5.13 shows the levels of 3 decomposition VOCs when sand was used as a substrate. Day 0 had no peaks detected. It shows a less complex profile than that of no substrate (Figure 5.12) but decomposition is still observed to be underway by day 5 and DMDS can be observed in significant quantities throughout.

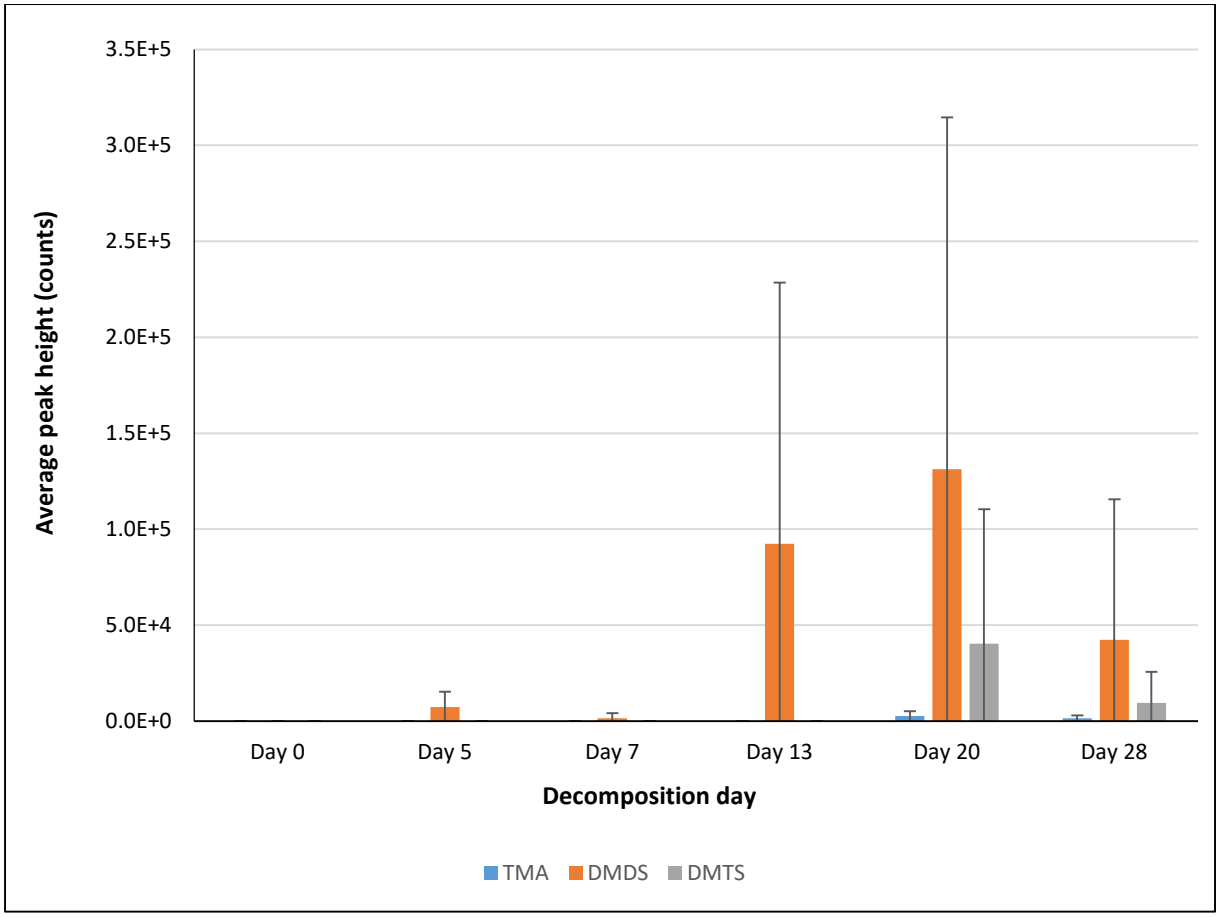

Figure 5.14 Levels of decomposition chemicals seen over 28 days with soil as a substrate

Figure 5.14 shows the levels of 3 decomposition VOCs when soil is used as a substrate. It shows a less complex profile compared to no substrate (Figure 5.12). Day 0 had no peaks detected. Decomposition is apparent on day 5 but DMDS is not seen in significant quantities until day 13. Error bars for figures 5.12-5.14 were large.

5.4.4 Discussion

Error bars were large for this experiment. Indeed, it has been observed throughout this project that decomposition as a natural event can give rise to great variation in terms of profile and levels even between boxes at the same stage and under the same controlled conditions. This supports work by previous authors about the complexity of decomposition and the difficulty observed in variable control.[1, 3, 6, 8, 9, 12, 13, 15, 17, 18, 23, 26-28, 33, 34, 46-53] Although multiple boxes under the same condition have been treated previously as a repeat or replicate, it is probably more appropriate for these to be thought of as simply population number (n). Subsequent experiments during the SOP development and testing therefore will have repeats taken from the same box, allowing for regeneration time.

The results confirmed the importance of substrate and its absence altered both the rate of decomposition and the number of VOCs detected. Although a more complex matrix than the sand, the soil gave the simplest VOC profiles of the two. Conceivably, the soil contained or developed microflora

that degraded the chemicals further or entrapped them through adsorption. The sand would disperse liquids but its general chemical inertness would be less likely to degrade chemicals directly.

Although soil is a more realistic substrate than sand in terms of a 'real life' decomposition event the latter was adopted for subsequent experiments, it was felt that sand would allow for drainage, possibly the key factor in impact on decomposition when relating to substrate, but not add additional variables, (for example soil microbiology, pH, oxygen levels) that the use of soil would bring. [2, 6, 12, 14, 23, 25, 33, 34, 36, 38, 45, 46, 54] It is thought for profiling VOCs, reducing the complexity of profile would not have a negative effect as it is likely one or two key compounds e.g. DMDS would be used.

One other point to note is the presence of long chain alkanes later in decomposition (not presented) which may be due to accumulation of bio-macromolecule breakdown. These are in much higher levels with no substrate indicating the possibility that as they cannot drain away they may affect the decomposition process. Some further investigation into substrates and how decomposition liquid interacted with them was undertaken as part of a 10 week MSc project under the supervision of the author and Dr Gareth Parkes (supervisor). [55] This work indicated that more chemical reactions occurred in the soil than in sand but that more conclusive results would require a more detailed study.

5.5 Chapter conclusion

During scope investigation using pork, it was indicated it may not be necessary to further investigate the link of VOC profile to PMI for the development of SOPs, although this could be possible future work. What is more important when developing SOPs is to enable the fundamental study of decomposition using TD-GC-MS and HS-GC-MS to enable this work to be accurately compared to similar studies and applications to allow their evaluation.

Work so far in this thesis has been focussed on method development and establishing sampling parameters appropriate for use of TD-GC-MS and HS-GC-MS for the SOP development.

This chapter allowed the rationale for choice of type and size of box, size of meat, presence of insects and presence and type of substrate to add to work necessary for SOP development.

In conjunction with sampling parameters (Chapter 4) and earlier method development (Chapter 3) it was possible to establish the scope of methodology to move onto development of SOPs for study of decomposition.

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5.6 References

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6. DEVELOPMENT AND VALIDATION OF STANDARD OPERATING PROCEDURES (SOPs)

6.1 Introduction

This chapter describes the process and rationale for the SOPs development by the author. There are two SOPs (one for TD-GC-MS and one for HS-GC-MS) embedded within the chapter itself. These are given a different font for clarity. The methodology for the development of the SOPs has been evaluated and implemented as an iterative process (using draft versions of the SOPs which fed back into SOP development) throughout this thesis to allow the testing and validation of the 'working versions' of the SOPs given below (see section 6.3).

6.2 Development of the SOPs

The overall aim of the research was to develop SOPs that would provide a more consistent approach to the collection and analysis of VOCs given off during decomposition under laboratory conditions. A longer term goal is that these SOPs would provide a framework for the study of decomposition VOCs in the field for the forensic community involved in taphonomy research including victim recovery (VR) and PMI estimation. The underlying basis of the SOPs was the idea that if conditions were controlled as much as possible for pure chemicals and on small scale pork studies in an 'ideal' laboratory setting then any insights into VOC collection and analysis could eventually be extended to the more complex 'real life' cases.

Following initial studies to establish the necessary instrumental and sampling parameters, preliminary versions of the SOPs were devised. These were continuously developed iteratively until 'working versions' were in sufficiently advanced stage for validation testing. Validation is the process of checking that an analytical procedure is fit for purpose in terms of quality, reliability and consistency.[1, 2] This is an important part of good analytical practice. Validation of the SOPs here, by applying the principles of repeatability, reproducibility and associated accuracy and precision, would give them more weight for their implementation in the further scientific community.[3, 4]

The basis of the 'working version' of the SOPs comprised the methods developed in earlier chapters and was adapted and modified throughout where appropriate and necessary by the author. The SOPs in section 6.3 were the versions used for testing SOPs for development and robustness by the author and the version given to validation participants.

The actual SOPs themselves were based on the format for BSI (British Standards Institution) standards [5] using a method most similar to the techniques used here. The SOPs are designed to stand alone but, as is common, cannot replace laboratory experience and training and are intended for use by competent analysts only. For any more detailed methodology queries it is possible to refer to the thesis in which this chapter is contained (particularly Chapters 2, 3 and 4). Any Standard Operating Instructions (SOIs) that were used in the laboratory with the particular instrumentation are discussed elsewhere in this thesis and could be obtained from there as supplementation to the SOPs if required.

Health and safety is always an important consideration but is not included in the SOP format chosen here as it is assumed that analysts would adhere to good laboratory practice and would carry out their own COSHH and risk assessments as required. However, it is important to note that participants in the validation stage (see section 6.5) were made aware of the odourous nature of the chemicals and were encouraged to dispose of chemical contaminated waste accordingly, double bagged where possible.

6.3 SOPs

The two SOPs given below are the versions used for testing and validation purposes ('working versions').

STANDARD OPERATING PROCEDURE FOR DETECTION OF DECOMPOSITION PRODUCTS BY THERMAL DESORPTION GAS CHROMATOGRAPHY MASS SPECTROMETRY (TD-GC-MS)

1. Scope

Decomposition products in the scope of this method are VOCs given off during the decomposition process using pork as a human model and standard chemicals for method evaluation and validation.

2. Principle

Decomposition standard chemicals are pipetted into vials and placed in sampling boxes and then the sample is pumped onto TD tubes to test the methodology and instrumentation. VOCs evolved are measured using TD-GC-MS. Evaluation of the method is done using standard chemicals and then applied to controlled amounts of pork loin as human model with a view to apply to field decomposition samples.

3. Reagents

Use only reagents of recognised analytical grade. Methanol for dilution to be of HPLC grade.

- **Pentanol** (CAS 71-41-0)
- **DMDS** (CAS 624-92-0)
- **2-methyl butanal** (CAS 96-17-3)
- **Butanoic acid** (CAS 107-92-6)
- **Methanol** (CAS 67-56-1)
- **Mix**
    - Place 50 µl each of pentanol, DMDS, 2-methyl butanal and butanoic acid into a 5 ml lidded sample vial using a 20-200 µl autopipettor.
    - Mix the contents well using a vortex mixer (>10 secs).
    - The solution is to be made fresh on each day of analysis.

4. Materials

Controlled laboratory decomposition studies are to use pork loin as a human model.

- **Pork loin-** food grade and to include flesh/meat and fat. Not in nitrogen protective atmosphere.

  Cut pork into approximately accurately 500 g pieces. Any string from butcher preparation to be removed.

- **Sand-**British play sand (with particle size range of 63 microns to 1 mm/majority 125-355 range with sub-rounded grain shape to avoid abrasion) to be dried in an oven at 105° C for 5 hours.

5. Apparatus

Usual laboratory glassware and equipment and, in particular, the following:

- Sampling boxes - Plastic boxes with clip lock lids (3 l Really useful 22 x 17 x 15 cm) or equivalent with 6 mm and 2 mm holes drilled
- Laboratory balance capable of masses up to 2 kg (2.d.p)
- Laboratory grade drying oven
- 20-200 µl autopipettor
- Vortex mixer
- Markes International ActiVOC electric TD sampling pump or equivalent.
- Flow meter
- Gas chromatograph with mass spectrometer detector coupled to GC and with Thermal Desorption autosampler unit (Agilent Technologies 6890N GC system and 5975 MSD with Markes international TD-100 or equivalent).
- Column – fused silica capillary column suitable for analysing VOCs
    - Zebron ZB-5MSi Dimensions - 30 m x 0.25 mm x 0.25 µm
    - Phase chemistry – 95 % dimethylpolysiloxane, 5 % phenyl
    - Temperature range – isothermal - -60 ˚ C to 360 ˚ C, TPGC - - 60 ˚ C to 370 ˚ C or equivalent.
- Gases – analytical cylinder grade
    - Carrier gas: Helium
    - Purge gas: compressed air
- TD tubes - Markes international Tenax TA/Carbograph or equivalent.
- Laboratory thermometer: -range -10-110 °C
- Stopclock with count up timer

6. Procedure

6.1 Preparation of standard chemicals for evaluation

No certified reference materials (CRMs) exist for decomposition studies so standards are chosen from previous preliminary studies and literature searches.

Standards are to be prepared for evaluation in the laboratory setting to show the effectiveness of the sampling and analysis techniques for use with laboratory samples and consequent application to field study. Standards are to either be separate or mixed prior to their release into the sampling box headspace.

For separate, transfer 50 µl of each standard chemical into 4 x 5 ml sample vials, using a 20-200 µl autopipette and place all 4 vials unlidded in the same sampling box. For mixed, use 200 µl of mix. Both conditions are to be produced in triplicate. Allow 2 hours for reaction and equilibration of chemicals in the sampling box headspace prior to sampling and a further 1 hour regeneration time for subsequent sampling

Standards are to be analysed and both conditions compared in triplicate and with 3 full repeats to check repeatability using TD-GC-MS

Blank samples of the box are to be taken.

6.2 Controlled laboratory human model studies

6.2 1 Preparation of samples

Pork loin is to be purchased from a butcher as soon as practicable (ideally on the day) before experimentation commences. Sampling boxes are to contain an approximately 1 cm layer of dried sand and be weighed including the lid before and after addition of pork. Pork loin to be placed meat side down and fat side up in the centre of box on top of the sand. Blank box; box plus sand. Day 0; box, sand and pork. Samples for TD to be taken.

Boxes are to be made in triplicate and to include protection from scavengers, placed outside in an area appropriate for decomposition studies and with the ability to receive weather station data. Boxes are to be weighted down with bricks. Blank box is also to be placed outside and an environmental blank to be taken for TD analysis. Temperature is taken.

6.2.2. Decomposition studies

On each sampling occasion temperature and general weather observations are noted. Observations are taken for the general condition of the pork and presence of any insects. Olfactory observations may be made by the analyst. An environmental blank and sample are taken for TD analysis. This is to be done in triplicate for each box. Due to the requirement for regeneration time these are done 3 hours apart, ideally 9 am, 12.30 pm and 4 pm. These are to be assumed as replicates.

6.3 Sampling procedure for TD

6.3.1 TD pump calibration

Using a calibrated flow meter, attach to sampling end of ActiVOC pump and set to 100 ml/min following the manufacturer's instructions.

6.3.2 TD pump sampling

TD tubes are uniquely identifiable by number and that should be noted. Remove both brass storage ends from the TD tube and attach the non-notched end to the sampling end of the ActiVOC pump and present the notched (sampling) end to the sample via the 6 mm hole in the sampling box. Set the pump to run for:

A) 5 mins (500 ml) for standard chemicals

B) 20 mins (2000 ml) for samples.

The samples shall have brass ends replaced following sampling and be refrigerated if they are not to be immediately analysed. If overload found or peaks at too low level sampling volume to be adjusted accordingly.

6.4 Detection of VOCs using TD-GC-MS

The VOCs associated with decomposition are to be identified, detected and analysed but not quantified.

Brass ends are removed from TD tubes and replaced with Difflok caps (Markes international) just prior to instrumental analysis and placed in the autosampler trays according to manufacturer's protocol.

Thermal desorption parameters:

In combination with the TD tubes (as in 5.0) the following parameters have been proven optimum:

- Trap flow: 42 ml/min
- Split flow: 10 ml/min
- Pre-desorption: Purge time 1.0 min
- Tube/sample desorption: 30 mins at 90 °C, trap in line
- Trap settings: 25 °C to 280 °C with max heating rate and 3 min hold, split off

GCMS parameters

In combination with the recommended column (as in 5.0) the following parameter settings have been proven optimum:

- GC-column flow: 1.0 ml/min helium (constant flow)
- Gas saver: off
- Oven temperature programme (total run time 43.5 minutes):
  - Initial isothermal hold at 35 °C for 5 minutes
  - 3 °C/min to 50 °C
  - 6 °C/min to 110 °C
  - 10 °C /min to 245 °C for 10 min
- Coupling to MS: direct

MS parameters:

- Ionisation energy: 70eV
- Mode: EI
- MS source temperature: 230 °C
- MS quad temperature: 150 °C
- MS transfer line: 225 °C

Data analysis

ChemStation software with NIST library

Peaks corresponding to standard chemicals as searched against the library to be noted in terms of GC retention time (RT) and peak height. This can also be applied to components of interest in pork (as human model) and field study. Any peaks used have to be above approximately 3000 abundance (in accordance with suggestions from Agilent Technologies for LOD). Overload is defined as clipped peaks or above 10^7. Quantification is not possible using this SOP.

7. Precision for standards

This is tested using the same analyst doing test repeats and on multiple occasions.

7.1 Repeatability

The difference between two single test results found on identical standard material by one analyst using the same apparatus in the shortest feasible time showed repeatability to be variable. This is variable between chemicals and also between conditions.

The following observations were made from the data obtained: -

a) Repeatability was generally better for the 'mix' than for 'individual' (between 4 and 20 % RSD as compared to 21-48 respectively).
b) Repeatability varied between the different chemicals with DMDS giving the best result for both 'individual' and 'mix' (with just over 1 % RSD in the mix).
c) % RSD was overall higher for the regenerated headspace than for the original equilibrated headspace. However, there was less variation in % RSD between the individual and mix and also between each chemical after regeneration. In addition, there was variation in % RSD between the chemicals.

This variability is a product of the complexity of the chemicals and adsorbant involved. It is important to note if this degree of variability is seen using standards that conclusions when applied to field decomposition matrices require caution.

7.2 Reproducibility

The absolute difference between two single test results found on identical test material reported by two laboratories.

This was not possible as no inter-laboratory testing was undertaken.

8. Limitations

Limitations to note:

- Inability to quantify
- No true LOD just accepted levels (see section 6.3)
- No defined upper limit just overload
- Acidic peaks are broad as column is general purpose

----------End of TD-GC-MS SOP----------

STANDARD OPERATING PROCEDURE FOR DETECTION OF DECOMPOSITION PRODUCTS BY HEADSPACE GAS CHROMATOGRAPHY MASS SPECTROMETRY (HS-GC-MS)

1. Scope

Decomposition products in the scope of this method are VOCs given off during the decomposition process using pork as a human model and standard chemicals for method evaluation and validation.

2. Principle

Decomposition standard chemicals are pipetted into headspace vials to test the methodology and instrumentation. VOCs evolved are measured using HS-GC-MS. Evaluation of the method is done using standard chemicals and then applied to controlled laboratory pork studies as human model with a view to applying to field decomposition samples.

3. Reagents

Use only reagents of recognised analytical grade. Methanol for dilution to be of HPLC grade.

- **Pentanol** (CAS 71-41-0)
- **DMDS** (CAS 624-92-0)
- **2-methyl butanal** (CAS 96-17-3)
- **Butanoic acid** (CAS 107-92-6)
- **Methanol** (CAS 67-56-1)
- **Mix**
    - Place 50 µl each of pentanol, DMDS, 2-methyl butanal and butanoic acid into a 5 ml lidded sample vial using a 20-200 µl autopipettor.
    - Mix the contents well using a vortex mixer (> 10 secs).
    - The solution is to be made fresh on each day of analysis.

4. Materials

Controlled laboratory decomposition studies are to use pork loin as a human model.

- **Pork loin-** food grade and to include flesh/meat and fat. Not in protective atmosphere. Cut pork into approximately accurately 500 g pieces. Any string from butcher preparation to be removed.

- **Sand-**British play sand (with particle size range of 63 microns to 1 mm/majority 125-355 range with sub-rounded grain shape to avoid abrasion) to be dried in an oven at 105 °C for 5 hours.

5. Apparatus

Usual laboratory glassware and equipment and, in particular, the following:

- Sampling boxes - Plastic boxes with clip lock lids (3 l Really useful 22 x 17 x 15 cm) or equivalent with 6 mm and 2 mm holes drilled
- Laboratory balance capable of masses up to 2 kg (2.d.p)
- Laboratory grade drying oven
- 0.5-10 µl autopipettor
- 20-200 µl autopipettor
- Vortex mixer
- Agilent 20 ml headspace vials and septum cap.
- Agilent 20 ml vial crimpers
- Gas chromatograph with mass spectrometer detector coupled to GC and with Headspace Sampler Unit (Agilent Technologies 7890B GC system and 5977A MSD with 7697 A HS Sampler)
- Column – fused silica capillary column suitable for analysing VOCs
    - Zebron ZB-5MSi Dimensions - 30 m x 0.25 mm x 0.25 µm
    - Phase chemistry – 95 % dimethylpolysiloxane, 5 % phenyl
    - Temperature range – isothermal - -60 ˚ C to 360 ˚ C, TPGC - - 60 ˚ C to 370 ˚ C or equivalent.
- Gases (analytical cylinder grade)
    - Carrier gas Helium
    - Purge gas compressed air.
- Laboratory thermometer range -10-110 °C
- Stopclock with count up timer

6. Procedure

6.1 Preparation of standard chemicals for evaluation

No certified reference materials (CRMs) exist for decomposition studies so standards are chosen from previous preliminary studies and literature searches.

Standards are to be prepared for evaluation in the laboratory setting to show the effectiveness of the sampling and analysis techniques for use with laboratory samples and consequent application to field study. Standards are to either be separate or mixed prior to their sealing in the headspace vial.

For separate, transfer 0.5 µl of each standard chemical into 20 ml headspace vial, using a 0.5 - 10 µl autopipette and seal immediately using the Agilent crimping tool. For mixed, use 2 µl of mix. Both conditions are to be produced in triplicate.

Standards are to be analysed and both conditions compared in triplicate and with 3 full repeats to check repeatability using HS-GC-MS

6.2 Controlled laboratory human model studies

6.2 1 Preparation of samples

Pork loin to be purchased from a butcher as soon as practicable (ideally on day) before experimentation commences. Sampling boxes are to have approximately 1 cm layer of dried sand and weighed including lid before and after addition of pork. Pork loin to be placed meat side down and fat side up in centre of box on top of sand.

Sand of approximately 2cm height in headspace vial to be sealed into a headspace vial. This is to be done in triplicate for HS-GC-MS analysis.

Boxes are to be made in triplicate and to include protection from scavengers, placed outside in an area appropriate for decomposition studies and with the ability to receive weather station data. Boxes are to be weighted down with bricks. Blank box is also to be placed outside and an environmental blank to be taken for TD analysis. Temperature is taken.

6.2.2. Decomposition studies

Samples of the sand under the pork are to be taken for HS-GC-MS analysis. This may not be possible throughout the study and may only be performed when the study is deemed complete in terms of TD-GC-MS.

6.3 Sampling procedure for HS

Sand to reach approximately 2 cm height in headspace vial is taken using a spatula and sealed immediately into the headspace vial. These are to be refrigerated if not to be analysed immediately.

6.3.1 Detection of VOCs using HS

The VOCs associated with decomposition are to be identified, detected and analysed but not quantified.

Headspace parameters:

- Oven: temperature 37 °C
- Loop: temperature 47 °C, size 1 ml
- Transfer line: temperature 52 °C
- Timing settings: vial equilibration 2 min, injection time 0.5 min
- Vial shaking: 18 shakes/min with acceleration of 60 cm/s^2
- Fill pressure: 15 psi

GCMS parameters

In combination with the recommended column (as in 5.0) the following parameter settings have been proven optimum:

- GC-column flow: 1.0 ml/min helium (constant flow)
- Gas saver: off
- Oven temperature programme (total run time 43.5 minutes):
  - Initial isothermal hold at 35 °C for 5 minutes
  - 3 °C/min to 50 °C
  - 6 °C/min to 110 °C
  - 10 °C /min to 245 °C for 10 min, run time 43.5 min
- Coupling to MS: direct

MS parameters:

- Ionisation energy: 70eV
- Mode: EI
- MS source temperature: 230 °C
- MS quad temperature: 150 °C

- MS transfer line: 225 °C

Data analysis

MassHunter (conversion to ChemStation for comparison to TD-GC-MS) with NIST library

Peaks corresponding to standard chemicals as searched against the library to be noted in terms of GC retention time (RT) and peak height. This can also be applied to components of interest in pork (as human model) and field study. Any peaks used have to be above approximately 3000 abundance (in accordance with suggestions from Agilent Technologies for LOD). Overload is defined as clipped peaks or above 10^7. Quantification is not possible using this SOP.

7. Precision for standards

Detection levels of standards and their interaction were observed for information on precision.

7.1 Repeatability

This was tested using the same analyst with different volumes of standard chemicals.

The difference between two single test results found on identical standard material by one analyst using the same apparatus in the shortest feasible time showed variability.

a) All individual chemicals could be seen in significant levels when using 0.5 µl. There was still some peak overload but the lowest peak (butanoic acid) was always seen in acceptable levels.
b) The 'mix' showed all four chemical peaks in significant levels including pentanol, which was absent when analysed using liquid GC-MS 'mix'
c) In addition, there were also several other peaks seen that were not detected in experiments using other techniques. This included esters that could have been reaction products but most were probably attributed to storage.

This variability is a product of the complexity of the chemicals and their interaction in the headspace. It is important to note if this degree of variability is seen using standards that conclusions when applied to field decomposition matrices require caution.

7.2 Reproducibility

The absolute difference between two single test results found on identical test material reported by two laboratories.

This was not possible as no inter-laboratory testing was undertaken.

8. Limitations

Limitations to note:

- Inability to quantify
- No true LOD just accepted levels (see section 6.3.1)
- No defined upper limit just overload
- Acidic peaks are broad as column is general purpose

----------End of HS-GC-MS SOP----------

6.4. Repeatability testing of SOPs using pork by the author

6.4.1 Introduction

After the SOPs had been developed and tested using standard chemicals, it was necessary to further test the SOPs by the author using the above 'working versions'. The first test consisted of an evaluation of repeatability based on the pork experiments of Chapter 5 for both SOPs.

6.4.2 Aims

- To further evaluate the SOPs in terms of repeatability
- To confirm that the sampling parameters and scope held true

6.4.3 Method

Instead of triplicate being 3 boxes, repeatability was evaluated by measuring a single box 3 times a day together with a blank. Sampling events took place 3 hours apart to allow *regeneration*. These samples taken on the same sampling day are, for the purposes of this study called 'repeats'. Samples were analysed as detailed in the TD-GC-MS SOP.

Samples of sand for HS-GC-MS analysis were taken at the end only and run against control sand using the appropriate SOP.

6.4.4 Results

The results are shown in the form of a bar chart (Figure 6.1) of the mean levels (as given by the height of the peaks on the TIC) of the 5 most abundant chemicals on each collection day. The error bars are ± 1 SD. The Relative Standard Deviation (RSD) % are given numerically in Table 6.1

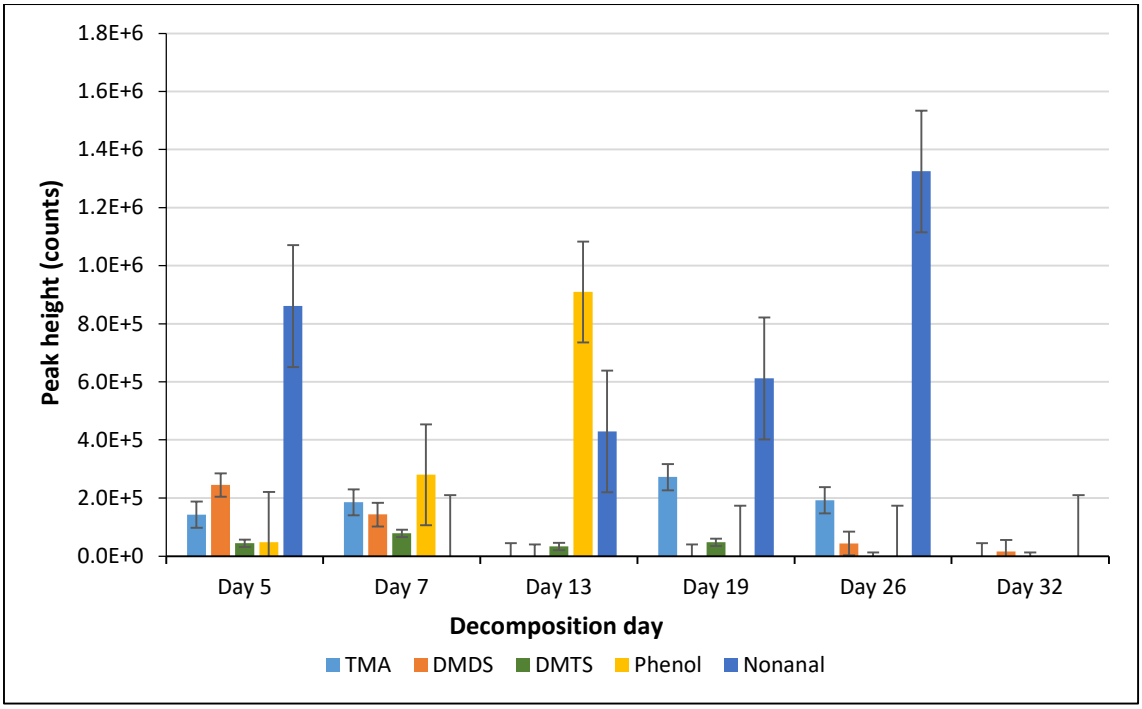

Figure 6.1 showing the chemicals monitored over time for the repeatability experiment (with error bars) using TD-GC-MS

	TMA	DMDS	DMTS	Phenol	Nonanal
Day 5	16.2	13.4	39.4	93.9	102.5
Day 7	37.1	86.9	118.5	104.4	0
Day 13	0	0	86.6	84.2	150.7
Day 19	11.8	0	37.3	0	94.3
Day 26	86.9	68.3	0	0	89.6
Day 32	0	108.2	0	0	0

Table 6.1 RSD (%) for repeatability experiment for each chemical monitored over time using TD-GC-MS

6.4.5 Discussion

It can be seen from the results that the principle VOCs vary over time (as would be expected) but not always in a consistent way. In some cases, chemicals seem to appear and disappear only to reappear later in the decomposition. Although this raises questions, it does support the findings of previous researchers who also saw inconsistency in VOC detection throughout the decomposition process [6-11] including a researcher working in the same decomposition location as the author but using.[12]

DMTS and phenol are seen earlier but not later and DMDS is seen early and late. This variation may be due to the dynamic nature of decomposition where the same chemical may be formed at different times due to variation in the rate of autolysis of different tissues. [7, 13-20] Both previous literature and

findings from this work demonstrate how difficult decomposition is to follow and difficult to apply consistent conclusions due to the complex matrix, hence the need to attempt at least consistency of measurement using an SOP.

The error bars are generally large. It was hoped these would be smaller, with multiple sampling of the same box as a repeat, in comparison to the 'repeats' of 3 boxes done previously (where each sampling was really a population number, n). However, this was not the case. This may have been due to the need for regeneration of headspace for TD sampling so the repeats were separated temporally by several hours and were subject to change in temperature. In future work using 3 TD tubes in one box simultaneously may be a better measure as each would sample an identical VOC environment although, presumably, the amount adsorbed per tube would be less.

RSD values, a reflection of the error bars, were generally large showing variation between sample repeats. Indeed, in some cases they were over 100 % meaning variability was very high. Obviously, these are far from ideal but from the quantity of experiments undertaken by the author it is believed to reflect the combination of air sampling using TD-GC-MS analysis and the complexity of matrix. Thus it can be concluded the methodology is acceptable and that decomposition study truly shows that much variation. A key point to note is that if this much variation between repeats is observed within a controlled environment, it is questionable the reliability of some of the conclusions in the literature with regard to PMI and decomposition stage based on low numbers of samples obtained in the field. For example, a case study type investigation like that of Statheropoulos *et al* (2005)[21] with low population number makes it difficult to draw conclusions of decomposition stage or PMI. Even extensive studies like the Decompositional Odour Analysis (DOA) databases work of Vass and colleagues, [9, 20, 22-27] with numerous chemical findings would be questionable due to lack of repeats and laboratory control.

TMA RSD values were the lowest and this is probably due to its presence across all 3 repeats on one day i.e. if it was seen one day, it was seen on all repeats. Some of the other chemicals with particularly high RSD for one day was probably due to high levels in one repeat and not seen at all on another. This just adds to the questions in the field if it is not possible to even control these factors in the laboratory. This data could be added to the SOP for repeatability measurement values.

It was intended to repeat this whole experiment several times but due to issues with accessibility to the decomposition location, lab infrastructure and instrumentation, there was only one complete experiment available from four attempts. However initial data processing for the measurements available on the sets not presented showed similar results to those discussed here.

HS-GC-MS analysis of the sand substrate at the end of the experiment only showed DMDS in significant levels at the end of the experiment which was consistent with that seen on the last day (day 32) for TD-GC-MS. Ideally it would be desirable to be able to have matching pairs of analyses (air and substrate VOCs) throughout the experiment for more information. However, this would either involve disturbing the decomposing meat to extract sand or having multiple parallel experiments operating.

6.5 Repeatability testing of SOPs using standard chemicals by other analysts

6.5.1 Introduction

The author carried out testing and self-validation on multiple occasions as part of this study. It was decided to challenge the testing of SOPs further to include different analysts which would allow some extra testing of its repeatability and some validation using *intra-laboratory* testing. This was achieved with the assistance of two colleagues both of whom were experienced in laboratory analysis.

6.5.2 Aims

- To check the SOPs for ease of use and errors.
- To test and validate the SOPs using data from multiple analysts

6.5.3 Methodology

- **Analyst 1**

  An experienced technician who has worked in chromatography and mass spectrometry for over ten years.

- **Analyst 2**

  An experienced laboratory technician who has used various analytical instrumentation but with little chromatography experience.

Analyst 1 and 2 were given the 2 SOPs in the format of section 6.3 and asked to perform the standard chemical trials. Time did not allow their carrying out of pork trials. They were given no explanation of the SOPs or supplementary information other than brief training on the instrumentation for Analyst 2. This was not done by the author to ensure no bias. Both analysts carried out the SOPs in the same laboratory, using the same chemicals and instrumentation.

6.5.4 Results

The results for the two analysts are presented in the following tables which show the 3 repeats and the associated RSD (%) for four chemicals investigated using SOPs for TD-GC-MS and HS-GC-MS

Chemical	DMDS	Pentanol	Butanoic acid	2-methyl butanoic acid
rep 1	136647	1511705	292398	216209
rep 2	88158	1579003	278364	216814
rep 3	32480	1798435	512861	192626
Mean	**85761.7**	**1629714.3**	**361207.7**	**208549.7**
SD	**52124.8**	**149940.8**	**131522.9**	**13793.6**
RSD (%)	**60.8**	**9.2**	**36.4**	**6.6**

Table 6.2a showing results as peak heights for' individual' vials using TD-GC-MS for Analyst 1

Chemical	DMDS	Pentanol	Butanoic acid	2-methyl butanoic acid
rep 1	449425	320151	30952	25499
rep 2	469111	295507	37603	59377
rep 3	595718	865633	56307	64409
Mean	**504751.3**	**493763.7**	**41620.7**	**49761.7**
SD	**79391.9**	**322283.9**	**13146.3**	**21162.2**
RSD (%)	**15.7**	**65.3**	**31.6**	**42.5**

Table 6.2b showing results as peak height for 'mix' vial using TD-GC-MS analysis for Analyst 1

Chemical	DMDS	Pentanol	Butanoic acid	2-methyl butanoic acid
rep 1	31712983	24332783	6645516	3401606
rep 2	31910857	24422569	8413013	4159846
rep 3	31337286	25347026	6225313	5418903
Mean	**31653708.7**	**24700792.7**	**7094614.0**	**4326785.0**
SD	**291343.5**	**561452.2**	**1160936.9**	**1018956.9**
RSD (%)	**0.92**	**2.32**	**16.3**	**23.5**

Table 6.2C showing results as peak height for 'individual' using HS-GC-MS for Analyst 1

Chemical	DMDS	Pentanol	Butanoic acid	2-methyl butanoic acid
rep 1	29470611	17596332	2687044	2907536
rep 2	29913633	17429694	2332294	2458603
rep 3	29610149	17952045	2688204	2835169
Mean	**29664797.7**	**17659357**	**2569180.667**	**2733769.333**
SD	**226510.4**	**266817.8307**	**205150.691**	**241032.423**
RSD (%)	**0.76**	**1.51**	**7.99**	**8.82**

Table 6.2d showing results as peak height for 'mix' using HS-GC-MS for Analyst 1

Chemical	DMDS	Pentanol	Butanoic acid	2-methyl butanoic acid
rep 1	949642	1612067	398600	229502
rep 2	30684	3139615	877811	344564
rep 3	31824	2509968	355558	238976
Mean	**337383.3**	**2420550.0**	**543989.7**	**271014.0**
SD	**530231.9**	**767689.7**	**289897.7**	**63872.1**
RSD(%)	**157.2**	**31.7**	**53.3**	**23.6**

Table 6.3a showing results as peak height for 'individual' vials using TD-GC-MS for Analyst 2

Chemical	DMDS	Pentanol	Butanoic acid	2-methyl butanoic acid
rep 1	544131	831640	83025	84981
rep 2	634135	1029968	100799	112411
rep 3	819129	1650363	251951	226672
Mean	**665798.3**	**1170657.0**	**145258.3**	**141354.7**
SD	**140206.6**	**427108.8**	**92824.9**	**75149.01**
RSD(%)	**21.1**	**36.5**	**63.9**	**53.2**

Table 6.3b showing results as peak height for 'mix' using TD-GC-MS for Analyst 2

Chemical	DMDS	Pentanol	Butanoic acid	2-methyl butanoic acid
rep 1	19755539	8774346	1334484	1733333
rep 2	19774354	8344349	1898744	908487
rep 3	18993798	8475454	2072192	1217711
Mean	**19507897.0**	**8531383.0**	**1768473.3**	**1286510.3**
SD	**445322.2**	**220386.9**	**385721.5**	**416704.6**
RSD(%)	**2.28**	**2.58**	**21.81**	**32.4**

Table 6.3c showing results as peak height for 'individual' using HS-GC-MS for Analyst 2

Chemical	DMDS	Pentanol	Butanoic acid	2-methyl butanoic acid
rep 1	10050812	3612316	440407	543363
rep 2	11756850	4208022	479769	666952
rep 3	14084493	5162299	664516	914481
Mean	**11964051.7**	**4327545.7**	**528230.7**	**708265.3**
SD	**2024807.4**	**781873.6**	**119656.2**	**188976.8**
RSD(%)	**16.9**	**18.1**	**22.7**	**26.7**

Table 6.3d showing results as peak height for 'mix' using HS-GC-MS for Analyst 2

6.5.5 Discussion

Variation was seen between chemicals and 'individual' and 'mix' for both analysts which agreed with the earlier work from the author. Due to prolonged storage of the 2-methyl butanal, its peak was not seen by either analyst and thus 2-methyl butanoic acid, a peak consistently detected in its place was used instead. Although it has been presented here for completeness, it will not be included in discussion as it cannot be compared fairly to the earlier work done by the author in Chapter 4.

HS-GC-MS analysis gives by far more consistent data, shown by lower % RSD for both analysts. The variation for the TD-GC-MS data is much greater, in line with results given in Chapter 4. This strongly suggests that this variation is innate to a combination of the nature of what is being analysed and the technique. Again, this does pose questions about the reliability of some of work presented in the literature especially when using TD-GC-MS for decomposition study. [7, 28] [9, 19, 26, 27, 29, 30] Previous authors have made conclusions about VOCs levels that may be more reliable if they were to have fundamental analytical principles applied to their methodology like in the SOPs of this study.

This testing was designed to add to the *repeatability* data for the SOPs but unfortunately does not give much of an indication of *reproducibility* (as the definition in the SOPs above). For the SOPs to reach a 'final form' it would be desirable to carry out *inter*-laboratory testing as well as the *intra*-laboratory testing done here.

In terms of the SOPs, repeatability is to be determined by a single analyst (e.g. the author in Chapter 4). Thus each of these sets (Tables 6.2 and 6.3) gives the repeatability of each analyst. Overall, the combined results of three separate and competent analysts doing, in effect, an *intra-laboratory* study suggest that the method defined in the SOP is fit for purpose and produces 'true' results. Although getting an appreciation of accuracy was difficult with no existing validated method to compare data to, this 'trueness' has been shown through validation and repeatability studies, as well as with the use of multiple instrumental techniques, and by attempting peer review using the literature. Peer review of the literature took the form of comparing the chemicals found using the SOPs to those in the literature. Similar chemicals were detected in the current study and the findings in the literature including that of notable authors, for example Vass and colleagues and Forbes and colleagues, using comparable techniques. [9, 16, 17, 20, 23-28] This suggests the large variability in data would appear to be inherent rather than the result of any analyst error.

It can be seen that the RSD values were generally higher for Analyst 2 than Analyst 1 (and both higher than the author). This follows the expected trend of relative experience on the instrumentation and methodology. A significance test like a t-test was not undertaken as the aim was not to evaluate the proficiency of analysts. The key point was that they could follow the SOPs getting comparable data. It was encouraging to find that both analysts found the SOPs to be easy to follow and free from procedural error and also required no supplementary information or discussion with the author.

When using the SOPs an analyst would gain insight into expected error for their particular laboratory set-up prior to their analysis in the field.

6.6 SOP validation using pork doped with standard chemicals

As no quantitative work was attempted, validation in the strictest terms (e.g. determination of the limits of detection-LOD, use of a CRM etc.) *per se* was not possible. However, a more limited form of validation was undertaken using pork spiked with chemicals (those which were used as standards throughout this project to enable comparison).

6.6.1 Introduction

The validation was only attempted for the TD-GC-MS SOP for two main reasons. Firstly, the majority of the experiments used that technique and, secondly, it was most appropriate for the type of validation experiment undertaken.

6.6.2 Aims

- To attempt validation of the TD-GC-MS SOP

6.6.3 Methodology

In the absence of a Certified Reference Material (CRM), doping was done with the four standard chemicals studied in detail in Chapter 4 (2-methyl butanal, DMDS, pentanol and butanoic acid).

1 ml each of pure chemical was put into a mix to make a total of 4 ml for the purposes of doping. The following conditions were used:

A) Doped sand (no meat)

B) Doped sand then meat placed on top

C) Surface of meat doped (on sand)

D) Meat soaked to coat all surfaces for 10 mins (on sand)

E) Meat doped by injection with 3 injection ports (on sand)

A blank was set up and sampling was done according to the TD-GC-MS SOP. Sampling was carried out at time zero, after 24 hours and after 7 days.

6.6.4 Results

Results are presented as a bar chart (Figure 6.2) showing the levels (as peaks height) of the four chemicals used for doping at time zero, after 24 hours and after 7 days, for the above doping conditions (see section 6.6.3)

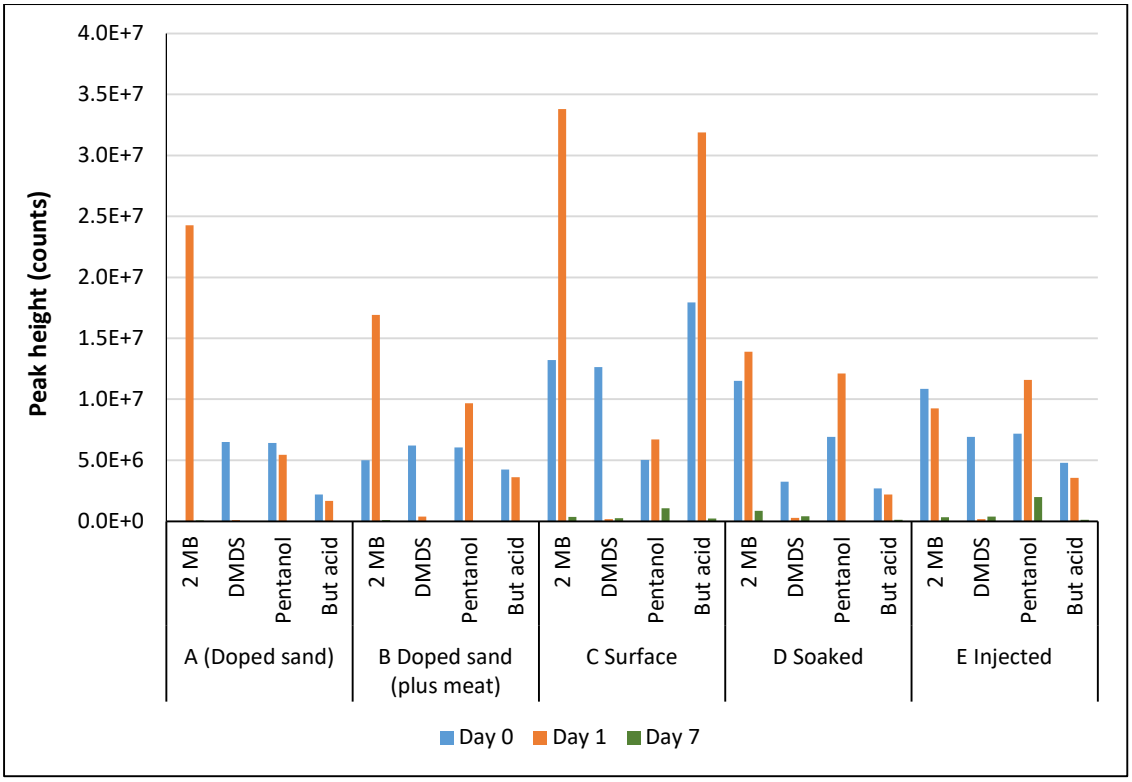

Figure 6.2 showing doping of meat with chemical standards over time

6.6.5 Discussion

It can be seen that there are generally lower levels of chemicals detected for the doped sand with meat on top and for the injected category. This could simply be a feature of accessibility of chemicals to the headspace- a requirement for detection.[31-33] The meat may act as a barrier blocking chemicals from leaving the sand and, in cases of injected samples, the chemicals may be effectively trapped at least until decomposition is in the later stages. Indeed, the highest levels were seen when the chemicals were simply doped onto the surface supporting access to detection. Chemicals may also be absorbed and subsequently released from the sand.

It was hoped that the doping study would act in a similar manner to the common analytical procedure of *standard addition* and that an overall increase of a chemical over time would be observed as those produced during decomposition would be in addition to the spiked ones. Levels of chemicals were indeed seen to rise by 24 hours (day 1) which may have supported this idea but alternatively, the longer time between sample collection may have simply allowed their accumulation in the headspace.

By day 7, levels were low across all doping conditions suggesting loss of spiked chemicals through evaporation combined with the decline of VOC formation as the decomposition process slowed. In contrast, the boxes containing injected meat still saw significant levels compared to the others which is consistent with the idea of trapped chemicals being released as the meat decomposes.

The experiment would have to be repeated multiple times for it to be definitively concluded that the differences seen were due to the way of doping or decomposition itself and, again, the innate variability of decomposition is apparent in the results.

The work would have also needed to be undertaken in comparable experiments with HS-GC-MS in a similar way to the type of work described in section 5.4.4. [34]

6.7 Chapter conclusion

The SOPs were tested by two independent analysts and found to be fit for purpose. The variation for the TD-GC-MS data is large but it can be concluded that this is due to the technique and the complex matrix of decomposition rather than analyst or method error.

It is hoped that in the future these SOPs could be used as a starting point for forensics applications including PMI determination. The idea is the analyst would use the SOPs (or a variant of them) with the standard chemicals and controlled pork decomposition studies as a basis to determine the variability in their own equipment and methodologies. The level of variability obtained could be then be used as a guide to the minimum level of uncertainty expressed in any results obtained from the field (where variations would almost certainly be greater).

This chapter focussed on testing the reliability of the SOPs and the results suggest that they are suitable for decomposition studies. However, a final evaluation of the SOPs in terms of *robustness*, the ability of a SOP to be still applicable if one, or more, experimental factors are changed was undertaken. This was based on experiments using plants which can produce VOCs similar to those encountered in decomposition and is described in the next chapter.

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7. TESTING THE ROBUSTNESS OF THE SOPS

Throughout this project, chemicals related to decomposition have been used for development of SOPs in conjunction with pork as an accepted human model. The robustness of the SOPs has been shown in their ability to withstand rigorous testing throughout. It would have been useful to continue to apply the testing of the SOPs using mammalian tissue, ideally whole porcine cadavers, but that was not possible due to restrictions at the decomposition location. However, using the SOPs developed and tested in this thesis (see section 6.3), their robustness was further tested by investigating their ability to withstand the changing of one or more conditions. This was done by using related applications with similar VOCs e.g. plants releasing VOCs mimicking those produced during decomposition.

7.1 Introduction to mimicry in the phyto-kingdom

Plants play a vital role in ecology and most aspects of their lifecycle and properties have been studied. Many plants emit VOCs to attract insects for either pollination or as a food source.[1] The latter, carnivorous plants, are known to produce VOCs that mimic those evolved during decomposition so as to attract blowflies and similar insects.[2]

The aim of the work presented in this chapter was to evaluate the scope and robustness of the methodologies and SOPs developed for the collection and analysis of decomposition VOCs by applying it to similar VOCs. The work on selected plants was facilitated by the author's father, Mr. Martin Brook, who specialises in the growing of carnivorous plants particularly *Sarracenia flava.*

7.1.1 Plant volatiles

Plants of many different species are known to release VOCs for various reasons e.g. as olfactory stimuli to pollinator attractants or as a chemical defence by attracting herbivore enemies. [2] [3] The extent to which these volatiles have been studied has largely depended on whether the species are of commercial interest. Plants used for the flavour and fragrance industry have been analysed in detail but little is known about the ecological role that VOCs may play for many plants from a purely botanical perspective [4]. One of the main target groups to receive olfactory stimulus from plants is insects. These can act as pollinators but also, in the case of carnivorous plants, can be attracted as prey [5] via simulation of naturally occurring products that would attract insects.

7.1.2 Olfactory stimuli

It is thought that most of the olfactory stimuli and plant volatile release is based on mimicry of nature. Different plants can release odours that mimic flowers, fruit, nectar and carrion depending on the target pollinator or prey. [2]

The subsequent recognition of these olfactory signals by insects depends on a number of factors including their olfactory systems' ability to detect certain compounds. Recent studies show that it is unlikely that individual chemicals are enough to act as a stimulus and that it is blends and combinations that exert the effect. [4]

Much of the previous work on plant volatiles has focussed on the major components emitted by plants but recent studies as well as work in this thesis have suggested it may be minor components and context in which the VOCs are released i.e. background odours that are also important. [4, 6]

7.1.3 Decomposition mimicry in plants

Odour mimicry has evolved as a pollination strategy in various plants. [7] Carrion mimicry sees flowers specifically exploiting groups of insects for example blowflies to allow their pollination. The release of decomposition associated VOCs for example oligosulfides including DMDS are thought to mimic oviposition sites of blowflies. This attracts the flies to the plants to enable their pollination. [6, 7] Some previous work has investigated whether their carrion odour is true mimicry i.e. are the same VOCs released in decomposition and in the flowers that are utilising this method of pollination and do they evoke the same response in blowflies? One notable example of this was work was on *Helicodiceros muscivorus* (Dead-horse Arum) which attracts blowflies with the release of oligosulfides. [7]

Other work has focussed on whether it is the major component VOCs that alone mimic decomposition and therefore attract insects or whether it is a subtler blend that is required. [4, 6]

7.1.4 Oligosulfide producers

Work studying various decomposition associated flowers e.g. *Amorphophallus* has shown one of the most characteristic classes of VOC to be the sulphur compounds.[6] Therefore, the choice of flowers for study here was focussed on these.

Perhaps the most famous example of carrion mimicry is *Amorphophallus titanium* or corpse flower and DMDS and DMTS have been recorded during its rare flowering.[8] However, as it takes over 10 years to reach maturity, only produces flowers every few years which last only 2-3 days when it does, it was impractical for study. *Drucunculus vulgaris* is another species that resembles the corpse flower.

7.1.5 Wasp and fly pollinators

Pollination systems can be mediated by different insects depending on the plant and its requirements. Wasps and flies are attracted to very different chemicals in nature and thus the plants attracting these have evolved to emit different chemicals to attract one or the other even amongst plants of the same species. *Eucomis species* have flowers closely related in colour with similar greeny-white flowers. However, where the species differ is in scent chemistry, which depends on pollinator attraction. *Eucomis* species have flowers that utilise both wasp and fly pollinators. [9]

7.1.6 Carnivorous plants

Carnivorous plants are known to release VOCs to attract various prey and pollinator insects.[5] These can vary between different species of plants but can also be released throughout the lifecycle of an individual plant which can then be separated by temporal and geographical factors.[2] One example of the use of temporal separation in VOCs release is the ‘yellow pitcher plant’ *Sarracenia flava*, studied in this work.

7.1.6.1 *Sarracenia flava*

S.flava or 'yellow pitcher trumpet' is a carnivorous pitcher plant. One of the earliest of its species to be cultivated, it has been studied by botanists and naturalists for years. This was so named by Mark Gatesby as far back as 1731.[10] *S.flava* originate from several areas of the North American Atlantic Coastal Plain region.[11]

The yellow pitcher plant shows great variation and although the actual taxonomy of sub-species is in contention, several variants have been labelled colloquially in the carnivorous plant community. These vary in colour, size and degree of veining in the pitchers. [11]

Carnivorous plants like the yellow pitcher plant release VOCs to attract pollinators but have the additional complication of also releasing VOCs to attract prey. This creates prey-pollinator conflict.[2, 4, 5]

- **Flowers** – these grow on tall stems and open whilst the pitchers are only in early growth, allowing the temporal separation of flower and pitcher. This makes sense from an evolutionary point of view as the flower attracts insects for pollination purposes whilst the pitchers aim for insect attraction as prey. Petals are green-yellow with the sepals darker in colour. The flowers of *S.flava* are amongst the earliest of this genus. In their native N. America this is from around late February. [5, 10, 11]

- **Pitchers** – These grow from spring into summer following the demise of the flower. Although some variants continue pitcher production as late as early autumn, the strongest are usually spring and summer [9, 10]. The main variation of this species is seen in the pitchers which display different colours, vein patterns and height, although typical height is 50-70 cm.[10, 11] Exposure of pitchers to sunlight lightens and yellows the plant tissue.[10]

  S.flava has a variety of common prey to include flies, wasps, ants, beetles and moths.[11] It is thought that the particularly tall pitchers of some variants may have adapted to have specifically flies as prey.[5]

  The pitchers have an elongated funnel or trumpet-like opening that flares at the mouth, with a broad lip called the peristome. The pitcher narrows rapidly and has a smooth interior with downward pointing hairs deep within .[10, 11] The pitcher also has what looks like a lid which keeps out rain and can be used as a landing pad for insects.[11] Following pitcher growth, before this lid opens, there is an absence of bacteria in the pitcher. Bacteria are later introduced from the trapped insects' bodies.[10]

  Insect trapping by the pitchers – S.flava traps insects using the basic pitfall method.[10, 12] Where adhesion traps require a large surface area, pit-fall traps can be relatively small and attract prey using colour and specific odour, hopefully without attracting pollinators.[5]

Insects are seen to be intoxicated [10] when entering the mouth of the pitcher.[11] This indicates the release of a chemical component which somehow drugs them. The bottom of the pitcher contains digestive juices which drown the insects that have fallen down the pitcher and the combination of these digestive juices and bacterial action break down the soft tissue of the insects leaving only exoskeleton.[10] The juice secretion level increases as the pitcher fills with bodies and as this inevitably changes in bacterial and insect content, this may result also in chemical profile change.

- **Phyllodia –** as the pitcher production slows in late summer this leaf-like structure is produced during pitcher shrinkage and can remain green until new pitchers are formed the following spring. [10, 11] This leafstalk resembles an Iris-leaf and is called a Phyllode (Phyllodia plural) [11] and appears to keep the plant going through winter, it may be involved in nutrient uptake or photosynthesis.

Chemicals released during the lifecycle of *S.flava*

- **Flowers –** thought to release chemicals to attract pollinators and are known to smell of cat urine with a butter-sweet fragrance.[10]
- **Pitchers –** known to release intoxicating substance (coniine) to 'drug' insects [10, 11] as mentioned and the importance for the temporal separation of flowers and traps is noted. Composition of chemicals in the digestive juices and those VOCs given off will likely change over time.
- **Phyllodia –** not known to emit odour

It is the release of these chemicals for pollination and prey attraction and the possible change in composition of digestive secretions due to bacterial changes and decomposing matter (victims' bodies) over time that is worth further study here.

7.1.7 Previous work on the analysis of VOCs from plants

There are various methodologies available for the study of VOCs release from plants which each have their advantages and limitations.[1] Dynamic headspace sampling involves the pumping of headspace over time, collecting the resulting air volume. In combination with TD, used throughout this thesis and using the developed SOP, it gives the advantage of increased volume sampled which can be trapped and any trace volatiles that otherwise would be lost can be detected.[1] This can however cause humidity level issues that are particularly problematic with plants although this can be somewhat overcome with the purge option on the instrumentation.

GC-MS including TD-GC-MS has been used for VOC analysis in plant analysis including carnivorous pollinator-prey attraction systems in carnivorous plants and those releasing different volatiles depending on pollinator. Shuttleworth and Johnson (2010) investigated the possibility of shift from wasp to fly mediated pollination by the release of sulphur compounds.[9] Jurgens and colleagues (in 2009 and 2013) investigated the differential release of VOCs depending on what pollinator or prey a carnivorous plant desires and the conflict between the pre-pollinator systems using TD-GC-MS.[2, 5, 6]

TD-GC-MS use for plant analysis has the benefits of low prep times and storage capabilities not seen with Solid Phase Micro Extraction (SPME). The main advantage of TD-GC-MS for plant analysis is that the plant can remain *in situ*. Also the plant material itself and surrounding headspace volume can actually be quite low, allowing for increased sensitivity by using repeated airflow over the TD tube. However, this can result in increased contaminant background and problems from carbon artefacts which had to be accounted for during analysis.[1, 13-15]

HS-GC-MS analysis also lends itself well to plant analysis in its simplicity as it requires little sample preparation and material can be stored (if frozen) for analysis later. HS-GC-MS also allows the heating of original plant material to release volatiles where TD-GC-MS is limited to the volatiles given off in ambient conditions at the time of sampling. It is however destructive and limited to the amount of material sealed in the vial so has no sample concentration step. HS-GC-MS analysis was used in conjunction with TD-GC-MS in this study as previous chapters of this thesis have shown the powerful potential of combining these complementary techniques.[16]

It is hoped that the SOPs developed for decomposition analysis will also be applicable to the VOCs of plant systems.

7.1.8 Current work

The decomposition work in this thesis has shown the importance of a subtle blend of major compounds and key indicators as well as minor components in an odour profile for both olfactory and instrumental detection. A major compound for example DMDS may need to be present to indicate a decomposition event but it is only in combination with minor components that a unique profile can be achieved.

Mimicry of carrion in the phyto-kingdom with studying compounds like DMDS is the focus of this current plant work and is another interesting decomposition-related application of the main instrumental techniques and thus for the testing of the robustness of the SOPs.

Plants selected for study were chosen because they were known to give off VOCs related to decomposition for insect attraction, were commercially available to buy and did not involve too complex growing conditions or excessive germination/growth periods.

Fly-mediated pollination was of specific interest to this work as it was thought to be related to fly attraction and carrion-mimicry and by comparison to wasp-mediated pollinator species it was hoped to illustrate which chemicals were genus specific and what were involved in the attraction of individual species-specific pollinators. By doing so it was also hoped that this would confirm the reason for detection and thus production of certain VOCs linked to decomposition. Wasps are known to be attracted to sweet nectar associated chemicals and therefore plants aiming for their attraction should have vastly different floral scent chemistry to those mimicking the smell of death.

Pitcher plants were studied as their carnivorous nature links them to insect attraction and due to their availability at the sampling site. *Sarracenia flava* was prolifically available at the sampling site and the study of their life-cycle was thought to be particularly interesting as it has been postulated that VOCs change over time depending on prey or pollinator need.[5] Sarracenia *purpurea* is known to give off

DMDS so was useful for study for the same reasons as the plants chosen that give off known decomposition VOCs but was not as readily available at the sampling location.

The evaluating of the developed SOP for flexibility and robustness was carried out using the plants as a chemically similar application.

7.1.9 Aims

- To test the robustness of the SOPs developed in this thesis
- To investigate carrion mimicry in the phyto-kingdom

7.2 Materials and methods used for mimicry in the phyto-kingdom

SOPs ('working versions', see section 6.3) were used as a starting point. So as to test the robustness of the SOPs for a chemically similar but different applications, they were used as seen as far as possible. However, certain adaptations for use with plants and due to the sampling site not being local to the author, were required (as detailed below).

7.2.1 Plants used in this study

Plants were selected for study because they were known to give off VOCs related to decomposition for insect attraction, were commercially available to buy and did not involve too complex growing conditions or excessive growth periods.

The plants studied were divided into three main groups: -

- Fly attractors (sulphur producers): *Dracunculus vulgaris* (Dragon arum), *Eucomis bicolor* (Pineapple lily)
- Wasp attractors: *Eucomis pole evansii, Eucomis* 'twinklestars'
- Pitcher plants (carnivorous plants): *Sarracenia flava, Sarracenia purpurea*

Eucomis and *Dracunculus* were sourced from *Anglia Bulbs Ltd* and were grown in all-purpose garden compost in pots kept outside. *Sarracenia* were sourced from *P and J Plants* in Somerset as rhizomes and were grown in a mix of approximately 20 % horticultural grade vermiculite in peat in pots. These were kept in an ambient greenhouse insulated with bubble wrap and were watered according to the 'tray method' (as accepted by the carnivorous plant community). This has the plants in a tray with approximately 2.5 cm of water, preferably distilled or rainwater. All plants were grown by Mr. Martin Brook in Dumfries and Galloway, Scotland. It was originally intended to also study *Sauramatum venosum* (Voodoo lily) but unfortunately it failed to grow.

A.Dracunculus vulgaris

Figure 7.1 *Dracunculus vulgaris* (closed) in the growing and sampling location

Figure 7.2 *Dracunculus vulgaris* (open) in the growing and sampling location

D. vulgaris is related to the famous corpse flower, indeed the resemblance in appearance can be noted. The flower (see Figures 7.1 and 7.2) is large and purple with a phallus like the corpse flower and like the corpse flower has limited time for flowering and therefore sampling. This work noted the flower to bloom and shrivel in a 3-day period, giving a limited sampling period.

B. Eucomis bicolor

Figure 7.3 *Eucomis bicolor* in the growing and sampling location

Eucomis bicolor was chosen as the sulphur producing fly attractor for study and *Eucomis pole-evansii* as the main one for wasp-mediated pollination study. Fly-mediated pollination was studied using different variants of the *Eucomis* species *Eucomis bicolor*, known to attract blowflies for pollination purposes. [9]

Eucomis bicolor lives up to its name with two colours, often spotty (see Figure 7.3). It is known to release sulphur compounds to attract flies as pollinators. Other species of *Eucomis* e.g. *Eucomis pole-evansii* and *Eucomis humilis* 'twinkle stars' are known to attract wasps as pollinators and therefore release sweet smelling compounds. It is even thought possible to shift pollinators from wasp to fly by change in sulphur compound production.[9] This is interesting from an evolutionary and mimicry point of view and would be worth further investigation. Clearly sulphur compounds are important for carrion fly attraction but it may be the minor components in the profile context that may exert the full effect.[4]

Wasp-mediated pollination was studied using *Eucomis pole-evansii* and *Eucomis 'twinkle stars'*, known to attract wasps for pollination purposes.[9] The opportunity for study of these species was limited as these were not grown by the researchers and had to be sampled from Broughton House, National Trust for Scotland in Kirkcudbright by Martin Brook. These were sampled for comparison purposes and to investigate any similarities and differences between species with different target pollinators. Wasps are known to be attracted to sweet nectar associated chemicals and therefore plants aiming for their attraction should have vastly different floral scent chemistry to those mimicking the smell of death.

C. *Sarracenia flava*

This plant was studied in the most detail due to availability at sampling site and because it produces flowers and pitchers at different times during its lifecycle which release different VOCs. This may have evolved to reduce the prey-pollinator conflict.[5]

Figure 7.4 *Sarracenia flava* in the growing and sampling location. This shows plants at both the flower and pitcher stage

D. *Sarracenia purpurea*

This was available at the sampling site but not in the prolific quantities of *S.Flava*. It was only sampled on one occasion for pitchers only using TD-GC-MS analysis.

Figure 7.5 *Sarracenia purpurea* in the growing and sampling location

7.2.2 Plant sampling

The SOPs for the sampling and analysis of VOCs given off during decomposition (see section 6.3) were used for this study in order to test its flexibility and robustness. However, there needed to be certain adaptation to allow its application to the analysis of plants. For both TD and HS, sampling was carried out *in situ* by Martin Brook. For TD, samples were collected by TD tubes adjacent to the plant or by using a sampling hood placed over the entire plant/section of plant. For HS, plant samples were collected and sealed in 20 ml HS vials.

In following the lifecycle of the *S.flava* plant different components of the plant were isolated during different stages throughout. These were: -

- Flowering (absence of pitchers)
- Early pitcher production (prior to flies/insects)
- Mature pitchers (with/without flies)
- Phyllodia

Regeneration time – Some work was carried out with the objective of determining how long it would take for sample regeneration. It was observed that regeneration time was generally much longer than that of pure standard chemicals and also longer than that of active decomposition samples that are highly odourous. Therefore, it was decided that the best sampling technique for pitcher plant analysis was to leave the sampling hood on for at least an hour (preferably overnight) and to not sample sooner than another hour following.

Temporal effects – it was noted that time of day seemed to have an effect on sampling. Sampling was also carried out at different times of day to determine if there was any difference between diurnal and nocturnal chemical production. Diurnal samples were taken when the sampling greenhouse was hotter and moisture was observed on the sampling hood. This was not fully investigated but interesting to note.

7.2.2.1 Sampling for TD analysis

To allow headspace accumulation an in-house 'sampling hood' (see Figure 7.6) comprising a modified 2 litre plastic still water bottle with the bottom removed was used. This also had an extension piece if required. The bottle, with the original lid, was placed over the entire plant/plant section with lid left in place for a period of approximately 1 hour. After this period the lid was replaced with one that had a 6 mm hole to allow for insertion of the TD tube. The use of the hood was not possible for *Dracunculus vulgaris* due to its size. For *S.flava* the hood was used where possible for different plant sections but was sometimes difficult without compromising the plant itself. For pitcher sampling it was attempted to hood only an individual pitcher but sometimes it was necessary to sample groups of pitchers from the same plant.

Collection on the TD tube was done using a Drager hand bellows pump capable of 100 ml air collection for each squeeze. It was not possible to send the electric pumps to the remote sampling site. Preliminary experiments indicated that, although a sampling volume of 1000 ml produced detectable

levels of compounds, a sampling volume of 2000 ml (effectively exhausting the headspace of the sampling hood) produced better results. Tubes were sealed using the brass ends and posted by Royal Mail in standard packaging to Huddersfield for analysis. The aim was to analyse tubes within 2-3 days of sampling but if this was not possible they were stored according to section 2.4.

Pitchers of *S.flava* are thought to be sterile before they open which may yield a different olfactory signature to when bacteria are present. Although this was not specifically investigated, samples were taken for TD and HS analysis on open pitchers that did not yet have insects in the pitcher (pre -insects) but would likely have bacteria in the digestive juices as well as after insects are trapped (post-insects).

Figure 7.6 'In-house' sampling hood with extension piece

7.2.2.2 Sampling for HS analysis

HS was only carried out on *S.flava*. Sections of the plant were collected, cut and dissected using nail scissors and sealed into 20 ml headspace vials. Where possible different parts were taken from the actual same plant but sometimes it was necessary for a sample to be taken from one of the same species or variant.

Flowers were cut into the following sections:

- petals
- stigma hood
- stamen
- ovary
- sepals

Pitchers were taken at different stages and cut into sections:

- lid
- first 1 cm of pitcher throat (after lid)
- second 1 cm of pitcher throat (after lid).

The liquid from the bottom of pitcher was also collected by cutting the pitcher bottom and collecting the fluid in a vial.

Individual Phyllodia were used which were also dissected to fit into the vials.

Generally, vials were analysed within 2-3 days of sampling. However, where it was not possible to send the samples or analyse them within this time frame they were frozen prior to sending and/or once received.

7.2.2.3 Pitcher fluid experiment

To investigate if pitchers actively produce the smell given off from the pitcher or if it is due to the presence of decomposing insects in the pitcher some pitcher fluid pre-insects was set aside. The following were set up for HS-GC-MS analysis: -

- Pitcher fluid
- Pitcher fluid plus flies
- Pitcher fluid plus wasps

The insects were collected from one of the author's decomposition experiments when available. These vials were also run using deionised water for comparison. These were sealed into 20 ml HS vials and left for 1 week before running on the HS-GC-MS.

7.2.3 Instrumental analysis

As the aim of this work was to evaluate the application of the standard sampling and analysis protocol but using the SOPs developed earlier to a different, but chemically related, system (i.e. by using phyto-mimicry) it was intended to keep the protocol as unchanged as possible. However, certain additional considerations and adaptations needed to be made when studying plants and these are discussed below.

7.2.3.1 TD-GC-MS

The conditions used for the TD-GC-MS analysis were as in the standard SOP (section 6.3) but with a modification of utilising the recollection step. Although recollection should be done with care and has drawbacks (see section 3.4.6) it was decided that in light of the complexity of obtaining a sample, limited flowering of plants, importance of capturing each life-cycle stage etc. this was offset by being able to re-run a sample should there be an instrumental issue.

If the peak levels seen were low or not many peaks seen using the standard 20:1 split, then using splitless was an option on the re-run. This must be also used with caution when anlysing unknown samples to avoid system overload.

7.2.3.2 HS-GC-MS

The conditions used for the HS-GC-MS analysis were as in the standard SOP (see section 6.3).

If peaks were at low levels, then the HS oven was successively increased from 37 °C (as dictated by the SOP) to 60 °C and then 80 °C. Even if increased abundance and variation was seen with increased temperature, it was stopped at 80 °C to avoid moving too far from adherence to natural conditions and

limited to the 3 heating cycles as repeated heating damages plant material and peaks may no longer be representative of the original plant material.

7.3 Results for mimicry in the phyto-kingdom

Phyto-mimicry was an interesting area of research in its own right and once the SOPs' robustness was successfully noted, the author continued investigation yielding results presented as a combination of observations and tablature. VOCs are shown to be distinct for different plants but with some commonalties across species. Some VOCs such as sulphur-containing DMDS are common to both plants and decomposition.

7.3.1 Observations

***Eucomis bicolor* beheading**

Two *Eucomis* flowers were inadvertently beheaded during one aspect of the study so these were sampled following beheading. It was observed that although the high level compounds were still present in significant amounts, the subtler components were no longer seen when the flower was not growing. Also observed was DMTS in a beheaded flower but not when it was growing, where DMDS was observed.

***Sarracenia flava* regeneration time**

It was observed that regeneration time was generally much longer than that of pure standard chemicals and also longer than that of active decomposition samples that are highly odourous.

***Sarracenia flava* time of day**

It seemed that nocturnal samples had many less chemicals in their profile than diurnal and sometimes differences were observed.

7.3.2 Tables

Results from the experiments are presented in Tables 7.1-7.5. These show the types of VOCs detected for the different plants studied in this part of the project. It can be seen that there are VOCs common to plants and that there are also many differences in VOC profile.

Tables 7.1 and 7.2 show the presence of DMDS (also seen in decomposition) in plants known to be oligosulphide producers (*D.vulgaris* and *E.bicolor*) which supports previous work [8]

Plant	Technique	Chemicals of interest
Dracunculus vulgaris (sulphur producer)	TD-GC-MS	DMDS and DMTS were detected but at insignificant levels (<3000 abundance).

Table 7.1 showing the VOCs released by carrion mimicry/sulphur producing *Dracunculus vulgaris* using TD-GC-MS

Table 7.2 compares the VOC profile for two variants of the same species where sulphur compunds are observed in the one attracting flies as pollinators (*E.bicolor*) and sweeter smelling compounds in the one attracting wasps (*E.pole evansii*) which supports previous findings [8]. D-limonene can be seen to be common to both *Eucomis spp.*

Plant	Technique	Chemicals of interest
Eucomis bicolor (sulphur producer – fly mediated pollination)	TD-GC-MS	The following were detected from more than one sample: • ethyl acetate • DMDS • α-pinene • **D-limonene** The following were only detected from one sample: • DMTS • camphene • β-myrcene • β-phallandrene • carene • 1,3,6-octatriene,3,7-dimethyl (Z) (ocimene)
Eucomis pole evansii (wasp-mediated pollination)	TD-GC-MS	The following were only detected from one sample: • **D-limonene** • 1,3,6-octatriene,3 ,7 dimethyl (E) (ocimene) • 1,3,6-octatriene,3 ,7 dimethyl (Z) (ocimene) • 3-carene • 1,5,7 -octatrien-3-ol,3,7-dimethyl (Geraniol) • 3,5-dimethoxytoluene • benzene, 1,2,3,-trimethoxy-5-(2-propenyl) (elemicin)

Table 7.2 showing the comparison of VOCs released by *Eucomis* species that undergo fly (sulphur producer) and wasp-mediated pollination using TD-GC-MS. Note D-limonene is common to both

Table 7.3 shows a comparison of the VOC profile for the different stages of the lifecycle of *S. flava* using both TD-GC-MS and HS-GC-MS. It can be clearly seen that the profiles differ greatly which will have an impact on both pollinator and prey attraction. As is common throughout this thesis and dictated by the SOPs, these results were qualitative i.e. showed the presence of chemicals of interest but did not quantify their amounts

Lifecycle stage	Technique	Chemicals of interest detected
Flower	TD-GC-MS	The following were detected on at least four samples: • α – pinene • camphene • β-pinene • D-limonene • eucalyptol • 1,3,6 -octatriene,3,7-dimethyl (Z) (ocimene) • dodecane • benzene, 1-(1,5-diemthyl-4-hexanyl)-4-methyl (curcumene) • cyclohexene,1-methyl-4-(5-methyl-1-methylene-4-hexyl) (β-bisabolene)

		The following were detected on at least two samples: • 3-hexen-2-one • 2-pentene 4,4-dimethyl- (Z) • β -phellandrene • benzene, 1-methyl-2-(1-methylethyl) • 1,3,6-octatriene3,7-dimethyl (E) (ocimene) • acetophenone • benzoic acid, methyl ester • 1,6-octadien-3-ol3,7-dimethyl (geraniol) • borneol • cis a-bisabolene • 1H-3a,7-methanoazulene,2,3,4,7,8,8a-hexahydro-3,6 (α-patchoulene) The following were only detected on one sample: • 3-penten-2-one,4-methyl (mesityl oxide) • caryophyllene • linalool oxide trans
	HS-GC-MS	Petals: • D-limonene • α-pinene • β-pinene • camphene • ocimene Stigma hood: • D-limonene • α-pinene • β-pinene • camphene • ocimene • eucalyptol Stamen: • D-limonene Ovary: • D-limonene Sepals: No compounds detected
Pitcher	TD-GC-MS	Pre-flies. Seen on at least 2 samples: • benzaldehyde • 1,3,6-octatriene, 3, 7dimethyl- (Z) • 1,4-cyclohexadiene-1-methyl-4 (1-methylethyl) • benzene (1-ethylbutyl) • caryophyllene • 1,6-cyclodecadiene, 1-methyl-5-methylene-8-(1-methylethyl) Post-flies. Seen on at least 2 samples: • DMDS
	TD-GC-MS (splitless)	Post flies. Seen on at least 2 samples (*splitless)*: • triethylamine • ocimene • caryophyllene
	HS-GC-MS	No compounds detected
Phyllodia	TD-GC-MS	No compounds detected

	TD-GC-MS (*splitless*)	The following were seen *(splitless*): • eucalyptol • α-cedrene oxide
	HS-GC-MS	No compounds detected

Table 7.3 showing the VOCs detected at different stages during the lifecycle of *Sarracenia flava* using TD-GC-MS and HS-GC-MS

Table 7.4 shows the presence of sulphur-containing VOCs in the species of pitcher plant known to be associated with the smell of insects and decomposition [5]

Plant	Technique	Chemicals of interest
Sarracenia purpurea	TD-GC-MS	DMDS and DMTS were detected

Table 7.4 showing the VOCs detected for *Sarracenia purpurea* using TD-GC-MS

Table 7.5 shows the presence of DMDS in water for both flies and wasps. This suggests this is something given off during decomposition of the insect bodies themselves rather than something actively produced from the pitcher fluid

Condition	Chemicals of interest
Pitcher fluid	None seen
Water	None seen
Pitcher fluid plus flies	None seen
Pitcher fluid plus wasps	None seen
Water plus flies	DMDS (peak height 936223)
Water plus wasps	DMDS (peak height 64913)

Table 7.5 showing the VOCs detected for the pitcher fluid experiment using HS-GC-MS

The below table (Table 7.6) shows the relevance in the literature of chemicals seen in the VOC profile of *S.flava* at different stages of the lifecycle

Chemical of interest	Chemical Information and scent	Stage seen	Relevance to current work
DMDS	(see Table 4.1)	Flower	DMDS has been observed to be heavily associated with decomposition both in this and previous work and therefore makes sense as a component in carrion mimicry and fly attraction.
Ethyl acetate	Ester of ethanol and acetic acid and has a sweet pear drop smell	Flower	Ethyl acetate has been seen in nature to enhance the attraction of insects to plants (palms) and is used as an entomological anaesthetic [17]
D-limonene	Cyclic terpene that smells of citrus fruits	Flower	
Ocimene (both isomers)	Monoterpenes with a pleasant sweet odour	Flower, pitchers (post-insects)	

3,5-dimethoxytoluene	Phenolic derivative known to be associated with floral scent chemistry particularly that of the rose	Flower	
Geraniol	Monoterpenoid alcohol associated with essential oils and perfumes and has a rose-like smell [18]	Flower	It is known to be a stimulus for honeybees and hornets preying on honeybees [18]
Elemicin	benzene, 1,2,3,-trimethoxy-5-(2-propenyl), spicy flowers	Flower	
Carene	Pungently sweet	Flower	Associated with plants [9]
α-pinene	Monoterpene associated with the smell of pine and turpentine.[2]	Flower	
Camphene	Bicyclic monoterpene associated with pine, camphor and industrially related to α-pinene	Flower	
β-phallandrene	is a cyclic monoterpene associated with eucalyptus and with a pleasant minty smell	Flower	
β-bisbolene	is a sesquiterpene, like most terpenes associated with essential oils	Flower	Associated with pheromones of stink bugs and fruit-flies so reasonably could be involved in insect attraction
Borneol	is a terpene alcohol that can be oxidised to camphor (its ketone).	Flower	
Caryophyllene,	a sesquiterpene associated with cannabis, rosemary and hops	Flower, pitcher (pre and post-insects)	
Benzaldehyde	Aldehyde with an almond-like smell	Pitcher (pre-insects)	
Triethylamine	Amine associated with smell of decomposition	Pitcher (post insect)	Anaesthetises *Drosophila*
3-hexen-1-ol (Z)	Alcohol whose aldehyde is involved in the smell of leaves	Pitcher (post-insects)	

Table 7.6 Odour information of detected chemicals (unless otherwise stated references [19-21])

7.4 Discussion for *Dracunculus vulgaris*

The detection of oligosulfides DMDS and DMTS supported previous work on the amorphophallus-related plants. [7] In the same way that *Amorphophallus titan* blooms very infrequently so does the *Dracunculus* studied here, albeit to a lesser degree. Where the *Amorphophallus titan* takes years for a

few days flowering [8] this simpler plant was observed to take months of growing for approximately two days of full bloom, where the scent was observed to be pungently strong.

Perhaps further study to see how the levels change in relation to each other over time; before, during and after flowering would be interesting. Also investigating any interplay of subtler VOCs would be worthwhile.

7.5 Discussion for *Eucomis* spp.

7.5.1 Fly-mediated pollination system

Eucomis bicolor sampling yielded the expected DMDS [9] which was seen in the majority of samples. The levels were high, approaching overload for the instrument. In addition, ethyl acetate and D-limonene were also seen in comparatively high levels for most samples. Many other chemicals were observed with only α-pinene seen in more than one sample but some chemicals detected were known to be decomposition or plant associated so were listed for interest (see Table 7.2)

DMDS was expected from previous literature and is thought to be the main attractant for flies required for pollination. [9] DMDS is thought to attract *Diptera, Calliphoridae* and *Muscidae*. [2] The current work confirmed the work of Shuttleworth and Johnson (2010) by detecting the sulphur compound DMDS when observing the presence of blowflies during sampling. Interestingly DMTS also thought to be involved in this attraction was only observed in one sample.

Ethyl acetate, seen in high levels, could aid in pollinator attraction or may be a by-product of other compounds present. Ethyl acetate has been postulated to attract *Diptera, Muscidae, Sarcophagidae*, and *Drosophilidae*, [2] a wide range of fly genera, which would make sense as a general fly attractant with other compounds being combined for individual needs and specificity. Its use as an entomological anaesthetic may suggest its use in nature has evolved to utilise these properties of ethyl acetate to sedate the insect thus prolonging the stay for flower pollination using appropriate levels.

D-limonene, is present in high levels both in the fly and wasp pollinators indicating it a chemical widely present across *Eucomis* species and perhaps the wider plant community. Incidentally D-limonene was also observed in this work when studying *Sarracenia flava* as well.

DMDS, ethyl acetate and D-limonene seem to be associated with all variants of the species sampled, regardless of if the flower is actively growing or not and thus perhaps could be used as markers for *Eucomis bicolor*. The other, subtler components seem to be more variant specific with differing VOCs observed between variants of different colour. It may be that these are more subtly involved in mimicry depending on the fly species desired by different variants for pollinator attraction. In the same way that colour and UV-activity are known to have an impact on plant pollinators,[9] like in other plant mimicry systems where a subtle blend is required to simulate carrion for fly attraction, [6] perhaps different *Eucomis bicolor* variants have also evolved to have slightly different VOC production.

Following beheading (see section 7.3.1 observations) the presence of high level compounds may be due to them being in sufficiently high levels to not quickly volatilise and thus still be detected but the subtler ones were no longer seen. This indicates an active, continual production of VOCs from the plant and would make interesting future work.

DMDS, as well as ethyl acetate and D-limonene, was seen in high levels during flowering and flies were observed when sampling to be attracted to the flower.

Some compounds associated with plants were seen but in only one sample, these were noted for interest but would require further investigation as to their specific relevance.

The fly-mediated pollination was of specific interest to this work as it was thought to be related to fly attraction and carrion-mimicry and did show a link particularly with the presence of the decomposition associated oligosulfide, DMDS. [12, 22-28]

7.5.2 Wasp-mediated pollination system

Wasp-mediated pollination was studied using *Eucomis pole-evansii* and *Eucomis 'twinkle stars'*, known to attract wasps for pollination purposes.[9]

D-limonene was observed in high levels in both wasp-pollinator samples as well as in the fly-mediated pollination indicating a possible marker of not only species but perhaps genus. This may not be discrimatory enough alone as it was also observed in other plant systems (see *Sarracenia flava*) but may be able to be used in conjunction with other chemicals.

Ethyl acetate and DMDS, both seen in high levels in *Eucomis bicolor*, were notably not present here indicating these are heavily involved in fly attraction and not necessary for the attraction of wasps.

The chemicals detected for the wasp species (see Table 7.2) interestingly are conserved between the two species. The list shows all chemicals detected and was seen in both. This shows less variation between wasp-mediated pollination species but also much less complexity of VOCs present and not the same subtle variation between. This may be a product of the nature of pollinator attraction. Decomposition is innately a very complex matrix system and thus may require more complexity and subtle variation for its phyto-mimicry. Nectar attractive to wasps may be much more consistent with less variation between stimuli to different wasp species.

Ocimene (both isomers) and 3, 5 –dimethoxytoluene were detected here and were somewhat expected as they had been observed in previous literature. [9] The sweet floral nature of these compounds make their attraction to wasps and their clear absence from the *Eucomis bicolor* obvious.

Geraniol was detected and being an essential oil component indicates its importance in floral scent chemistry. Due to its association with honeybees it is reasonable it may also stimulate wasps for their attraction. Another essential oil-associated compound is elemicin (benzene, 1,2,3, -trimethoxy-5-(2-propenyl)), which was also seen in both wasp *Eucomis* species studied.

Carene has been seen in many plants. It is one of the overlaps with the fly species. Another overlap is the aforementioned D-limonene and where there is overlap this could indicate genus specificity or an association with the botanical scent of the wider plant community.

7.5.3 Conclusion for the comparison of fly and wasp attractors

It is clear from this aspect of the study that the floral scent chemistry of *Eucomis* is both varied and selective depending on pollinator. Wasps are known to be attracted to sweet nectar associated chemicals which were detected here and had vastly different floral scent chemistry to those mimicking the smell of death.

Wasp-pollinated *Eucomis* seem to share more chemicals inter-species where fly-pollinated vary even with intra-species variants. This may be an indication of the need for subtler evolutionary development when undergoing decomposition mimicry than that of sweet nectar. However, it may be that actually it is the complex combination of chemicals and the subtle blending that attract the pollinators and like in other plant systems one chemical e.g. DMDS is insufficient alone. [4]

Subtle blends with minor components may have an important role to play that is less essential for wasp attraction. However, it certainly seems that there is still an essential role for key components that are required for the attraction of flies as seen here and with previous work with the presence of DMDS. Indeed, it has been suggested that the mere presence of DMDS can shift pollinator from wasp to fly in certain *Eucomis* species [9] but it may be that the minor components have a less well understood role, perhaps of species specificity for attraction.

7.6 Discussion for pitcher plants - Lifecycle of *Sarracenia flava*

Sarracenia flava has been studied by previous workers in combination with the investigation of other carnivorous plants. [2, 5] However, there is little work on the separate study of the different stages of its lifecycle which is the focus of this work.

7.6.1 TD-GC-MS analysis of *S. flava* flowers

Flowers of *Sarracenia flava* were sampled from several different variants. There was some conservation of chemicals between variants, perhaps potential key indicators of species and some variation was also seen. For the main analysis of the flowers, samples of different variants were merely assumed as replicates, regardless of time sampled or variant used. However, it is also worth noting that there were observed temporal differences and regeneration time has an effect (discussed further below).

The chemicals seen in 4 or more samples (see Table 7.3) are all associated with plants and essential oils. [20] The levels at which they are detected are significant but different for all. The peak levels seen are sufficiently high for confident detection but the variation in levels between is also high.

D-limonene was seen in high levels throughout, suggesting its use as a possible indicator. However, this was also seen during the *Eucomis* analysis and perhaps could be a more generally plant associated compound. α-pinene, camphene, ocimene (Z) and eucalyptol are all seen in the majority of samples but in different levels and thus could be used in combination as a species indicator, perhaps with further investigation leading to the ability to differentiate between variants by their relative ratios.

α-pinene, camphene and ocimene were also seen in the other plant work, albeit in much lower levels so may only be suitable for indication of species in very specific ratios. Ocimene, also seen in the *Eucomis* work, was linked to *S.flava* in the literature, most notably in the work of Jurgens *et al* (2009) [2] where interestingly camphene and α-pinene were not, albeit their work focussed only on pitchers and not the whole lifecycle, as studied here. Eucalyptol seems to be more specific in this situation and perhaps it could be used as a key indicator in the context of α-pinene, camphene and ocimene to be indicative of *S.flava* flowers.

Other chemicals, observed in two or more samples were also of note. Of particular interest were β-phallandrene, ocimene (E), geraniol and borneol which are all plant associated terpene derivatives. Both isomers of ocimene as well as geraniol have been observed in this part of the study and the *Eucomis* aspect so may not offer enough discrimination. They possibly could be used in conjunction with other chemicals as a means to identify individual *S.flava* flowers.

The list seen in 4 or more (see Table 7.3) could be used for species determination and then the others used for variant floral chemistry investigation. β-phallandrene, β-bisbolene and borneol were all observed. β-bisbolene is associated with pheromones of stink bugs and fruit-flies [20] so reasonably could be involved in insect attraction. Indeed, it appears there are quite a few camphorous and eucalyptus associated compounds involved in the yellow pitcher plant floral chemistry. It was observed to smell piney, citrusy and woody as well as with the olfactory detection of cat urine. The cat urine smell has known association with pitcher plant flowers [29] and has been observed on numerous occasions, over years by Martin Brook.

Mesityl oxide (3-penten-one, 4-methyl) which smells of cat urine was only observed on one sampling occasion but this may be due to temporal release or trace levels making it detectable by olfaction but not instrumentation (due to odour threshold-see section 1.5.1). Another chemical only seen on one occasion was linalool oxide trans. Although this was not initially of interest, it was observed in the work of Jurgens *et al* (2009).[2] Although they stated pitchers as their sampling location there may have been neighbouring samples with open flowers present. Caryophyllene was also seen in one sample and could also be considered for individual variant discrimination.

Although it was decided to use all samples merely as replicates there are some individual observations worth noting: -

- **Regeneration time** –This was longer than pure chemicals or decomposition work. This may be longer with plant analysis than in the lab as the plant needs to synthesise the compounds for release. It may also be longer than for the decomposition work as the compounds may be

subtler and in lower concentrations especially for the individual ones. If samples were taken too soon then some chemicals were not detected in subsequent samples.

- **Temporal effects** –Time of day. It may be the plant does not need to release chemicals for insect attraction during the night as the insects are not present and this would be a waste of energy. Another postulation is the effect of temperature, humidity and vapour pressure. Increased temperature in the daytime may allow the volatilisation of chemicals not seen at night. Previous literature on the study of carnivorous plants (in that case Venus fly trap) has suggested that for certain chemicals, especially terpenes, their biosynthesis and emission is slowed by decreasing the plant temperature so there could be similar mechanisms at play here. [30] Temperature, moisture and other environmental factors would need to be investigated further to be sure.

Using TD-GC-MS the floral chemistry profile for *S.flava* shows some similarity between samples of different variants with some degree of difference. Key chemicals could be used to identify species and then profile further for variant determination. Evolutionary development of the plant for temporal separation of flowers and pitcher to avoid prey-pollinator conflict [5] probably means there will be a great distinction between that of the floral and pitcher scent chemistry profiles. These will depend on the insect target of interest for either pollination or for prey. Different carnivorous plants have different strategies for overcoming prey-pollinator conflict and these include geographical and temporal separation. [5, 31] The temporal separation seen in *S.flava* would account for the differences in scent chemistry observed between its flowers and pitchers.

7.6.2 HS-GC-MS analysis of *S. flava* flowers

Headspace analysis was used in an attempt to determine which part of the flower produced or at least presented the chemicals of interest. Splitting the flower into sections (see Table 7.3) yielded interesting results. No peaks were seen on the sepals which was not unexpected as these are located at the back of the flower structure and would have no insect access. All other portions of the structure presented chemicals.

D-limonene was expressed on all other structures but the main presentation of chemicals was the petals and stigma hood where D-limonene, α and β-pinene, camphene and ocimene were seen on both as well as eucalyptol on the stigma hood. These were also seen using TD-GC-MS analysis which gives a double confirmation of their presence. Their location is indicative of their release and/or presentation. To allow pollination an insect has to enter the flower and rub against the desired area to collect pollen to distribute it to the next plant. [6, 10, 11] Therefore if the chemicals detected were involved in pollinator attraction it would make sense to present them on petals and stigma hood.

The highest concentration of these chemicals was seen in the petals and they act as the entrance to the flower and thus pollinators would be attracted to this entrance. The stigma hood (where hopefully they will obtain/deposit pollen) also has significantly high levels of chemical and the additional eucalyptol. A subtle difference between petals and stigma hood may allow directional attraction through

the flower. It would not be beneficial to have chemicals emitted from the sections of flower not involved in pollination.

Further investigation with increased headspace temperature may yield subtler differences between sections.

7.6.3. Pitchers *of S.flava*

Sarracenia flava has evolved to have temporal separation of flower and pitcher to avoid any pollinator-prey conflict. [5] The release of different VOCs depending of life-cycle stage would allow for the attraction of different insects depending on pollinator and prey species desired. VOC profile has been shown to change from pre-insects following insect trapping and subsequent digestion in pitchers and it has also been postulated that this inevitable change in VOCs can shift prey attraction. [5] This study investigated all life-cycle stages and samples were taken of pitchers pre- and post-insect and stark differences were seen, most notably the flower scent chemistry is much more complex than that of the pitchers. Following the entry of flies into the pitchers the VOC profile changed from a variety of compounds to DMDS being the only one detected in very high levels. This is likely the product of decaying insects but may be self-perpetuating by allowing this prey shift discussed in the work by Jurgens and colleagues (2009, 2012).[2, 5]

7.6.3.1 TD-GC-MS analysis of pre-insect pitchers

Although sampling was done on different variants with the hope of comparison, population constraints meant they had to be taken merely as species replicates. Chemicals that were detected in 2 or more replicates (see Table 7.3) of particular note were ocimene and caryophyllene (both seen in flowers) as well as terpinene (terpene isolated from plant sources) and benzaldehyde.

Benzaldehyde is known to be involved in the stimulus in various insects. [2, 30] This was detected in this current study during the pitcher stage thus supporting the works of both Jurgens *et al* (2009) and Kreuzwieser *et al* (2014). [2, 30] Interestingly, benzaldehyde was not one of the VOCs emitted by *S.flava* flowers in the current study and thus may indicate one of the chemicals involved in the differentiation between pollinator and prey attraction. Conversely the pinene isomers and D-limonene chemicals, consistently present in flower analysis, were not seen in the pitchers. It is worth noting that Jurgens *et al* (2009) [2] did not isolate lifecycle stage so any differences seen in this study may be due to that.

The pitcher bouquet was generally less complex than that of the flowers. A possible reason for this could be that the pollinators required are quite specific and require a complex profile for attraction. Kreuzwiser *et al* (2014) studied the venus-fly trap and found some chemicals to be ubiquitous to certain plants including caryophyllene.[30] Although a very different carnivorous plant, perhaps *S. flava* also releases VOCs of a more general profile for prey attraction. Indeed *S.flava* is known to have a broad prey range and may not evolutionarily desire to limit genus or species by emission of a very specific and complex VOC profile.[10, 11]

Jurgens *et al* (2009) [2] investigated different carnivorous plants and whether they use volatiles to attract prey insects. They looked at *S.flava* pitchers but didn't study the individual pieces and lifecycle as was

done here. It was found that *S.flava* pitchers were seen to have alcohols, esters, benezenoids and terpenes/terpene derivatives. In this current work terpenes/terpene derivatives were the main compounds of note as these were known to be plant associated. However, these were not always the same as those found in the study by Jurgens *et al* (2009).[2] This may have been due to a number of factors with one possibility being the study and isolation of specific lifecycle stage (including pre and post insect pitchers) here where theirs was un-isolated pitchers.

It is worth noting that dodecane and other long-chain alkanes (also seen in decomposition) were noted as plant associated in previous literature. [2] These were observed in the current work in both flowers and pitchers but were not used for discussion or comparison as they were called into question during contamination and TD tube artefact issues.

7.6.3.2 HS-GC-MS analysis of pre-insect pitchers

There were no significant peaks (as defined by the SOP) seen for headspace analysis pre-insects. However only the standard 37 °C HS oven temperature was used. This prompted the increasing of the oven temperature for later analysis i.e. the post-insect samples.

7.6.3.3 TD-GC-MS analysis of post-insect pitchers

Using the standard experimental conditions for the post-insect TD-GC-MS analysis, the only significantly observable peak was DMDS. This is in stark contrast to the complexity of the pre-insect spectra. This decomposition associated compound has most likely been released following the decomposition of the soft tissue of the digested insects in the pitcher. Other decomposition associated compounds may be emitted but in quantities insufficient for detection. TD is known to preferentially adsorb DMDS so this could be a reason for its detection with nothing else. [13, 14] The fact that none of the VOCs thought to be involved in attraction are detected could be due simply to tube overload of DMDS, they are no longer required for release by the plant once prey is present, or they are emitted at levels detectable by olfactory senses in prey but not by the sampling technique/instrumentation.

It was thought that there could be other trace VOCs present but not seen so the instrumentation was run in splitless mode. Although this has to be used with care to avoid overload on the instrumentation, it did yield some interesting findings. Firstly, other decomposition related chemicals were observed including triethylamine which, like the DMDS, is probably from the decomposition of the insect bodies which may act as a secondary attractant for prey attracted to carrion. In addition, some of the chemicals seen during the pre-insects stage were also detected. These included ocimene and caryophyllene, which may just be associated with the plant and not actively released thus becoming background to the decomposition VOCs. *Sarracenia purpurea* is known to smell of decomposition and DMDS [2] and DMDS was detected from its sampling (see Table 7.4) thus supporting previous workers. [2] It would be interesting to compare in more detail this and *S.flava* to further investigate if the release of DMDS is active in *S.pupurea* and not in *S.flava*. Another possibility for the differences between the pre and post insect samples could be an active shift in volatile release to attract different prey. *S.flava* is known to have a wide prey range and previous work has suggested that there could be an evolutionary shift in prey simply from the inevitable change once decomposition of prey occurs. [5] It may be that early

attraction leads one form of prey and later release leads to those more attracted to carrion for example blowflies. It is important to determine whether the plant releases the DMDS etc. or if it is simply a convenient occurrence that *S.flava* can use.

When using pitcher fluid (pre-flies) to see if it actively gives off VOCs or it is due to decomposing prey in the pitchers, no VOCs were seen from the fluid categories (see Table 7.5). DMDS, an indicator of a decomposition event, was seen in the water with both flies and wasps. Incidentally, this was in higher levels with flies, probably due to their ability to be broken down more completely as the wasp has a harder exoskeleton. This may indicate that the pitcher fluid may not release the decomposition VOCS as easily as water and perhaps even that they do actively give off the VOCs. However, the experiment also showed that both flies and wasps give off DMDS and thus its detection could be due to their decomposing bodies in the pitcher. This would require further study to make a full conclusion.

7.6.3.4 HS-GC-MS analysis of post-insect pitchers

In addition to the study of volatiles emitted from opened post-insect pitchers, samples of sections of pitcher plant material and pitcher fluid were taken for HS-GC-MS analysis. This was with a view to determine if chemicals were released from the plant material or as more of an inadvertent release from the digestive fluid.

Headspace analysis for plant study had the limitations of no sample concentration (which TD allows), decomposition of plant material and being destructive. However, it did allow the ease of isolation of different components of the plant. It also allows the artificial increase of temperature in the lab releasing volatiles otherwise not seen in the field but because of this artificial nature, it does require caution in its use.

Unlike with flower analysis, very few volatiles were actually observed from pitchers using HS analysis. This may have been due to difference in levels present between plant parts or that those in the pitchers are less appropriate for HS study. Even with increased HS temp (not required with flowers and not carried out on pre-insect samples) only a few compounds were seen. The one of most note was 3-hexen-1-ol (Z).

3-hexen-1-ol (Z) is known to attract certain insects but it may be just simply being involved in the olfactory signature of the plant material and specifically involved in prey attraction. It is present in the pitcher but not the fluid or hood. The fluid would be the location for release of VOCs from decomposing matter where the hood or pitcher or indeed the fluid could be the original VOC location area. This would also require further study.

7.6.4 Phyllodia of *S.flava*

The phyllodia was mainly studied for completeness of life-cycle. It was not thought to be involved in plant scent or insect attraction. The phyllodia structure remains after the pitchers die back until the following spring of new growth and is thought to be involved in maintaining the plant perhaps through photosynthesis. [10, 11]

Using HS-GC-MS and TD-GC-MS analysis in split mode yielded no peaks so splitless mode for TD-GC-MS was attempted and used with caution. Using this eucalyptol and α-cedrene oxide were seen. Eucalyptol may be plant material associated for *S.flava*. α-cedrene oxide is associated with woody material and probably is a consequence of the nature of the phyllodia but irrelevant in terms of scent.

7.6.5 Conclusion for the lifecycle of *Sarracenia flava*

Carnivorous plants release VOCs to attract both pollinators and prey. It is important that they have evolved to separate these two processes to avoid-pollinator-prey conflict. *S.flava*, the yellow pitcher plant, has temporal separation of flowers and pitchers and therefore it would be logical that the associated volatile release would differ.

S.flava has evolved to attract insects through a variety of methods including vein pattern, colour, UV activity as well as VOC emission. Although there were differences seen throughout lifecycle and between samples it also appears there are some marked similarities as well. Kreuzwieser *et al* (2014) [30] during their study of carnivorous plants, suggested there are some VOCs observed widely through the plant kingdom[30] and other much more species specific. *S.flava* in this study has shown some core chemicals as well as suggesting there could even be variant specificity and indeed differences seen between aspects of the lifecycle of the same plant.

S.flava has a wide prey range and may have evolved to use a certain core set of chemicals for general prey attraction that can then be varied accordingly to need. These ubiquitous VOCs may act as a signal for attraction, either of pollinator or prey, from a relatively large distance away and once closer to the plant get more specific depending on purpose. These would have to be recognised by insects from a complex background of other plants so may have to be in relatively high concentrations with the VOCs adding to the individual complex signature in more trace levels.[4, 30, 32]

7.7 Discussion for *Sarracenia purpurea*

Sarracenia purpurea is known to release sulphur based compounds and DMDS was detected in this study.[2] This adds to the idea that this carnivorous plant like other sulphur-producers may specifically release DMDS to attract pollinators or in this case prey. Its narrower VOC range may indicate its preference for flies over other insects for prey or it may be due to the presence of flies decomposing in the pitcher. Perhaps this leads to self-perpetuation of attraction but this would require further study including pitcher fluid collection to be certain.

7.8 Conclusion of mimicry in the phyto-kingdom

Carrion mimicry in the phyto-kingdom has been studied both in the current work and previous literature and has shown the presence of decomposition associated VOCs, most notably DMDS, with this study supporting the work of previous literature.

Carnivorous plants have been shown to attract both pollinators and prey using VOCs release. Here *S.flava* has been studied in terms of lifecycle and volatile release and the link to insect attraction for pollination and prey from previous work. Similarities and differences were noted but one key observation is that the difference between flower and pitcher is not just that of time, the VOC profiles are also quite distinct.

The SOPs have been shown to be fit for purpose as the author has detected VOCs from plants previously seen in the literature. Most notable the sulphur compounds like DMDS that were also presented by Shuttleworth and Johnson (2010) [9] and the VOCs matching those detected by Jurgens and colleagues. [2, 5, 6] This comparison acted as a sort of peer review to ensure the validity of the SOPs. The SOPs are robust and flexible with the ability to be used for this different but related application and the application has itself yielded interesting and relevant results.

7.9 Odour reduction system-another application of the SOP

In addition to the detailed study of plant systems by the author using the SOPs developed in the thesis, the author also helped another researcher develop a method for odour reduction.

The researcher was using a patented product primarily as an anti-microbial for use on carpets. It was hoped this would also reduce odour. Using the authors SOP and methodology for TD-GC-MS analysis, the author and researcher adapted the SOP for use with spiking carpets with acetic acid, butanoic acid, ammonia (ammonium acetate) and a Volatile Fatty Acid (VFA) mix to see if their levels reduced over time following application of the product.[33]

The methodology used was the TD-GC-MS system and very little adaptation to the method in the SOP was required. This was another successful application of the SOPs to related VOCs.

7.10 Chapter conclusion

The method developed for the SOPs for decomposition studies has been transferred well to these different applications. The VOCs studied are similar in nature and indeed some overlap is seen. There has had to be some further method development for the plant studies but the results have been promising. TD-GC-MS is more appropriate as it allows sample concentration and ease of storage. The HS technique is destructive with no concentration step and alone would not be an adequate method. However, in combination with TD-GC-MS it shows potential for powerful and promising further study.

Both the plant systems, studied in detail by the author, and the odour reduction system they helped to develop, showed the robustness of the SOPs. The SOPs were robust as there had to be adaptations for these related systems as compared to the testing and development carried out for decomposition and the SOPs were able to withstand these changes whilst still being fit for purpose. This added to the rigorous testing carried out in earlier chapters. The SOPs were designed to be flexible to allow the necessary modifications both for the field of decomposition as well as any related but similar systems.

The methodologies were shown to be applicable to VOCs of a similar nature and the two instrumental systems could be used for these other applications with very little change to the SOPs. Chemicals of note were consistent with previous authors [2, 7-9, 30] also suggesting the appropriateness of methodology.

This shows great promise to apply the SOPs to other areas of research as well as full organisms and forensic field studies for PMI determination. However, researchers, analysts and forensic practitioners need to be aware of the limitations of the SOPs and the associated limitations of the techniques used, especially for the complex matrices seen for decomposition study.

7.11 References

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8. OVERALL CONCLUSION

8.1 Conclusion

Five project objectives were identified and these have been largely met.

1.To develop a methodology capable of studying the relevant chemicals evolved during decomposition process and compare the validity of such in terms of repeatability and reproducibility.

This was achieved through extensive method development for the instrumentation (TD-GC-MS and HS-GC-MS).

Standard decomposition chemicals were selected based on preliminary studies and a search of the literature. [1-12] These chemicals both individually and as a mixture were then used to further investigate methodology in terms of repeatability and reproducibility and for evaluation of sampling parameters.

2.To collect, identify and profile as a function of time the VOCs produced during decomposition using Thermal Desorption Gas Chromatography Mass Spectrometry (TD-GC-MS) Headspace Gas Chromatography Mass Spectrometry (HS-GC-MS) and compare and contrast these techniques.

The results show innate difficulties with respect to precision. Profiling VOCs as a function of time was undertaken as part of the SOPs development process but was not itself investigated.

The results from the controlled experiments in this project indicate the great variability that could be expected for time profiling in the field because of this innate low repeatability of the techniques which has implications for interpretation of results obtained in the field using similar instrumentation.

Both TD-GC-MS and HS-GC-MS were extensively used and thus comparison was possible. HS gives better repeatability due to the controlled nature of it but is less applicable to collection of VOCs in the field environment.

3.To investigate the effect of experimental parameters such as sample size, headspace volume and other factors, on the VOC profile

Experiments identified optimum controlled parameters of sample size, volume of headspace, regeneration time and presence or absence of insects and substrate. Even with optimisation and with carefully controlled conditions a high degree of variability was observed. Hopefully with a firmer basis for consistency of methodologies and procedures these principles can be extended from laboratory setting into the field.

It is worth noting that the results of a PhD project in the same decomposition location as the author but utilising porcine cadavers and SPME fibres for headspace collection, also showed considerable variability in the number and abundance of VOCs detected using GC-MS. [13] This supports the idea that there are innate complexities in the analysis of decomposition VOCs which are not attributed to a certain collection technique or procedure.

4. To develop Standard Operating Procedures (SOPs) for the analysis of the VOCs evolved during decomposition.

Two functional SOPs have been developed (for both the TD-GC-MS and HS-GC-MS). These SOPs, once developed were then evaluated by the author and further tested by two competent analysts.

True validation was not possible as quantification was not attempted. However, the author did validate these SOPs within the scope of this project by carrying out repeatability studies on decomposition samples (pork) and by doping these samples with known quantities of chemicals.

Although these SOPs showed great promise for the study of decomposition, difficulties were seen in terms of repeatability due to the complex matrices and the limitations of the techniques themselves. Nevertheless, it can be concluded that these SOPs could be used as a starting point for analysts and forensic practitioners to use to test their instrumentation and methodologies for decomposition studies.

5. To test the robustness of SOPs using the phyto-kingdom as a related system with similar chemicals as decomposition.

A measure of the robustness of the SOPs was undertaken using chemically similar VOCs but produced by plants rather than from decomposition. In addition, these were also applied to test an odour reduction system.

The robustness of the SOPs was evaluated by applying them, after some adaptations to the sample collection procedure, to plants that mimic decomposition by producing chemicals that attract insects.

The SOPs were found to the applicable to these systems and the robustness demonstrated by being able to withstand minor modifications while still being fit for purpose.

8.2 Future work

There are considerable opportunities for future work.

The results demonstrated the crucial importance of the substrate on the VOC profiles and this is an area that should be studied further. The two SOPs allow comparative studies of substrate (e.g. soil) and headspace (above sample) in one experiment. This could be fully utilised in future work. A true comparison of the techniques could be made using carbon and running the techniques in parallel. The VOCs evolved could be monitored using active sampling for TD-GC-MS analysis and passive sampling from the carbon using HS-GC-MS analysis.

Solid Phase Micro-Extraction (SPME) (as mentioned in this conclusion under point 3) is a sample introduction method for GC-MS analysis of VOCs that has its own merits and limitations. [14] It may be possible to develop another complementary SOP for its use in conjunction with the two techniques here.

Use of an Internal Standard (IS), for example bromobenzene [3] or chlorobenzene [5] used by other authors due to their similarity in terms of GC to decomposition chemicals but are not known to be evolved during the decomposition process, could be investigated. These could be used in 2 possible ways 1: injected directly onto the TD tube and 2: added to the pure chemical mix. This could be used for ratio analysis

(using it for normalisation study where complex profiles could be compared by Principle Component Analysis-PCA[15]) or to ensure adequate sampling levels and, of course, would be necessary for any future quantification.

The manufacturer of the TD (Markes International) recommended method for IS use would be injected onto the TD tube. This was considered in this project but was deemed unnecessary for SOP development and added another complication, in that the tubes would be subject to potential contamination when opened and the sampling itself was done in air. However, this would warrant further investigation as this is part of methodology for validation and is a requirement for reliable quantification.

In terms of SOP testing and validation, more data and repeats would be required as well as inter-laboratory testing for reproducibility data to be obtained. Whole organism study would also be beneficial to demonstrate the ability to follow on from chemicals and small-scale pork experiments.

The work on phyto-mimicry demonstrated the robustness of the SOPs and this interesting field is worthy of further investigation. The robustness studies suggest future work could have wider applications outside the area of decomposition.

8.3 References

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Glossary of terms

- **BP** – Boiling Point
- **BSI**- British Standards Institution
- **CRM** – Certified Reference Material
- **DMDS** - Dimethyl disulphide
- **DMTS** - Dimethyl trisulphide
- **DOA** - Decompositional Odour Analysis (database)
- **FAMEs** – Fatty Acid Methyl Esters
- **FID** – Flame Ionisation Detector
- **GC-MS** – Gas Chromatography Mass Spectrometry
- **HS -** Headspace
- **HS-GC-MS** – Headspace-Gas Chromatography Mass Spectrometry
- **LOD** – Limit of Detection
- **MS** – Mass spectrometry
- **PMI** – Post Mortem Interval
- **RSD** – Relative Standard Deviation
- **RT**-retention time
- **SOIs** – Standard Operating Instructions
- **SOPs** – Standard Operating Procedures
- **SPME** – Solid Phase Micro-Extraction
- **TD -** Thermal Desorption
- **TD-GC-MS** – Thermal Desorption- Gas Chromatography Mass Spectrometry
- **UKAS** – United Kingdom Accreditation Service
- **VOCs** – Volatile Organic Compounds
- **VFAs** – Volatile Fatty Acids
- **VR** - Victim recovery