An investigation into the recovery of ignitable liquid residues from entomological samples using solid-phase microextraction

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Somehow I can't believe that there are any heights that can't be scaled by a man who knows the secrets of making dreams come true. This special secret, it seems to me, can be summarized in four C s. They are curiosity, confidence, courage, and constancy, and the greatest of all is confidence. When you believe in a thing, believe in it all the way, implicitly and unquestionable.

Walt Disney

Certificate of Authorship and Originality

I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree except as fully acknowledged within the text.

I also certify that the thesis has been written by me. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

Lisa Mingari

25/03/2011

Signature of Candidate

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Abbreviations

Standard Abbreviations

ACS	Activated Charcoal Strip
ASTM	American Society for Testing and Materials
e.g.	Latin: *exempli gratia*, "for example"
EIC	Extracted Ion Chromatogram
et al.	Latin: *et alia*, "and others"
GC	Gas Chromatograph
GC-MS	Gas Chromatograph-Mass Spectrometer
i.e.	Latin: *id est*, "that is"
MS	Mass Spectrometer
N.B.	Latin: *nota bene*, "note well"
NFPA	National Fire Protection Authority
NSW	New South Wales
RT	Retention Time
SIM	Selected Ion Monitoring
TIC	Total Ion Chromatogram
UTS	University of Technology, Sydney
LPD	Light Petroleum Distillate
m/z	mass-to-charge ratio
MPD	Middle Petroleum Distillate

Unit and Quantity Abbreviations and Symbols

amu	atomic mass unit
°C	degrees Celsius
g	gram
hr	hour
L	litre
m	metre
km	kilometre
min	minute
mL	millilitre
s	second
eV	electron volts

Prefix Abbreviations and Symbols

α	alpha
β	beta
μ	micro (10^{-6})
m	milli (10^{-3})
c	centi (10^{-2})
k	kilo (10^{3})
%	percentage
®	registered trademark

Definitions

There are words and phrases used in this thesis that have specific meanings in relation to this work. These have been described in the text; however, some of these definitions have been clarified below.

Absorption: The process in which a fluid permeates or is dissolved by a liquid or solid.

Accelerant: A substance, often an ignitable liquid, used to initiate a fire or increase the rate of growth or spread of fire.

Adsorption: Is the adhesion of atoms, ions, biomolecules or molecules of gas, liquid, or dissolved solids to a surface.

Ambient temperature: The fluctuating levels of heat in air.

Ant: An insect in the family Formicidae (order- Hymenoptera) which preys on carrion feeding arthropods.

Ante-mortem: Prior to death.

Arson: The act of deliberately and maliciously setting a fire to destroy property or to take a life.

Arthropod: Any of a large group of segmented invertebrate animals, such as insects and spiders, with jointed legs and sometimes a hard, external skeleton.

Blow fly: A higher fly in the family Calliphoridae, also known as bottle flies.

Calliphoridae: The insect order commonly known as blow flies.

Carrion: Decaying animal flesh.

Chromatography: Is the collective term for a set of laboratory techniques for the separation of mixtures. It involves passing a mixture dissolved in a mobile phase through a stationary phase, which separates the analyte to be measured from other molecules in the mixture based on differential partitioning between the mobile and stationary phases.

Coleoptera: The insect order commonly known as beetles.

Combustible liquid: Is a liquid that vaporises and forms flammable mixtures with air when in an open container or when heated. An important characteristic of a flammable liquid is its flashpoint. Flashpoint is the minimum temperature at which the vapour concentration near the surface of the liquid is high enough to form an ignitable mixture. Any liquid with a flashpoint between 38^{o}C to 93^{o}C is considered combustible.

Decomposition: Post-mortem degenerative rotting of the corpse.

Desorption: The phenomenon whereby a substance is released from or through a surface. This process is the opposite of sorption (either adsorption or absorption).

Diptera: The insect order commonly known as true flies.

Egg: The characteristic reproductive unit of an adult female. Most carrion insects deposit eggs, but a few deposit first-instar larvae.

Entomology: The study of insects.

Entomotoxicology: The study of foreign substances in insects.

Flammable liquid: Any liquid with a flashpoint less than 38^{o}C is considered to be a flammable liquid. (See combustible liquid).

Gas chromatography: The separation of organic liquids or gases into discrete compounds seen as peaks on a chromatogram (which indicates the relative amount of each specific component). Separation is done in a column that is enclosed in an oven held at a specific temperature or programmed to change temperature at a reproducible rate. The column separates the compounds according to their affinity for the material inside the column (stationary phase) and their boiling point.

Headspace: The gas phase in a container above the sample.

Ignitable liquid: Any liquid that is capable of promoting the spread of a fire, including a flammable liquid, combustible liquid, or any other material that can be liquefied and burned.

Incendiary fire: A fire demonstrated to be deliberately lit when referring to statistical data.

Instar: Larval growth stages.

Larva: Soft-bodied, sexually immature, feeding stage of an insect life cycle; otherwise known as a maggot for flies; plural is 'larvae'.

Larviposition: Eggs hatch within the adult female and are born as an active larva.

Maggot: See larva.

Maggot-mass: The collective, closely packed mass of higher fly larvae occurring in decomposing carrion.

Mass chromatograph: A representation of mass spectrometry data as a chromatogram, where the x-axis represents time and the y-axis represents signal intensity.

Mass spectrometry: A method of chemical analysis that vaporises then ionises, the substance to be analysed, and then accelerates the ions through a magnetic field to separate the ions by molecular weight. Mass spectrometry can result in the exact identification of an unknown compound, and is a very powerful analytical technique, especially when combined with chromatography.

Metamorphosis: Change in insects from larva to adult form.

Mobile phase: The part of the chromatographic system which carries the solutes through the stationary phase. The mobile phases are either liquids or gases.

Moult: Shedding of the skin of one life phase.

Mouth hooks: The paired maxillary oral structures of a maggot.

Muscidae: The insect order commonly known as house flies.

Necrophagous: Carrion-feeding insects; feeding on dead bodies.

Omnivorous: A tendency to eat anything and everything.

Oviposition: Egg-laying.

Post-mortem: After death.

Post-mortem interval (PMI): Time elapsed since death.

Prepuparium: Inactive post-feeding larval stage in which the body is contracted.

Pupa: That immature stage between the larva and adult in insects having complete metamorphosis. This is a stage of major transformation. Among higher flies the pupa is inside the puparium.

Pupariation: The immobilisation of the post-feeding maggot with the shrinking, hardening and darkening of its outer skin.

Puparium: A globular, barrel-shaped container formed from the retained, hardened skin of the third larval instar and inside which the pupa is formed.

Pupation: To change form into a pupa in the process of transforming from the larval stage to the adult.

Putrefaction: The foul smelling, anaerobic decomposition of moist or wet organic matter by micro-organisms.

Pyrolysis: A process where thermal energy (heat) breaks chemical bonds in polymeric materials. The resulting fragments are often volatile. Pyrolysis provides the fuel for matrices that do not undergo unassisted combustion. Wood burns because it pyrolyses into gas phase volatiles.

Pyrolysis products: The products formed as a result of the pyrolysis process.

Sarcophagidae: The insect order commonly known as fly flies.

Sheep strike: The condition produced by the development of blow fly maggots on living sheep.

Solid-phase microextraction (SPME): A sample preparation technique that involves the use of a fibre coated with an extracting phase, that can be a liquid (polymer) or a solid (sorbent), which extracts different kinds of analytes (both volatile and non-volatile) from different kinds of media that can be in liquid or gas phase.

Species: A group of potentially interbreeding individuals that will produce reproductively viable offspring.

Stationary phase: In chromatography, the stationary phase is the non-mobile phase that is contained in the chromatographic bed.

Succession: Groups of species successively occupying a given habitat as the conditions of that habitat change.

Suspicious fire: A fire, demonstrated to be possibly deliberately lit, where no other cause is evident when referring to statistical data.

Volatility: The ease with which a substance passes from being a solid or liquid to being a vapour.

Wasp: Any winged insect in the order Hymenoptera, generally possessing a sting. These may be scavengers, predators or parasites of organisms at carrion.

Weathering: The evaporation of the more volatile compounds of an ignitable liquid resulting in a greater concentration of the less volatile compounds. This may be due to environmental conditions or due to exposure to extreme heat of a fire.

Abstract

The analysis of fire debris can indicate the presence of an ignitable liquid, but the volatility of these substances means that the likelihood of detecting them diminishes over time. It is proposed in this thesis that when a scene contains burnt human remains, entomological samples can be analysed for the detection of ignitable liquids, as an alternative to fire debris. It is hypothesised that a larva's ability to invade areas protected from the external environment, such as the natural body openings of cadavers, and accumulate substances present in the tissue in which they are feeding, will extend the period in which ignitable liquids can be detected.

In small-scale experiments conducted under controlled laboratory conditions, petrol and kerosene were detected in larvae of the blow fly *Lucilia cuprina,* (Wiedemann) (Diptera: Calliphoridae) that had been fed on meat burnt using these ignitable liquids. Four sample sets of meat, each with six replicates (24 meat samples in total) were prepared. The first and second sets were burnt using petrol and kerosene, respectively. The final two sets were control groups. Six larvae were collected daily from each of the 24 meat samples for a period of five days. Once the adults had emerged, six adults and six puparia were also collected from each meat sample. All of the entomological samples collected were analysed using solid-phase microextraction gas chromatography-mass spectrometry (SPME-GC-MS). It was found that larvae of the blow fly *Lucilia cuprina* can be used in a small-scale setting to detect both petrol and kerosene from burnt substrates for at least five days. Positive results for the ignitable liquids of interest were also obtained for a limited number of adult flies and puparia.

Given these findings, further research was conducted using a more realistic experiment (conducted in duplicate) that mirrored a casework scenario more closely. Sample sets identical to those in the small-scale experiments were prepared using 24 piglets, each approximately 1.39 kg in weight, instead of the meat samples. These piglets were placed a minimum of 51 m apart at the Holsworthy Military Area in New

South Wales, Australia, for three days. After this time, the piglets were transferred to a controlled laboratory. It was found during these fieldwork experiments that petrol and kerosene could be successfully detected in larvae for as long as eight days, and in the adult and puparia samples for at least one month. These findings confirm the significant advantage of using entomological samples as an alternative to fire debris, in that they extend the period available for sampling volatile ignitable liquids by at least one month. In particular, puparia can withstand changing climatic conditions, and unlike the larvae and adults, are immobile and hence could be found close to human remains even after considerable time has elapsed.

Chapter 1:

Introduction

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1.1 Preamble

The work described in this thesis investigates the analysis of fly larvae (as an alternative to fire debris) for the detection of ignitable liquids when a scene contains burnt remains. Therefore, this chapter reviews two distinctly different areas of science, namely forensic entomology and fire investigation.

1.2 Forensic Entomology

Forensic entomology can be defined as the study of insects and other arthropods as applied to legal proceedings [1-5]. Insects are extremely adaptable creatures as they can be found in almost any habitat [5]. Hence, it follows that insects encompass approximately three-quarters of the estimated three to 30 million animal species on the planet [6]. Despite the vast population of insects, there are a relatively limited number of experts in the area of forensic entomology. As a result, the quantity of research carried out in this field, and the number of cases requiring this type of expertise, falls behind many other scientific disciplines [3,7,8].

Forensic entomology comprises three main branches: stored products forensic entomology, which focuses on insect infestation on food and other stored products, structural forensic entomology, which is concerned with legal issues stemming from insect infestations of structures, and medico-legal entomology [9,10]. It is the latter branch of forensic entomology that will be discussed further in this thesis.

1.2.1 Medico-Legal Forensic Entomology

Medico-legal forensic entomology is the branch of forensic entomology that deals with arthropod involvement in events surrounding a death scene [11]. This includes ascertaining the possible movement of a cadaver, the presence of drugs or toxins, the cause of death, and a determination of the minimum time since death, or post-mortem interval (PMI) [3,12-15]. Forensic entomologists are frequently called upon to make estimations in regards to the PMI.

1.2.2 Post-Mortem Interval (PMI)

If a post-mortem is held within three to four days of death, the PMI can normally be calculated through pathological indicators by the pathologist [16]. However, it is when this period is longer that entomological techniques become important. There are two methods available to forensic entomologists to calculate this timeframe. The first is based on identifying larval species collected at a death scene or autopsy and estimating their age according to known growth rates and extrapolating backwards to estimate the time of oviposition and hence the approximate time since death. To achieve this, it is important to note the temperatures recorded at the site where the samples were located [17,18]. The second method works on the principle that insects colonise decomposing remains in a predictable, successive pattern, which consists of a series of overlapping waves of different arthropods [1,18-22]. Hence, the changes in the insect community can provide an estimate of the PMI [17]. Both of these methods work on the premise that insects are attracted to remains almost immediately after death, depending on seasonal availability and weather conditions [7,11].

1.3 Insects

All insects belong to the class Insecta, which comprises approximately 20 orders [23]. The order which is most often associated with remains during the early stages of decomposition is the Diptera or true flies [5,24]. There are three sub-orders of Diptera: Nematocera, Brachycera and Cyclorrhapha [23]. The sub-order Cyclorrhapha (on which this study will focus) is further divided into three families of forensic importance: Calliphoridae (blow flies), Sarcophagidae (flesh flies) and Muscidae (house flies) [5,24].

1.3.1 Calliphoridae (Blow Flies)

The Calliphoridae, or as they are more commonly termed, blow flies, are a large family of medium-sized flies (adults 6 to 14 mm in length). They are found throughout the world with at least 140 species in Australia. These species differ predominantly on whether the species is oviparous (egg-laying) or ovoviviparous (eggs hatch immediately after being deposited). They can be blue, green, black or bronze in colour. The majority of the species have a metallic appearance, while some have a dull metallic sheen as the presence of a fine powder obscures the metallic appearance. This family of insects, together with the sarcophagid and muscid flies are the most significant species used by forensic entomologists and play a significant role in the PMI calculation as they are the first insects to arrive and colonise remains [2]. Calliphorid larvae are attracted to carrion, excrement and in the case of some species, open wounds.

1.3.2 Sarcophagidae (Flesh Flies)

The Sarcophagidae, or (more commonly) flesh flies are a large family of medium-sized flies (adults 2 to 14 mm in length). Much like the Calliphoridae, the Sarcophagidae are also found throughout the world, with at least 67 species in Australia. This species differs greatly in appearance to the Calliphoridae as they have grey and black longitudinal stripes on the thorax and a tessellated pattern on their abdomen. Sarcophagid larvae are also attracted to carrion and excrement, but may also feed on other insects

Female sarcophagid flies deposit first-instar larvae on carrion rather than eggs like many of the other families in this sub-order, and are therefore viviparous [2,11].

1.3.3 Muscidae (House Flies)

The muscids or house flies are a large family of small to medium-sized flies (adults 3 to 10 mm in length) that have a dull grey appearance. They are found throughout the world and are closely associated with domestic settings. Muscid larvae feed on a variety of materials including carrion, decaying plant material, excrement (both human and animal), pollen, blood and garbage [2].

1.3.4 Necrophages

Decomposing remains offer a temporary and progressively changing habitat and food source for a wide variety of organisms [10,13,20]. Arthropods are a major part of this community. Members of the Calliphoridae (blow flies) and Sarcophagidae (flesh flies) families are usually the first and most significant group of arthropods to colonise remains following death, with some arriving within minutes (given appropriate conditions) [2,25].

The life cycle of these insects involves complete metamorphosis, where an abrupt change in body structure occurs from the immature to the adult stage [26]. This process is initiated when the female blow fly oviposits (lays eggs) or the female flesh fly larviposits (lays live larvae) on carrion. She skilfully seeks out areas that offer protection from predators and are moist. This reduces the occurrence of dehydration and optimises the chance of eggs hatching. It is for this reason that body orifices [23] or wounds [27,28] are selected. Once the larvae hatch, they feed voraciously. Fly larvae digest their food externally by releasing enzymes onto the surrounding food and ingesting the resulting liquid. The presence of mouth hooks allows them to pull food into their mouths [23]. Larvae often tunnel into their food source. When this occurs, their posterior spiracles (the main breathing apparatus of the larvae) point outwards ensuring availability of air.

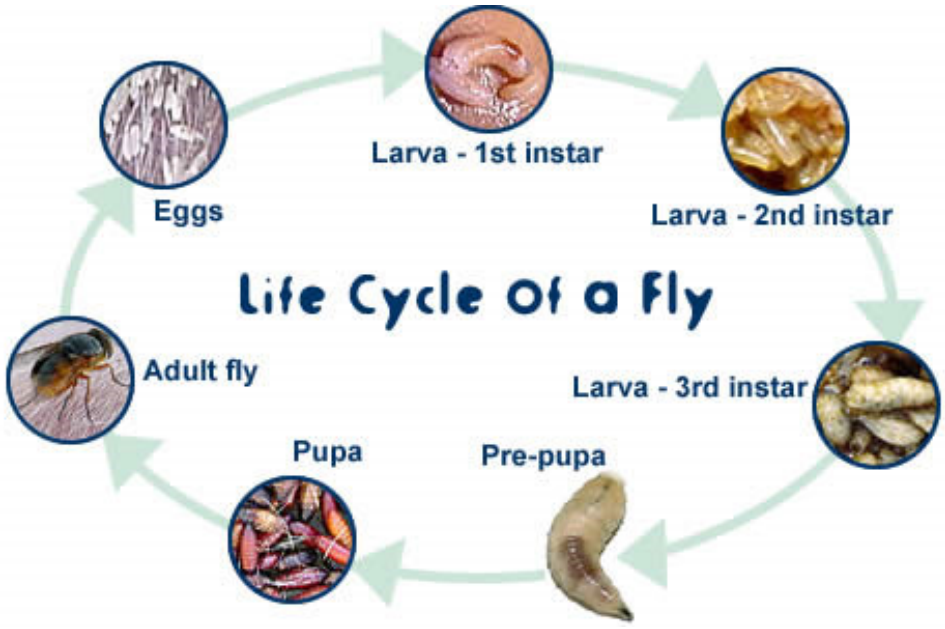


Figure 1.1: *The life cycle of a blow fly: the multiple stages from egg to adult (Photo: [29]).*

The duration of the life cycle of each species varies and is influenced by a number of factors, some of which include temperature, humidity, the presence of foreign substances on the substrate in which they are feeding, the type of substrate in which

they are feeding, and exposure to direct sunlight [2,24,30]. All species experience the same stages of development (see Figure 1.1), although the egg stage is sometimes, as in flesh flies, completed within the female (as first-instar larvae rather than eggs are deposited on carrion). The first-instar larval stage occurs prior to the first moult, when the cuticle is discarded. The second-instar larval stage occurs between the first and second moults. The third-instar larval stage occurs between the second moult and pupation. After reaching their maximum size and finishing feeding, third-instar larvae, then as pre-pupae, empty the contents of their crop and typically begin to wander from the carrion. Larvae migrate to a drier area where they are able to burrow and begin pupation. At this stage, the exoskeleton of the third-instar larva will harden and darken to form the puparium. The final immature stage occurs inside this structure, where the larva will transform into a fly. Once the fly has emerged, it takes approximately 24 hours for it to become completely expanded and pigmented. Following this, the fly requires food to survive. A female must obtain a meal rich in protein in order to mature her eggs and thus continue the cycle [2,16,23].

1.3.5 Waves of Insects

Members of the Calliphoridae and Sarcophagidae are routinely observed during the very early stages of decomposition, when the remains are relatively fresh. However, these families contribute to merely two of the eight waves of carrion insects that invade decomposing remains (see Table 1.1). The stage of decomposition (including the odours and liquids emitted) will determine the type of fauna associated with these remains. However, the succession pattern would differ if the remains were buried or in an aquatic environment [31].

Waves of Insects			
	Fauna	**State of remains**	**Age of remains**
First wave	Calliphoridae (blow flies)	Fresh	Fresh
Second wave	Calliphoridae (blow flies) Sarcophagidae (flesh flies) Muscidae (house flies)	Strong odour	
Third wave	Coleoptera (beetles) Lepidoptera (moths)	Fats become rancid	
Fourth wave	Piophilidae (cheese flies), Muscidae (house flies) Coleoptera (beetles)	Butyric fermentation and protein matter begins to ferment	3-6 months
Fifth wave	Muscidae (house flies) Sphaeroceridae (dung flies) Phoridae (scuttle flies) Coleoptera (beetles)	Ammoniac fermentation begins	4-8 months
Sixth wave	Acari (mites)	Remaining body fluids are absorbed	6-12 months
Seventh wave	Lepidoptera (moths) Coleoptera (beetles)	Desiccation complete	1-3 years
Eighth wave	Coleoptera (beetles)		> 3 years

Table 1.1: *Main groups of fauna associated with decomposing remains and the characteristics of these remains for each of the eight waves of carrion-feeding insects [11].*

The various fauna that arrive on decomposing remains (as shown in Table 1.1) can be divided into three groups. The first group is the necrophagous insects that feed on the remains. This group predominantly comprises Diptera (true flies). The second group is the predacious insects which feed on the necrophagous insects. This group predominantly comprises Coleoptera. The third group comprises insects that use the remains as an extension of their normal habitat such as, spiders, ants, bees and wasps [32].

1.3.6 Stages of Decomposition

The vast ecology of insects that attend decomposing remains contributes greatly to the process of decomposition. The presence of insects on these remains will accelerate liquefaction and disintegration through the distribution of bacteria, the excretion of digestive juices and the mechanical tunneling and burrowing action of larvae into the food source [21,32]. Further to this, insects feeding on carrion will develop their own microenvironment within this carcass. This in turn affects the succession of insects and the physiological development of fauna [33]. Therefore, it follows that decomposing remains that lack insect activity decompose and dry out at a much slower rate [18,21]. This was demonstrated experimentally when 20% of a piglet that lacked insect activity remained 200 days following initial exposure, whereas merely 10% of a piglet containing insects still remained six days after placement [21].

Although decomposition is a continuous process, decomposing remains that are exposed to arthropods will experience distinct stages representing physical changes of the carcass that can be observed [18,34] (see Table 1.2). These stages include: fresh, bloated, active decay, advanced decay, dry and remains [18,21,25]. The latter stages can be difficult to identify and therefore several studies have failed to differentiate between the dry and remains stages [20,28,35,36].

Decomposition		
Stage of decomposition	**Characteristics**	**Age of remains**
Fresh	Occurs when remains are first exposed	Days 1-2
Bloated	Putrefaction Odours of decay noticeable Liquids begin seeping out	Days 2-7
Decay	Penetration of abdomen wall Deflation of remains	Days 5-13
Post-decay	Diptera larvae depart Bones, cartilage, small portions of tissue and wet viscous material remains	Days 10-23
Remains	Bones and little cartilage remain By-products of decay have dried	Days 18-90 and beyond

Table 1.2: *The distinctive characteristics of the different stages of decomposition [18,20,21,25,28,35,36].*

Factors that influence the rate of decomposition include (but are not limited to); temperature, insect activity, scavenging activity, direct sunlight and freezing-thawing of remains [37]. Moreover, it has also been shown that carcasses are colonised at a faster rate outdoors compared with indoors [38].

1.4 Entomotoxicology

Insects are valuable tools in a post-mortem investigation. As was discussed earlier, insects present on a cadaver can be utilised to calculate the PMI (Section 1.2.2). Insects can also be used as reliable specimens for toxicological analysis [27]. This specialised branch of forensic entomology is termed *entomotoxicology*.

Entomotoxicology is the determination of toxic substances such as drugs, environmental pollutants and pesticides in carrion feeding insects [12,39]. Research in this field was initially aimed at investigating the transfer of environmental pollutants, such as mercury, to insects feeding on contaminated fish. A number of studies have confirmed the presence of this heavy metal in blow fly larvae, pupae and adults which had been feeding on contaminated fish [40-43]. It was also revealed that, although mercury was present in all of the larval growth stages, the levels detected following adult emergence were considerably lower. In one case, the mercury levels had dropped by 50% two days post-emergence, compared with the other growth stages [41]. This led to the hypothesis that flies possess a mechanism to excrete foreign substances, which may be achieved through moulting, larval defecation or voiding of meconium (a metabolic waste product from the pupal stage) by adults [42,43]. In addition, blow fly larvae were also found to contain far greater mercury levels than the tissue in which they were feeding (in one instance, the levels were recorded to be as high as 4.3 times greater than the tissue). Bioaccumulation of mercury in blow fly larvae was proposed as the most likely explanation for this observation [40,41].

Subsequent studies have confirmed the accumulation of other metals in adult houseflies, including copper, iron, zinc and calcium [44,45]. Hence, insects have been shown to be reliable toxicological specimens, and their analysis in order to detect drugs and other toxic substances in human tissue is now a valid area of investigation.

In the early stages of decomposition, there are many specimens available to the toxicologist for testing. However, as a cadaver reaches the latter stages of decomposition, traditional toxicological specimens such as blood, urine, organs and tissue are no longer available [12,27,39,46-50]. In these situations, fly larvae are typically abundant [50] and, thus have emerged as a useful tool for casework.

Fly larvae proved to be important in a case involving skeletonised remains, where the deceased was found to have a bottle of phenobarbital (used as an anticonvulsant) in their purse. Due to the state of the remains, there was a complete lack of toxicological samples. As a result, fly larvae were analysed and phenobarbital was detected in their tissues [51]. Had the fly larvae not been analysed, phenobarbital would not have been confirmed and this case may not have progressed further.

In another case, fly larvae were analysed together with traditional toxicological specimens after a bottle of malathion (an insecticide) was found adjacent to extensively decomposed human remains. In this case, traditional toxicological specimens were tested and malathion could only be confirmed in the fat and gastric contents. Fly larvae present on the deceased were also tested and malathion was also confirmed in these samples [52]. Conversely, malathion could not be confirmed in the blood, urine or fluid from the chest cavity. Similarly, in another case, where human remains were located and found to be in the latter stages of decomposition, both traditional toxicological specimens and fly larvae were tested. A total of five drugs were detected in the heart, lung, liver and fly larvae, but only four drugs could be confirmed in the spleen or kidney samples [53]. Furthermore, in two other cases, the presence of drugs was confirmed in the cerebral material, skeletal muscle and in the fly larvae feeding on the remains [47,48]. These case studies reveal that fly larvae are toxicological specimens worthy of examination, and at times may be able to confirm substances when the analysis of traditional specimens fails [52,53].

The use of insects in casework is a relatively new practice. One of the advantages of using fly larvae for toxicological purposes is that they can be analysed in the same

manner as other toxicological specimens. That is, they do not require a specific extraction technique tailored to them [12,54]. It has also been reported that results obtained from specimens of fly larvae exhibit less interference from decomposition by-products compared with other traditional specimens [47,48,50]. This may allow the results to be reported with greater confidence, particularly when a cadaver is extremely decomposed.

The most significant advantage of using insects as alternative toxicological specimens is that they extend the sampling period. This is achieved in three ways: firstly, fly puparia can withstand changing climatic conditions, and, unlike the larvae and adults, are immobile, and hence can be found close to human remains even after considerable time has elapsed [12,55,56]. In addition, if the foreign substances present in tissue are volatile (that is, evaporate quickly), bioaccumulation of these substances in fly larvae could enable them to be identified later than would otherwise be possible. Furthermore, fly larvae often feed in protected areas, and hence the tissue in which they are feeding may be less vulnerable to the external environment, which may be a significant advantage when the substance is highly volatile. These positive aspects of entomotoxicology will be explored throughout this thesis.

An important factor to consider when using insects as toxicological specimens is the potential effect any given foreign substance may have on the growth rate of the larvae, which in turn may affect the accuracy of the PMI calculation [27]. For example, it has been shown that malathion delays the invasion of insects [52], cocaine accelerates the larval growth rate [57], heroin accelerates the larval growth rate and extends the pupal stage [58] and, finally, diazepam increases the larval growth rate and extends both the pupal and adult stages [19]. These are merely a few of the substances that are commonly encountered. Hence, when a PMI calculation is to be performed and the presence of a foreign substance is suspected, further research should be carried out to ensure the PMI calculation is as accurate as possible.

In some cases, fly larvae have provided positive results for substances when traditional toxicological specimens could not confirm them. Conversely, the use of insects for quantitative extrapolation has proved challenging and unreliable [59-61]. As a result, there is no evidence to suggest that a correlation exists between larval concentration and human tissue concentration [47,48,53,54,62]. This is a significant downfall, particularly when it is important to confirm the concentration of drugs and toxins in human tissue to conclude that these substances contributed to the cause of death. Hence research in this area continues.

To date, studies in the area of entomotoxicology have focused on the detection of substances that may have contributed to the cause of death. However, this type of analysis has not been utilised for the detection of substances that contribute to the destruction of a cadaver. That is, foreign substances that are introduced to destroy evidence, using methods such as burning, have not been considered. Therefore, this is potentially a new area of entomotoxicology that warrants further investigation. This concept will be discussed in further detail in **Chapter 3** and **Chapter 5**.

1.5 Fire Investigation

Fire has been perhaps the most important and empowering technology facilitating the evolution of primitive man into a more sophisticated species. Evidence of the use of fire has dated back as early as one million years ago [63]. Over that time, it has provided humans with heat, light, and a weapon. In modern day society, technology is responsible for the production of far more sophisticated weaponry than fire. Nevertheless, crimes involving fire, such as arson, are still prevalent in society [64].

1.5.1 Arson

Arson, the act of deliberately and maliciously setting a fire to destroy property or to take a life is a serious crime. In New South Wales alone, there were 7262 recorded

criminal incidents of arson from January to December 2008 [65]. The high number of incidents highlights the need for an efficient investigation process for fire scenes. However, the scene of a fire can be inherently difficult to investigate. Valuable evidence that may aid in determining the cause of death is potentially destroyed [66]. Conversely, the damage resulting from the fire leaves patterns that are indicative of the cause and origin of the fire [67]. It is the role of a fire investigator to examine these patterns (together with eye witness accounts) to decipher the mechanism that started the fire, and to determine whether the fire was an accident, or was ignited under suspicious circumstances [66].

1.5.2 Fire and Death

Fire is a destructive process that necessitates an in-depth investigation to locate vital evidence. The investigation may be complicated further when the fire scene involves fatalities, because evidence that may assist in determining the manner of death may be destroyed both by the fire itself, and through fire suppression efforts [66].

Fire may be used post-mortem in order to conceal a homicide, destroy evidence, dispose of a body and prevent identification [68,69]. According to the Australian Institute of Criminology, there were 100 homicides where fire resulted in deaths or where deaths occurred in association with arson in the periods 1989-1990 and 2004-2005 [69]. In 29% of these reported cases, fire was a secondary event to the homicide.

There is limited knowledge in the area of fire-associated homicide, as there have been few studies performed in the area. However, autopsies carried out at the Institut de Médecine Légale, Lyon, France between 1993 and 2003 were reviewed and post-mortem burning was recorded as the cause of death in 31% of cases. In these cases it was noted that the fire was secondary to the homicide and was introduced as a means to destroy evidence [70]. Similarly, post-mortem burning was also reported in two cases where it was utilised as a means to conceal homicidal strangulation [71] and a head trauma, respectively [72]. According to the Fire and Explosion Investigation

Branch of the Victoria Police (Australia), from the fires that this unit attended there were 557 fire fatalities recorded between 1993/94 and 2009/10, with 50 of these events taking place outdoors. It is worth noting that out of these 50 scenes, 70% involved an ignitable liquid. Also, 49 of the 557 fatalities were considered to be homicides. Interestingly, 59% of these homicides involved the use of an ignitable liquid [73]. These figures reveal that fire may be utilised in homicides, and, more specifically, ignitable liquids may be introduced to facilitate the intensity and spread of the fire.

Fire has multiple uses, and can be utilised as a method of suicide (termed *self-immolation)*, for the purposes of political protest, religious or cultural ritual, or punishment [66]. Although suicide by self-immolation is rare in Western culture, it continues to occur [74]. In Ontario, Canada there were 32 cases of self-immolation from 1986 to 1988 [75]; it represented 18% of deaths in patients admitted to the Birmingham, (U.K.) burns centre from January 1979 to December 1998 [76]; in Berlin, Germany there were 46 cases of self-immolation between 1990 and 2000 [77]; and in Washington, United States of America, there were 25 suicides by burning reported from 1996 to 2009 [78]. The studies conducted in Berlin and Washington also noted that the most commonly selected location for this type of suicide was outdoors [77,78]; however, another reported a case in a vehicle trunk [75]. Interestingly, a number of studies have reported that the most commonly encountered ignitable liquid was petrol (gasoline) [75-78]. Collectively, these studies reveal a number of factors that are significant in the context of this project: firstly, acts of self-immolation can involve ignitable liquids; these events occur in areas that are open to the external environment; and finally, these events occur in areas where a delay in locating the body may be experienced.

1.5.3 Fire Debris Analysis

The chemical analysis of fire debris is an important aspect of the investigation process, as it seeks to determine if any residues are present from a substance that may have

been introduced to facilitate the spread of a fire. A common example of such a substance is an ignitable liquid [79,80]. Confirming the presence of an ignitable liquid can be indicative of a deliberately lit fire [68]. However, the presence of an ignitable liquid does not necessarily mean the fire was ignited under suspicious circumstances, as there may be legitimate reasons for such a result, just as absence of such a substance does not necessarily mean that none was present initially [81-83].

There are numerous factors that may influence the detection of ignitable liquids. These include the type of ignitable liquid involved, the sample matrix, duration of the fire, the availability of air post-fire, the sample preparation technique utilised, the analysis technique selected, the presence of pyrolysis products, naturally occurring volatile components and decomposition products of combustion and how the data interpretation step is carried out [67,84]. The following situations are not considered to be optimal and therefore would affect the detection of an ignitable liquid: the analysis of materials that do not retain fluid; material that is quickly consumed in fire and produces significant amounts of pyrolysis products; the use of only general methods to analyse samples and performing visual inspection of chromatograms alone.

The recovery of ignitable liquids has been investigated on a number of different types of materials to determine which sample matrix retained the residues most. It was found that carpet, fabric and plastic items retain ignitable liquids well, whereas ashen debris and soil samples have a limited retention of these residues [85]. Clearly, there are countless sample matrices available for testing at a fire scene. Therefore it is of no surprise that research in fire debris analysis has not previously utilised entomological samples as a sample matrix to confirm the presence of ignitable liquids.

Petroleum distillates are the most commonly encountered ignitable liquids, most likely due to their low cost and availability in the community. Therefore, it is important to have a chemical analysis process to confirm the presence of these distillates [79]. Petroleum distillates are composed of hundreds of compounds that differ based on

their physical and chemical properties. One of the most challenging aspects of fire debris analysis is selecting the most appropriate sampling technique that will be suitable for a range of ignitable liquids, encompassing the compounds that dominate their profiles [68,86]. These compounds create patterns that are individual to particular ignitable liquids [79,81].

Fire debris analysis is performed by first extracting and concentrating volatile residues from the sample matrix [87]. This is followed by the separation, analysis and possible identification of the compounds present in these residues. The first step is the most critical, as ineffective extraction of the ignitable liquids will affect possible identification. There are a number of different separation techniques available to chemists.

1.5.4 Steam Distillation

Steam distillation has been largely superseded by other separation techniques, but it is still utilised in some laboratories. Steam distillation is used to isolate water soluble, low molecular weight alcohols and ketones, such as ethanol and acetone. This technique offers limited sensitivity, is rather complex to perform and requires large volumes [67]. To carry out this technique, distillation equipment is required including: a round bottom flask, a heating mantle and a condenser. In order to achieve separation, a sample of the fire debris, together with some water, is placed in a round bottom flask. Heat is then applied in order to boil the mixture. The resulting steam will contain any volatiles present. The steam that has evolved is condensed and trapped [88]. Thus, the accelerant is recovered as a pure liquid; however, the lighter components may be lost and the heavier components may not be recovered effectively. A major advantage of this technique is that it allows for physical isolation of relatively pure liquids [67].

1.5.5 Solvent Extraction

Solvent extraction involves the fire debris being washed with a solvent in order to remove any ignitable liquids. Typically, this requires a large volume of solvent. Hence, the extracted liquid must be concentrated prior to analysis. This is achieved by evaporation in a stream of warm air until a small quantity remains for testing. The advantage of this technique is that it allows large compounds with a high boiling point (low volatility) or highly evaporated petroleum distillates to be separated from the sample matrix [67]. Despite this, solvent extraction does not exhibit the same level of sensitivity as the adsorption techniques (see Section 1.5.6). One of the factors that contribute to this level of sensitivity is that the solvent used to wash the fire debris is not selective. That is, any soluble compounds present in the fire debris will dissolve together with any soluble compounds of interest. Therefore the final extract may be a complex mixture with compounds derived from the ignitable liquids, potentially interfering with peaks from the sample matrix [67].

1.5.6 Headspace Analysis

Headspace sampling techniques are the most widely used separation techniques [67], and produces the 'cleanest' samples. That is, compared with other more traditional separation techniques, the extract contains the least interference from the sample matrix. Headspace analysis requires the fire debris to be stored in sealed containers, such as metal cans, gas-tight containers or air-tight polymer bags. The basic principle of this technique is that volatiles present in the fire debris will vaporise (this process may or may not involve heating the sample container) into the area above the sample termed the *headspace*. The disadvantage of this technique is that it is not selective of the type of volatiles it separates. That is, if volatiles are present in the sample matrix rather than the fire debris, it will separate these compounds irrespective of their origin. These compounds are often similar to several compounds of interest, and thus

they can result in misidentifications of ignitable liquids (discussed further in Section 1.6).

Headspace analysis can be achieved through direct analysis, whereby an aliquot of air above the sample is withdrawn from the sampling container either a) dynamically, whereby the volatiles are sampled externally to the sampling container; or b) passively, whereby the volatiles are sampled in the sealed sampling container. Passive headspace analysis can further be carried out in two ways: (i) the gas phase above the sample can be sampled - this technique is effective for extremely volatile ignitable liquids but is not ideal for compounds with a higher boiling point; (ii) separation can take place after volatile vapours are first concentrated onto the surface of a sorbent (either a porous polymer or carbon). These volatiles are then removed through the use of a solvent or heat (this process is known as *desorption)*. Passive headspace analysis is largely dependent on temperature and time. However, it can be used together with most sample matrices. In addition, multiple separations can be conducted and it is a non-destructive technique [89]. Commonly used sorbents include activated charcoal, which is placed above the sample in the form of a strip, and Tenax TA, which is manufactured as a powder and placed adjacent to the sample. Both of these techniques require a solvent for the desorption process. The most recent advancement in sorbents for passive headspace analysis involves the adsorbent material coated to the surface of a fibre. This technique is termed *solid-phase microextraction* (SPME). In order to adsorb (the adhesion of molecules of gas, liquid, or dissolved solids to a surface) the volatile residues, the fibre is inserted above the sample, or immersed in the sample if it is in liquid form. The desorption process differs from the aforementioned techniques as it takes place directly in the injection port of a gas chromatograph, and thus does not require a solvent. However, because this process is conducted in the GC, it is closely related to the efficiency of the chromatographic separation [90]. These headspace techniques will be discussed further in **Chapter 2**.

1.6 Chromatographic Analysis

The analysis of petroleum distillates is almost exclusively carried out on a gas chromatograph (GC) [67]. This technique can be used in order to separate the volatile components of a mixture, which are then seen as peaks in a chromatogram. The solute mixture is carried through a column by an inert gas (the *mobile phase*). The column (containing a *stationary phase*) is enclosed in an oven and the components are detected as they elute from the column. Separation is dependent on the boiling points of the sample components (those with lower boiling points elute first), and interactions with the stationary phase. The period that a compound will be retained in the stationary phase is dependent on the type of column selected and specific parameters, such as column temperature and flow rate (these parameters will be discussed further in **Chapters 2 and 4**).

The chromatograms produced from a GC are interpreted through pattern recognition. Unknown and reference samples are assessed compared by noting any correlating peaks based on retention time and shape of peak. For ignitable liquids, the presence of multiple peaks corresponding to common petroleum distillates is sought [82,91]. However, several factors may make the recognition of specific patterns difficult, including the presence of interferences derived from the sample matrix and distortion of the chromatogram due to weathering (evaporation of the volatile components of an ignitable liquid). Instrument-specific issues may also affect the chromatographic resolution and therefore have a significant bearing on the separation and shape of peaks, and thus in turn how well the specific pattern of a petroleum distillate can be identified.

When this powerful separation technique is integrated with a mass detector, its sensitivity is heightened. This is achieved when compounds elute from the GC column into the mass spectrometer. The compounds are ionised, which causes them to dissociate into smaller fragments. These fragments are detected based on their mass-to-charge ratio (*m/z*). The abundance of these fragments is plotted, producing a mass

spectrum. The fragmentation pattern produced is predictable for a given compound. Therefore, a significant advantage of this technique is that it provides structural information which allows for the identification of individual compounds from the mixture [92]. This information, together with a library database search, makes identification of each compound possible. However, this task can be challenging, particularly for isomers, as they may not differ based on their fragmentation pattern.

To assist in the identification of components from a mixture, an Extracted Ion Chromatogram (EIC) can be produced by plotting only selected ions from the sample. This has a filtering effect on the data as it reduces the chromatogram to include only key chemical classes and therefore peaks that do not contain certain ions are eliminated, thus simplifying the chromatogram [93]. This is important in the identification of ignitable liquids as ions can be extracted that are indicative of particular chemical classes (see Table 1.3) [81,94]. This can be applied to any class of compound to produce a less complicated profile, with a reduced background, allowing for an easy comparison of patterns produced (see Figure 1.2) [83]. When this information is interpreted together with retention time information gained from the mass chromatogram, specific components can be identified [81,93].

Ions for Specific Classes of Compounds	
Class of compound	**Mass-to-charge ratio (m/z)**
Alkanes	43, 57, 71, 85, 99
Cycloalkanes and alkenes	55, 69, 83
Aromatics	91, 105, 119
Naphthalenes	128, 142, 156

Table 1.3: *Characteristic ions for specific groups of compounds [82,87,92,94,95].*

Mass spectrometry also plays an important role in the identification of decomposition products of combustion and other naturally occurring volatile components that may be present in sample matrices [87,93,95]. These compounds are routinely present as they

are produced from the combustion of commonly encountered household items, such as carpet or treated surfaces. They can interfere with the identification of ignitable liquid residues, especially when the overall concentration of the residues is low, as they may share similar components to various ignitable liquids and thus obscure the ignitable liquid profile [95]. These interfering compounds and other contaminants may be removed through the post-analysis construction of an EIC, enabling the individual components to be visualised more easily and possibly improve the quality of the mass spectra produced [93] (Refer to Appendix A for the macro used
to construct the EIC throughout this project).

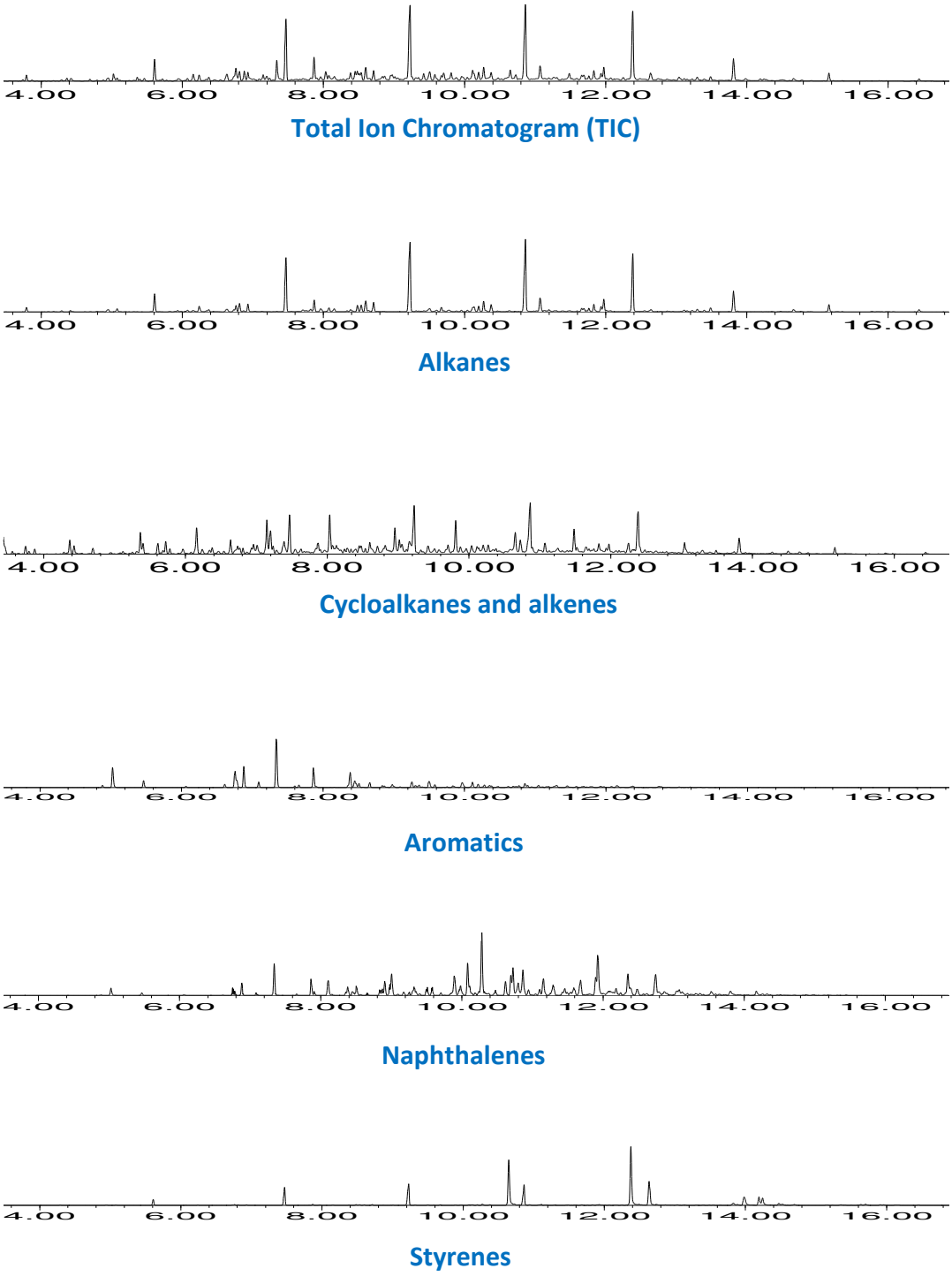


Figure 1.2: *Extracted ion chromatograms for kerosene indicating the classes of compounds separated from the total ion chromatogram (refer to Appendix A for macro used to construct this).*

The total ion chromatogram (TIC) in Figure 1.2 is a summary of the intensity of the entire range of masses being detected at every point throughout the analysis process. The TIC pictured is derived from a sample that has been burnt using kerosene. This ignitable liquid is dominated by an *n*-alkane pattern that spans at least five alkanes. The extraction of specific classes of compounds enables the specific groups present in this sample to be more clearly visualised and thus assists with the identification of this ignitable liquid.

1.6.1 Characterisation of Petroleum Distillates

The most common ignitable liquids are derived from petroleum distillates [86] and more specifically in casework, petrol and kerosene are the ignitable liquids most commonly used to accelerate suspicious fires [96]. Petroleum distillates can be divided into five classes: light, medium and heavy distillates, petrol (gasoline) and kerosene (see Table 1.4). Each of these five classes exhibits its own distinct chromatographic pattern and differs based on the types of compounds that dominate its profile [87].

Due to the prevalence of petrol and kerosene in casework, this thesis will concentrate on these distillates alone. These ignitable liquids have similar chemical properties but differ based on the components that dominate their profile and thus their boiling point ranges are quite different. As a result, they each have distinctively different chromatographic patterns (see Figure 1.3). Petrol is much more volatile than kerosene. It is highly abundant in aromatic components that include branch chained alkanes, cyclic alkanes, aromatics and aromatics with alkyl side chains. Aromatics are the most abundant hydrocarbon class in petrol and for this reason they provide the best criteria for its identification.

The minimum requirement in order to designate a sample as positive for petrol is the presence of the C_3 alkyl benzene group (listed in Table 1.4) and the C_4 alkyl benzenes (1,2,4,5-tetramethylbenzene and 1,2,3,5-tetramethylbenzene). However, the mere presence of these compounds does not justify an identification of petrol. These

compounds must be present at approximately the same relative concentration as that observed in known petrol samples [81,82]. The C_3 alkyl benzene group occupies the range between C_9 and C_{10} and is still present in petrol samples that have lost as much as 90% of their initial weight by evaporation or combustion [97]. Petrol, unlike many other distillates, does not give rise to equidistant *n*-alkane peaks [80]. Conversely, kerosene does exhibit an equidistant *n*-alkane distribution, which comprises approximately eight alkanes in the range of C_9 to C_{16} [94].

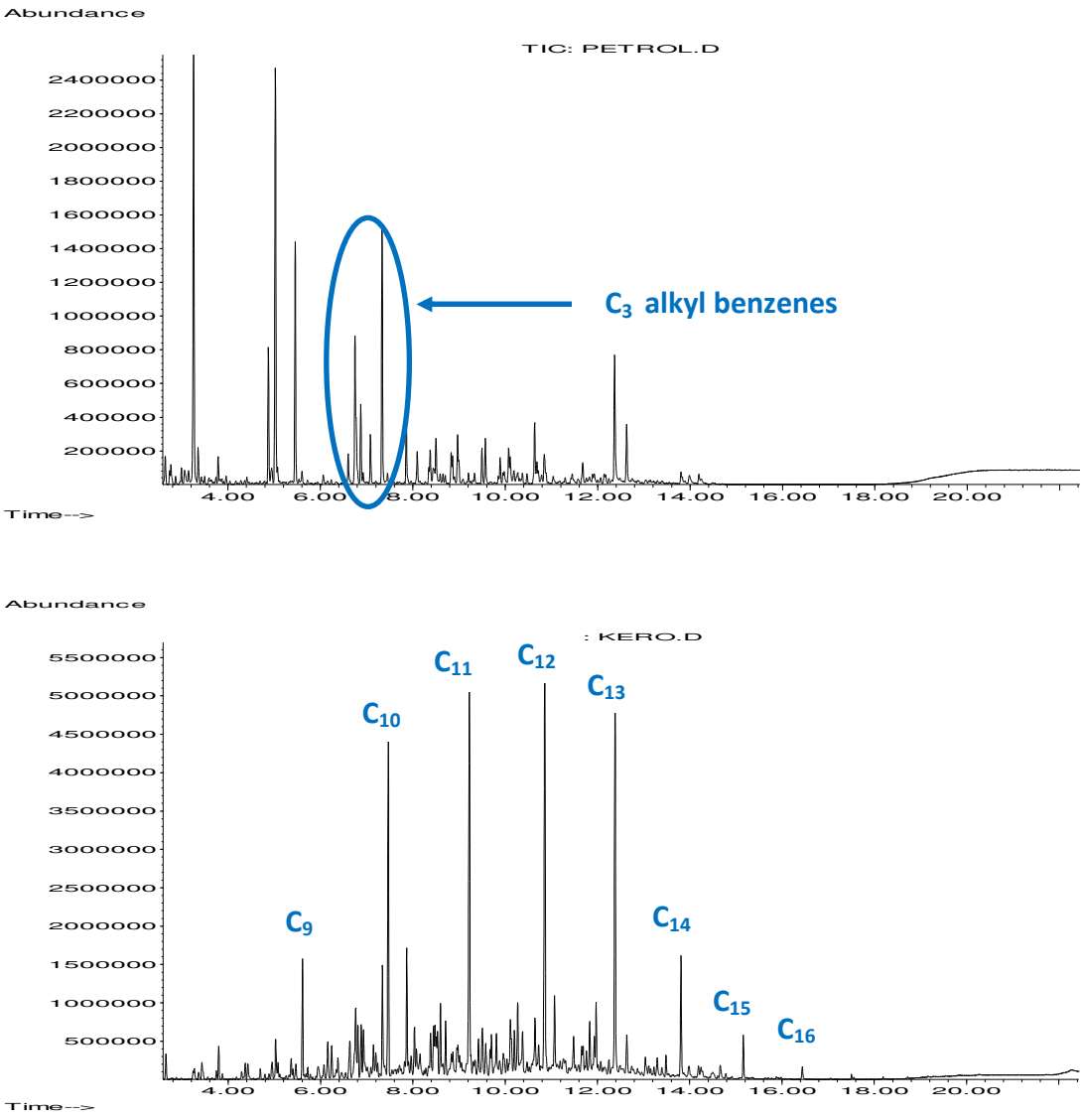


Figure 1.3: *Total ion chromatograms for petrol (top) indicating the C_3 alkyl benzene group, and for kerosene (bottom), indicating the equidistant* n*-alkane peaks.*

Petroleum Distillates	
Class of petroleum distillate	**Characteristics**
Light petroleum distillate	Majority of pattern in C_4 to C_9 range Cycloalkanes and aromatics present but less than alkanes
Medium petroleum distillates	Majority of pattern in C_8 to C_{13} range Cycloalkanes and aromatics present but less than alkanes Some naphthalenes present
Heavy petroleum distillate	Majority of pattern in C_9 to C_{20+} range At least 5 *n*-alkanes present Alkanes most abundant Pristane and phytane present
Petrol (gasoline)	Majority of pattern in C_4 to C_{12} range Aromatics most abundant Naphthalenes present C_3 alkyl benzenes (*m*-ethyltoluene, *p*-ethyltoluene, *o*-ethyltoluene, 1,3,5-trimethylbenzene, and 1,2,4-trimethylbenzene) must be present
Kerosene	Majority of pattern in C_9 to C_{16} range At least 5 *n*-alkanes present Cycloalkanes, aromatics and naphthalenes present but less than alkanes

Table 1.4: *Compounds characteristic to particular classes of petroleum distillates [94].*

1.7 Aim and Scope of Thesis

The experiments reported in this thesis aim to establish that ignitable liquids can be recovered from entomological samples that have been collected from burnt remains. This is achieved through the development of a novel technique using solid-phase

microextraction gas chromatography-mass spectrometry (SPME-GC-MS), and testing this in both small-scale and fieldwork settings.

The research presented in this thesis has direct implications for casework, as a protocol detailing the appropriate collection and storage of entomological samples recovered from burnt remains is presented. The techniques described in this study are not merely aimed at demonstrating a novel application of entomological samples to the field of fire investigation, but rather towards introducing a novel type of sample for future fire investigation protocols.

1.8 Outline of Thesis

This thesis investigates the use of entomological samples collected from burnt remains as novel substrates for the chemical analysis of fire debris. Hence, two distinctly different areas of science, namely, fire investigation and forensic entomology are integrated. **Chapter 2** details the development of a laboratory method for the recovery of ignitable liquids from entomological samples. In **Chapter 3** this method is tested in a small-scale laboratory controlled experiment, referred to herein as small-scale experiments. The results of these were evaluated in the context of the aim of the project. Although a method may work in the laboratory, its veracity in the field must also be examined. Hence, in **Chapter 4**, the laboratory method used in **Chapter 3** is developed further with the aim of increasing the overall sensitivity of the. Hence, **Chapter 5** details a larger-scale fieldwork experiment (run in duplicate), designed to mimic real life. These experiments will be collectively referred to as fieldwork experiments or individually as Experiment 1 and Experiment 2. The final chapter of the thesis (**Chapter 6**), includes comparisons between the major experiments carried out in the study, namely the small-scale laboratory experiments and the large-scale fieldwork experiments. The results obtained from these experiments are further explored to determine their usefulness. Further to this, the potential use of the

proposed method in fire investigation protocols is discussed. Finally, conclusions are drawn and recommendations given, including potential areas of future research.

Chapter 2:

Method Development

Chapter 2: Method Development

2.1 Overview

This chapter addresses a series of experiments designed to develop a method that can be used for the purposes of detecting ignitable liquids in entomological samples. A step-wise approach was taken for the optimisation of parameters (not a multi-factor approach) in order to examine the effect each parameter had on the chromatogram produced [98]. Parameters relating to the chemical analysis and sample preparation techniques used in this experiment were tested separately, so that each could be optimised. These included the GC oven temperature, flow rate, pressure, mass range, the type of sample preparation technique, fibre coating, inlet liner, fibre blanks, addition of adhesive tape and headspace sampling conditions.

2.2 Ignitable Liquids

There are many types of ignitable liquids. These can be divided into classes such as petrol, petroleum distillates, isoparaffinic products, aromatic products, naphthenic paraffinic products, normal alkane products, oxygenated solvents and others (miscellaneous ignitable liquids) [81,82]. Substances used to accelerate a fire are predominantly derived from petroleum distillates [86]. Therefore, it was concluded that petrol and kerosene were the most appropriate for the purposes of the current study. These ignitable liquids were selected due to their low cost, ready availability and especially their prevalence in casework [80,96,99].

2.3 Chemical Analysis

A gas chromatograph-mass spectrometer (GC-MS) was selected to carry out the analyses in these experiments. The GC is the instrument of choice for the purposes of fire debris analysis [67,93]. An integrated spectroscopy technique (where the composition, structure and properties of a sample are determined) was selected as there is no previous research in this field to indicate the extent to which fly larvae contribute to a GC profile, or whether any peaks introduced by fly larvae would interfere with the profiles of the ignitable liquids of interest. Therefore, GC-MS was selected for use throughout this study. GC-MS software has the ability to extract specific ions of interest and confirm the presence of specific components in a mixture. It has even been suggested that this technique is necessary for the identification of ignitable liquids [100]. Concise American Society for Testing and Materials (ASTM) methods are available for the analysis and interpretation of fire debris using GC-MS, and its use for fire debris analysis is widely accepted in the literature [80-82,95].

Prior to the selection of an appropriate sample preparation technique, GC-MS parameters were optimised to attain the highest sensitivity without compromising chromatographic resolution. This was performed using 10 µL standards of petrol and 1 µL standards of kerosene. The results from these tests were evaluated through visual comparison of the resulting chromatograms based on the pattern observed, separation and peak symmetry, and the presence of target compounds, which are necessary for identification purposes [82] (see Table 2.1).

Target Compounds for the Ignitable Liquids of Interest[82]			
Petrol		**Kerosene**	
1,3,5-trimethylbenzene	7.623	decane	8.209
1,2,4-trimethylbenzene	8.091	*n*-butylcyclohexane	8.828
1,2,3-trimethylbenzene	8.588	trans-decalin	9.322
indane	8.841	undecane	9.988
1,2,4,5-tetramethylbenzene	10.288	1,2,3,5-tetramethylbenzene	10.355
1,2,3,5-tetramethylbenzene	10.355	*n*-pentylcyclohexane	10.638
5-methylindane	10.701	dodecane	11.671
4-methylindane	10.849	*n*-hexylcyclohexane	12.337
dodecane	11.671	2-methylnaphthalene	13.256
4,7-dimethylindane	12.489	1-methylnaphthalene	13.497
2-methylnaphthalene	13.256	tridecane	13.235
1-methylnaphthalene	13.497	*n*-heptylcyclohexane	13.927
ethylnaphthalenes (mixed)	14.707	1,3-dimethylnaphthalene	14.876
1,3-dimethylnaphthalene	14.276	tetradecane	14.690
2,3-dimethylnaphthalene	15.057	*n*-octylcyclohexane	15.403
		pentadecane	16.056
		hexadecane	17.351
		heptadecane	18.287
		pristane	18.304
		octadecane	18.936
		phytane	18.979
		nonadecane	19.464
		eicosane	19.898
		heneicosane	20.290

Table 2.1: *The target compounds and corresponding retention times (min) for petrol and kerosene that were used throughout Chapters 2 and 3 for identification purposes.*

2.3.1 Initial GC-MS Method

The GC-MS method initially selected was derived from the literature [101-103]. This method was chosen as it was designed to test samples of low concentrations and was used with a number of different sample preparation techniques. This method was run using the splitless injection system whereby the whole sample is deposited onto the column with a flow rate of 1 mL/min. The GC oven was held at 35 ^{o}C for 2 minutes initially, then increased at a rate of 10 ^{o}C/min to 220 ^{o}C and held for 2 minutes, followed by a second temperature ramp of 30 ^{o}C/min to 300 ^{o}C, and finally held for 5 minutes. The final temperature was reduced to 290 ^{o}C (to produce the method referred to herein as Method 1). In order to minimise column artefacts and column bleed, this was the maximum temperature routinely used for the analysis of ignitable liquids in the laboratory where this research was conducted. A 10 µL petrol standard was analysed using an automated method to evaluate these conditions (see Figure 2.1). The chromatogram obtained did not exhibit optimum chromatographic resolution. The peaks of interest were overlapping, although they could still be identified and the lighter components (first few peaks to elute) exhibited peak broadening (ideally these peaks should be sharp), which could be a result of the flow rate or temperature program or a combination of the two parameters.

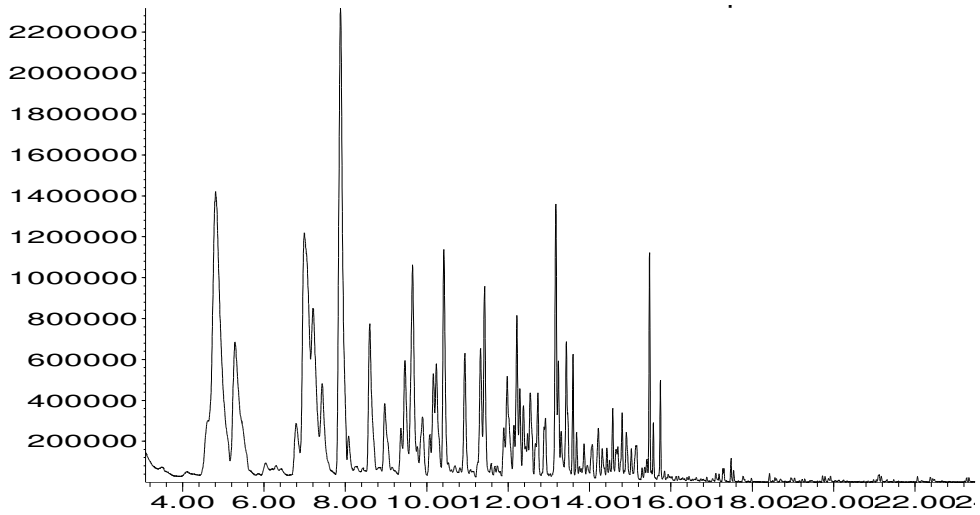


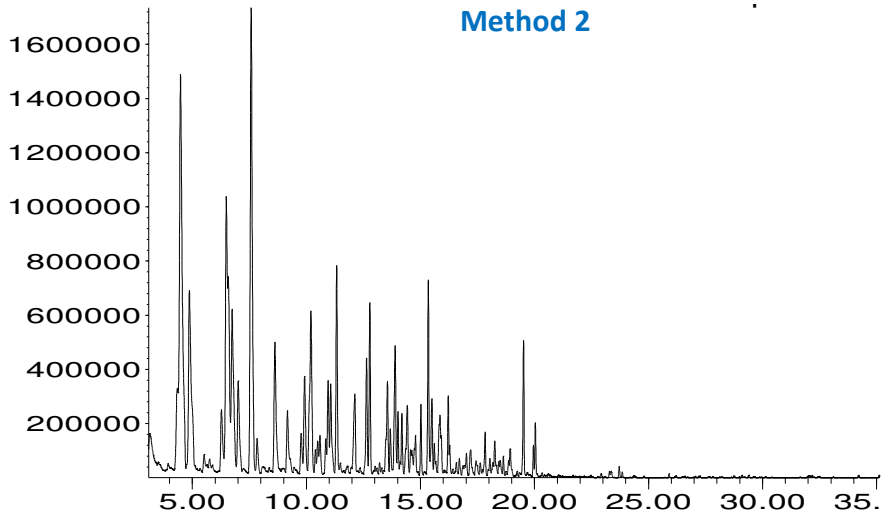
Figure 2.1: *Chromatogram obtained for a 10 µL petrol standard using Method 1.*

As a result, additional methods with different GC temperature programs were designed and compared with this original method in an attempt to improve the chromatographic resolution and decrease the total run time (see Table 2.2). For this experiment, 10 µL petrol standards and 1 µL kerosene standards were used.

Temperature Programs Tested	
Method number	**Conditions**
2[104]	35 °C, held 2 min 15 °C/min to 70 °C, held 5 min 4 °C/min to 195 °C, held 6.5 min 20 °C/min to 270 °C, held 3 min Total runtime: 52.83 min
3[103]	35 °C, held 2 min 10 °C/min to 220 °C, held 2 min 30 °C/min to 300 °C (changed to 290 °C), held 5 min Total runtime: 29.83 min
4[105]	35 °C, held 2 min 20 °C/min to 230 °C Total runtime: 10.75
5(Designed for this study)	35 °C, held 2 min 10 °C/min to 190 °C 70 °C/min to 290 °C, held 4 min Total runtime: 22.93 min

Table 2.2: *The temperature programs that were tested on petrol and kerosene standards to determine which provided the best chromatographic resolution in the shortest time.*

The chromatogram obtained from the petrol standards for Method 5 exhibited good chromatographic resolution; the peaks of interest were sufficiently separated to allow for identification and the peaks were sharp (no peak broadening evident) and symmetrical (see Figure 2.2). The chromatograms obtained for Methods 2 and 5 exhibited the best chromatographic separation for the kerosene standards (see Figure 2.3). Although Method 2 performed slightly better for the kerosene standards, this was not true for petrol. This coupled, with the total runtime of 52.83 minutes, led to a conclusion that Method 5 (with a runtime of 22.93 minutes) was also the preferred method for kerosene.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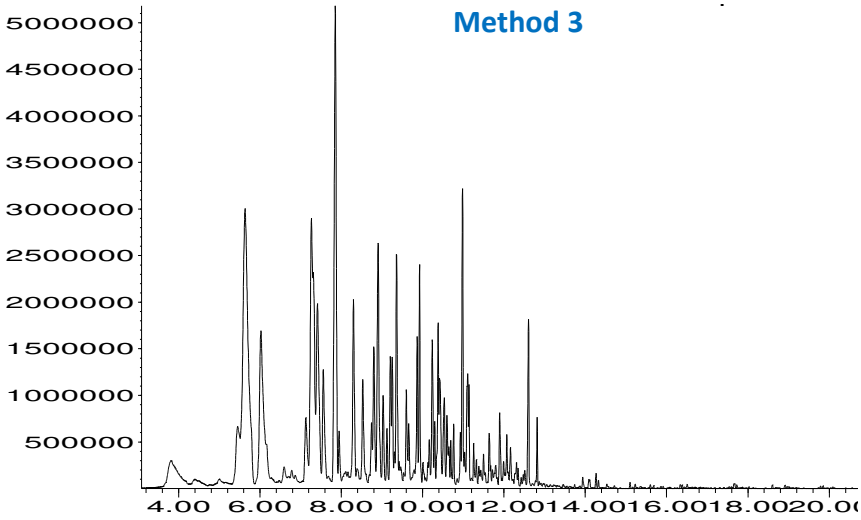
1200000
1000000
800000
600000
400000
200000
5.00
10.00
15.00
20.00
25.00
30.00
35.


Method 3
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4000000
3500000
3000000
2500000
2000000
1500000
1000000
500000
4.00
6.00
8.00
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12.00
14.00
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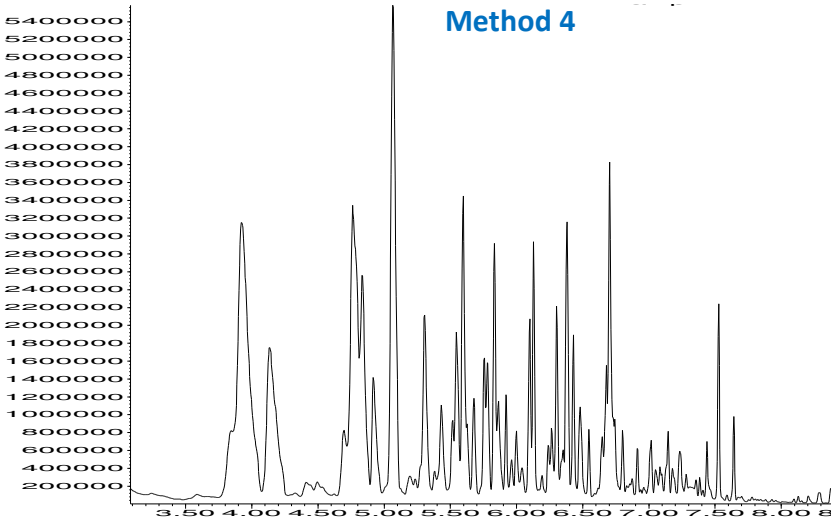
18.00
20.00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5000000
4800000
4600000
4400000
4200000
4000000
3800000
3600000
3400000
3200000
3000000
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2400000
2200000
2000000
1800000
1600000
1400000
1200000
1000000
800000
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6.50
7.00
7.50
8.00

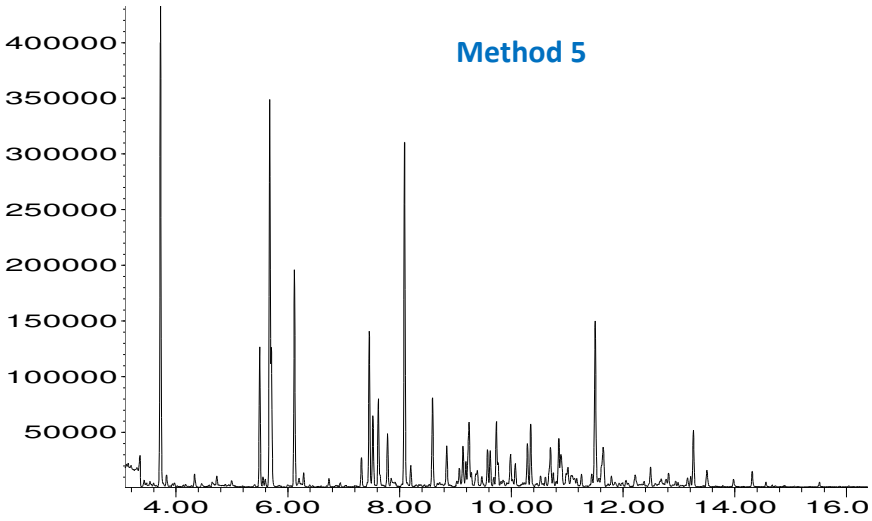
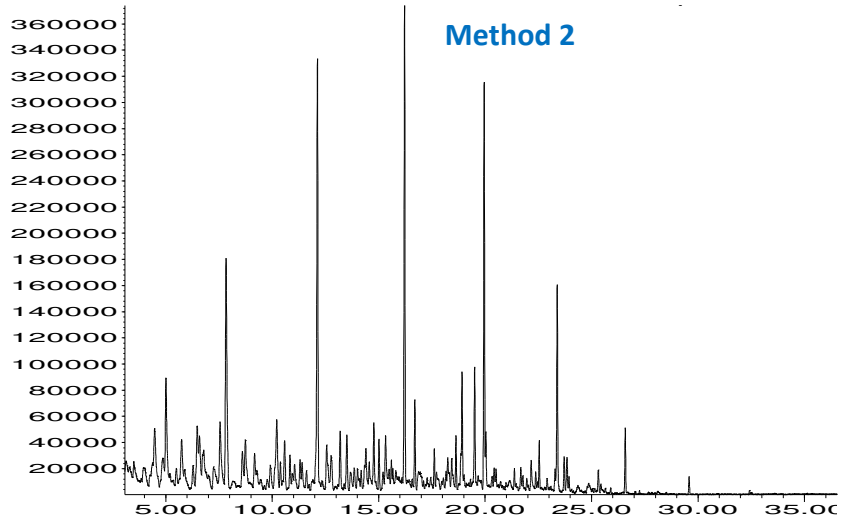


Figure 2.2: *Chromatograms obtained for petrol standards using the temperature programs from Methods 2 to 5.*


Method 2
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320000
300000
280000
260000
240000
220000
200000
180000
160000
140000
120000
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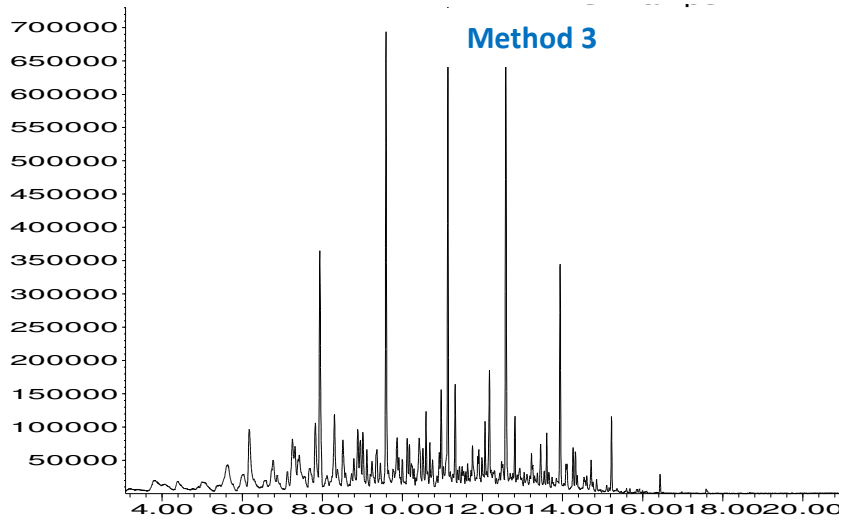
10.00
15.00
20.00
25.00
30.00
35.00


Method 3
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550000
500000
450000
400000
350000
300000
250000
200000
150000
100000
50000
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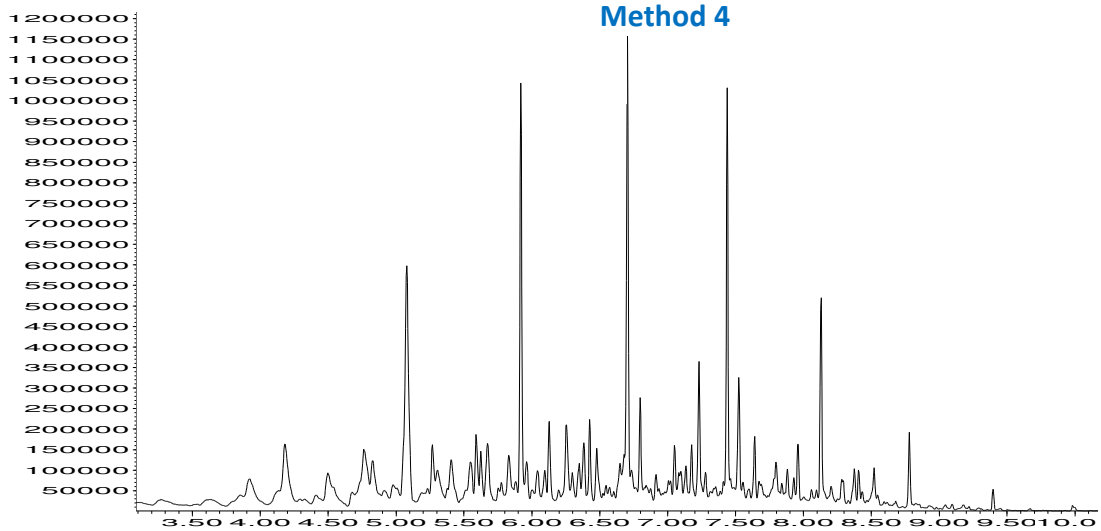
10.00
12.00
14.00
16.00
18.00
20.00


Method 4
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1000000
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900000
850000
800000
750000
700000
650000
600000
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200000
150000
100000
50000
3.50
4.00
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7.50
8.00
8.50
9.00
9.50
10.0

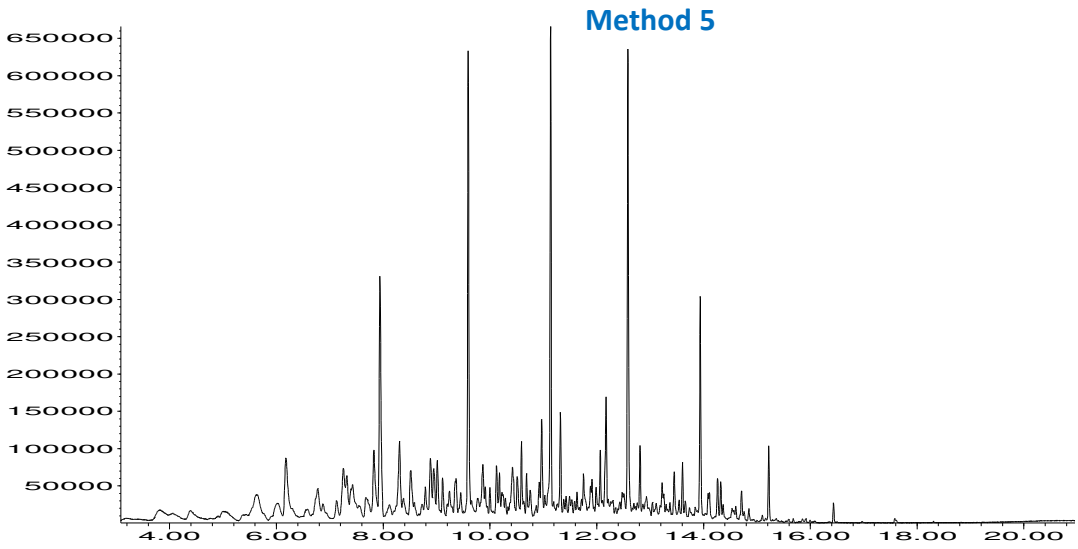
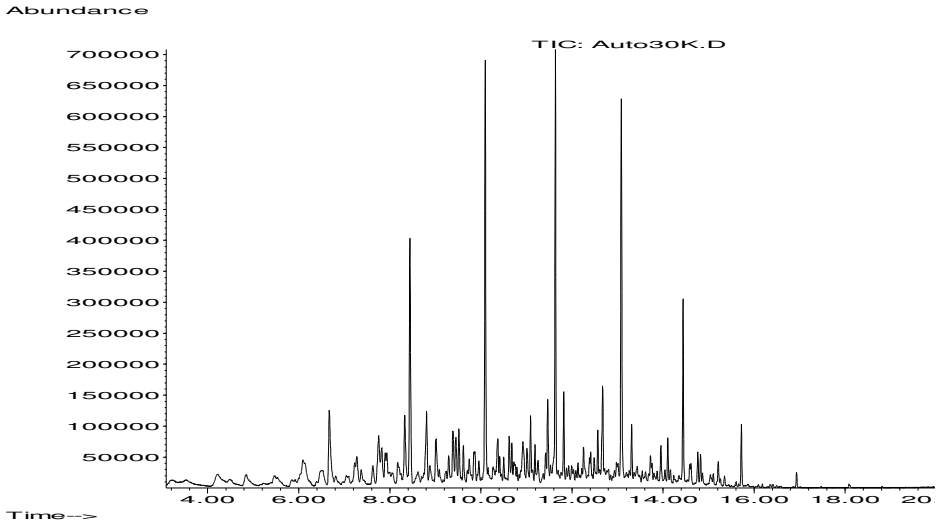


Figure 2.3: *Chromatograms obtained for kerosene standards using the temperature programs from Methods 2 to 5.*

2.3.2 Initial Oven Temperature

According to the original published method from which Method 5 was derived [101-103], the initial GC oven temperature was set at 35 ^{o}C, and held for 2 minutes. However, the method was designed for the analysis of petrol, not kerosene, which has a wider boiling point range. Experiments were performed to determine the optimal initial GC oven temperature for kerosene. A range of different initial GC oven temperatures (30 ^{o}C, 35 ^{o}C, 40 ^{o}C, 50 ^{o}C and 60 ^{o}C) were tested using 1 µL kerosene standards (see Figure 2.4). The most suitable parameter value was selected based on the same criteria as in the previous experiment.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600000
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500000
450000
400000
350000
300000
250000
200000
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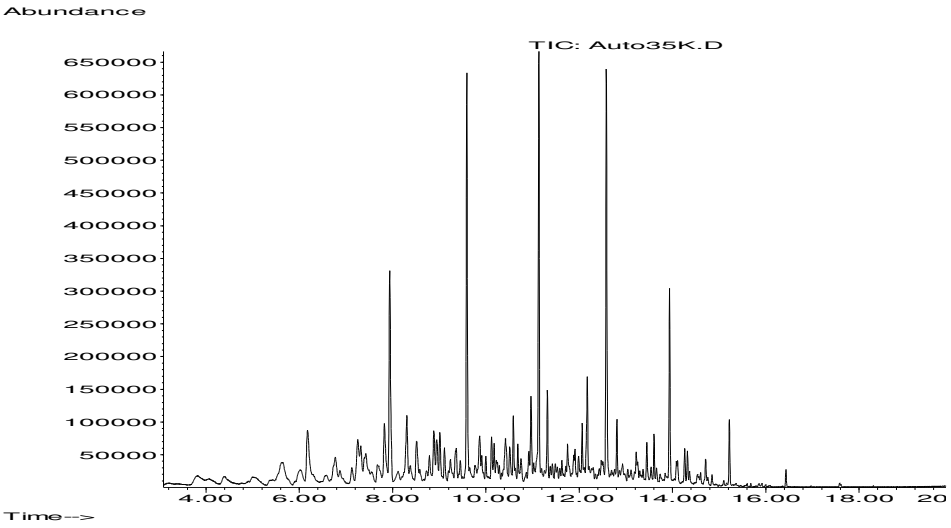
8.00
10.00
12.00
14.00
16.00
18.00
Time-->


Abundance
TIC: Auto35K.D
650000
600000
550000
500000
450000
400000
350000
300000
250000
200000
150000
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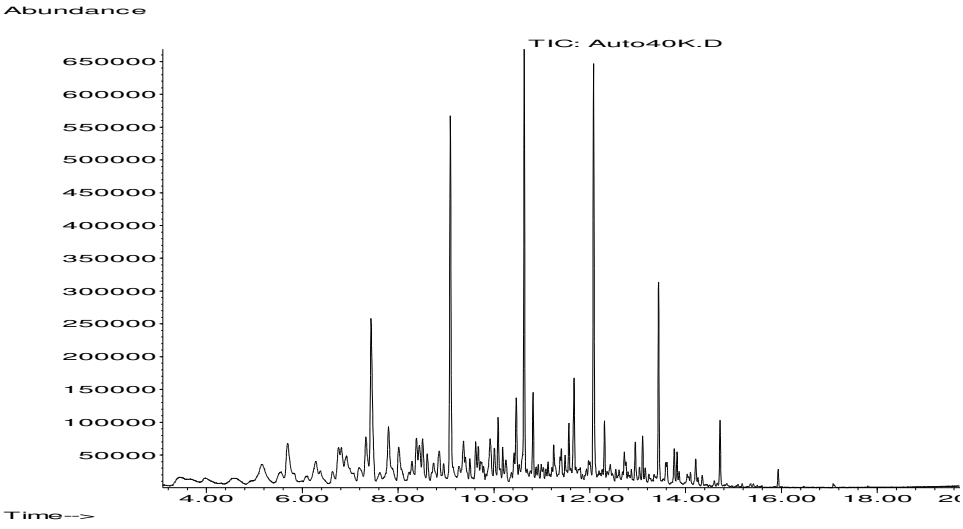
10.00
12.00
14.00
16.00
18.00
Time-->


Abundance
TIC: Auto40K.D
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550000
500000
450000
400000
350000
300000
250000
200000
150000
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10.00
12.00
14.00
16.00
18.00
Time-->

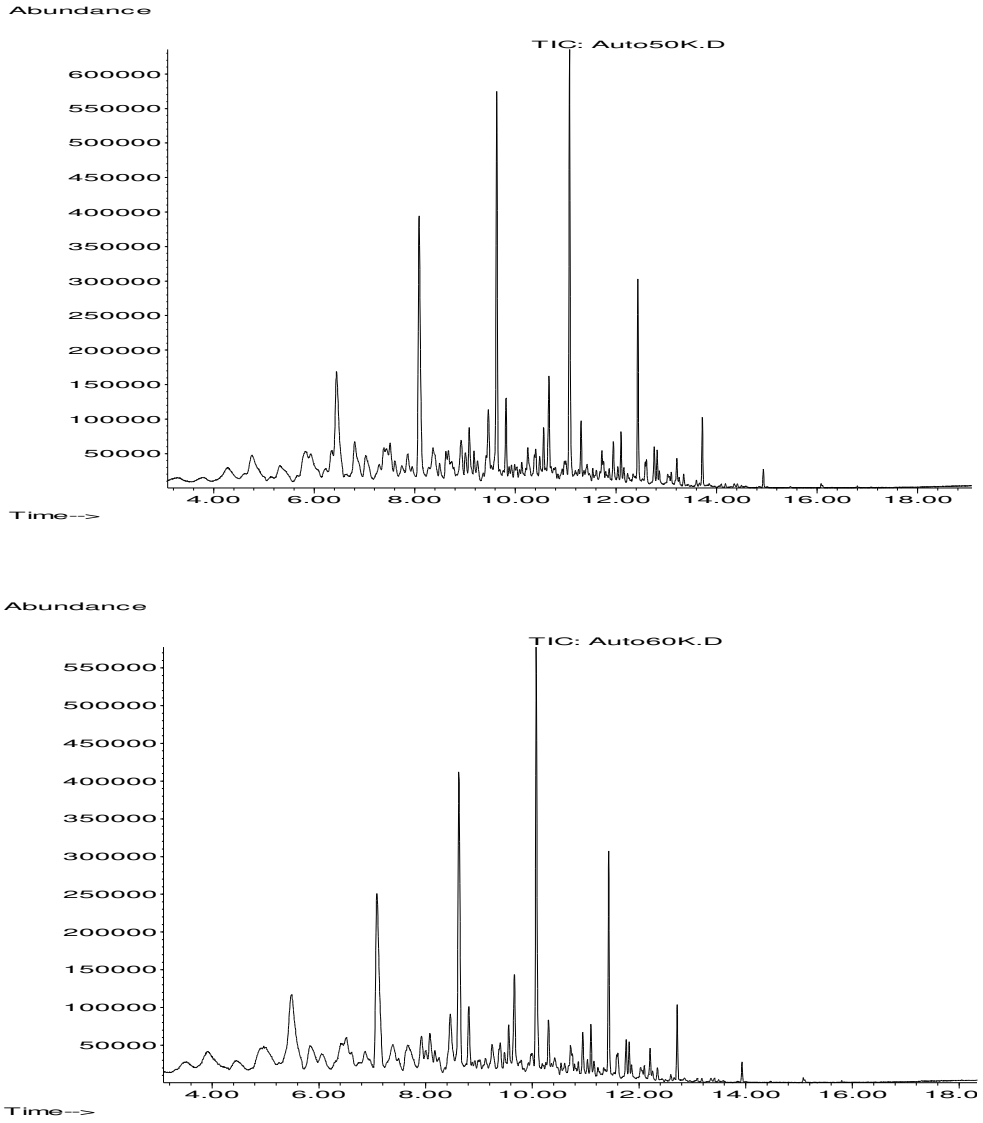


Figure 2.4: *Chromatograms obtained for a 1 µL kerosene standard using Method 5 with initial GC oven temperatures of (from top to bottom) 30 ^{o}C, 35 ^{o}C, 40 ^{o}C, 50 ^{o}C and 60 ^{o}C.*

It was difficult to determine the most suitable initial oven temperature for the kerosene samples as the chromatograms obtained for the initial oven temperatures of 30 ^{o}C, 35 ^{o}C and 40 ^{o}C could not be easily differentiated. That is, the peaks obtained in all three cases were sharp, well separated and symmetrical. Therefore an initial GC oven temperature of 35 ^{o}C was selected because (i) this was the temperature recommended in the published method; (ii) both ignitable liquids demonstrated

optimum chromatographic resolution at this temperature; and (iii) all the peaks of interest were present.

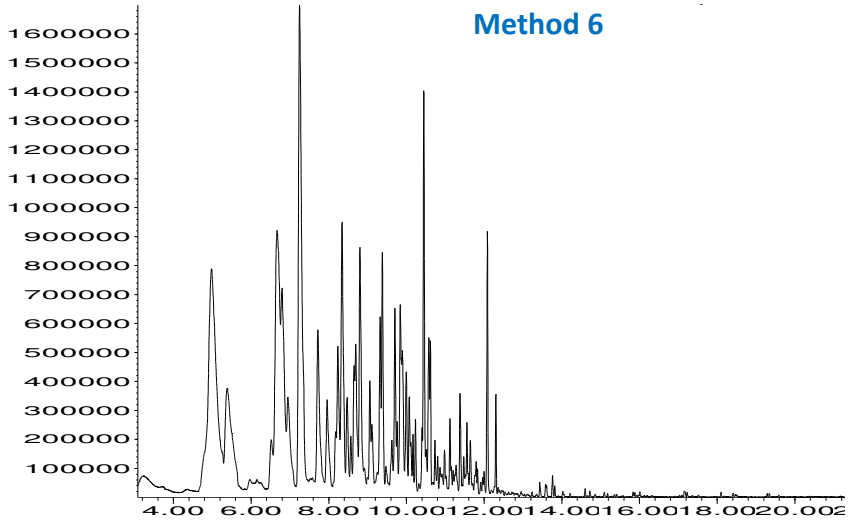
2.3.3 Temperature Programs

Section 2.3.1 describes the design and testing of a number of temperature programs (Methods 1-5, see Table 2.2), all of which had an initial oven temperature of 35 °C, held for 2 minutes. Further to this, a number of published GC-MS methods (see Table 2.3) were compared with Method 5, which had been determined to be the most suitable thus far.

Temperature Programs Tested	
Method Number	**Conditions**
6[98]	45 °C, held 2.5 min 10 °C/min to 215 °C 20 °C/min to 290 °C, held 7 min Total runtime: 30.25 min
7[106]	30 °C, held 1 min 7.5 °C/min to 110 °C 35 °C/min to 280 °C, held 2 min Total runtime: 18.52 min
8[107]	40 °C, held 2 min 9 °C/min to 280 °C Total runtime: 30.67 min
9[102]	35 °C, held 2 min 10 °C/min to 220 °C, held 2 min 30 °C/min to 290 °C, held 5 min Total runtime: 22.83 min
10[108]	Initial temperature: 40 °C 5 °C/min to 100 °C 10 °C/min to 260 °C, held 2 min Total runtime: 30 min

Table 2.3: *The temperature programs that were tested on standard petrol and kerosene samples to determine which provided the best chromatographic resolution in the shortest time.*

The chromatograms obtained from the published GC-MS methods (see Figures 2.5 and 2.6) did not exhibit the level of chromatographic resolution provided by Method 5. Therefore, Method 5 was maintained as the working method.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1500000
1400000
1300000
1200000
1100000
1000000
900000
800000
700000
600000
500000
400000
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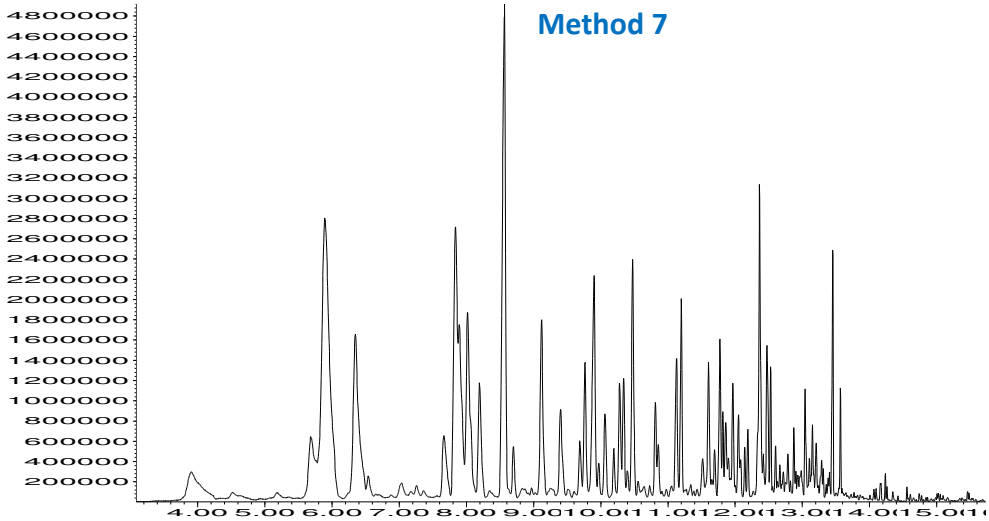
100000
4.00 6.00 8.00 10.00 12.00 14.00 16.00 18.00 20.00


Method 7
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4400000
4200000
4000000
3800000
3600000
3400000
3200000
3000000
2800000
2600000
2400000
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2000000
1800000
1600000
1400000
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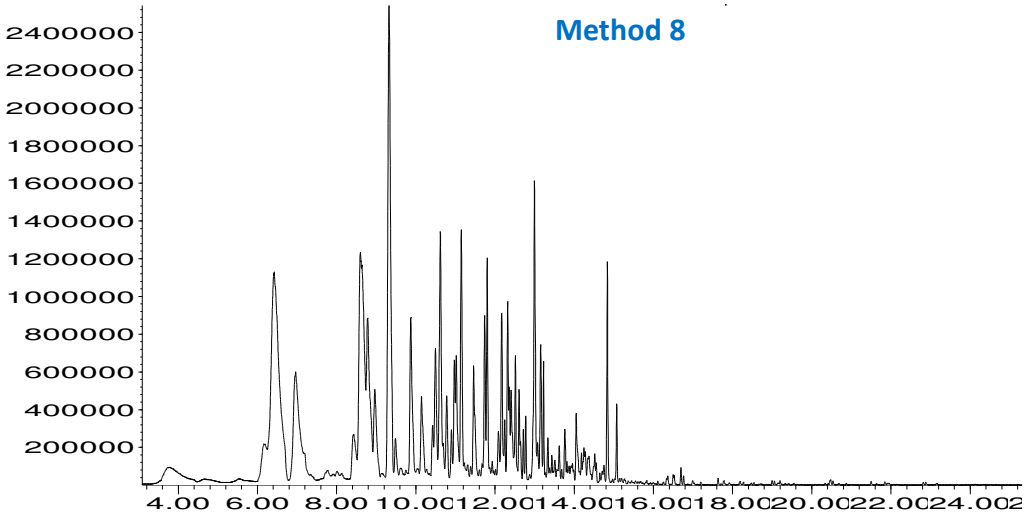

Method 8
2400000
2200000
2000000
1800000
1600000
1400000
1200000
1000000
800000
600000
400000
200000
4.00 6.00 8.00 10.00 12.00 14.00 16.00 18.00 20.00 22.00 24.00

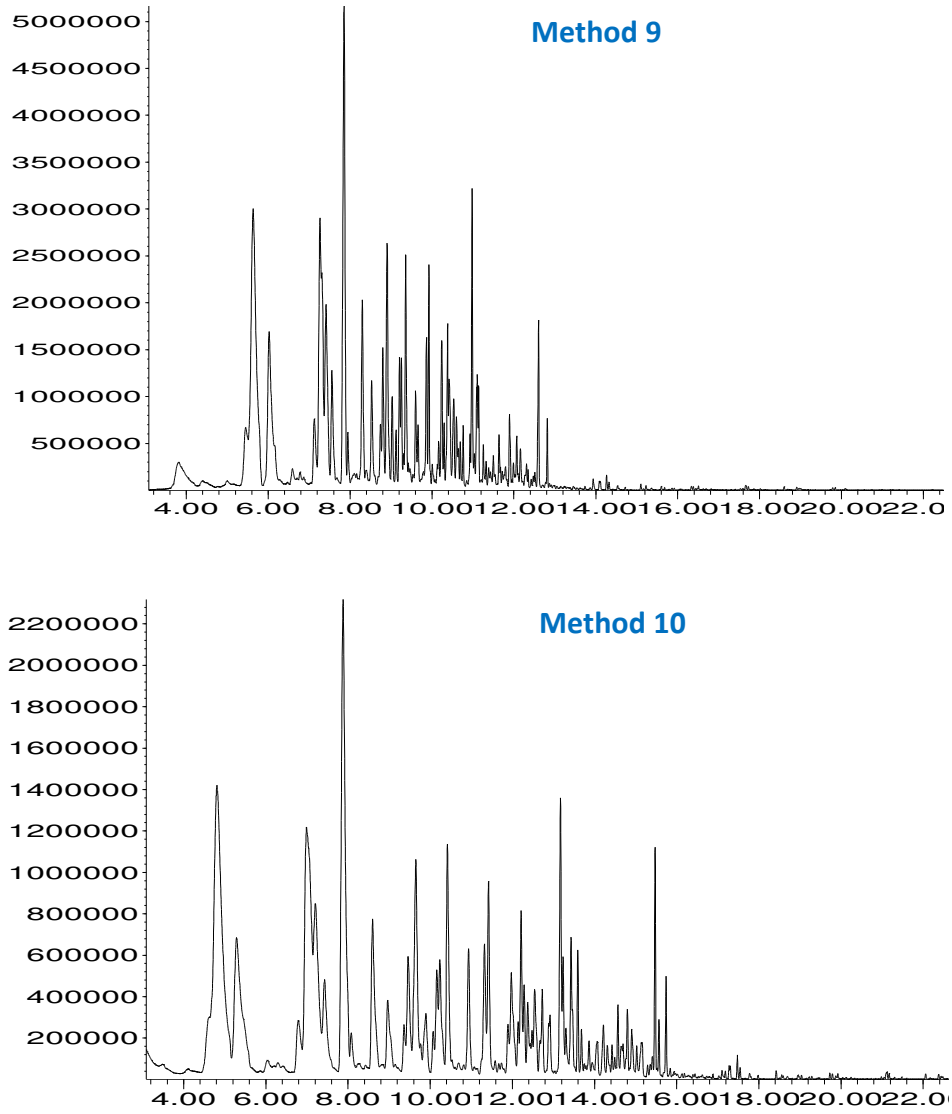
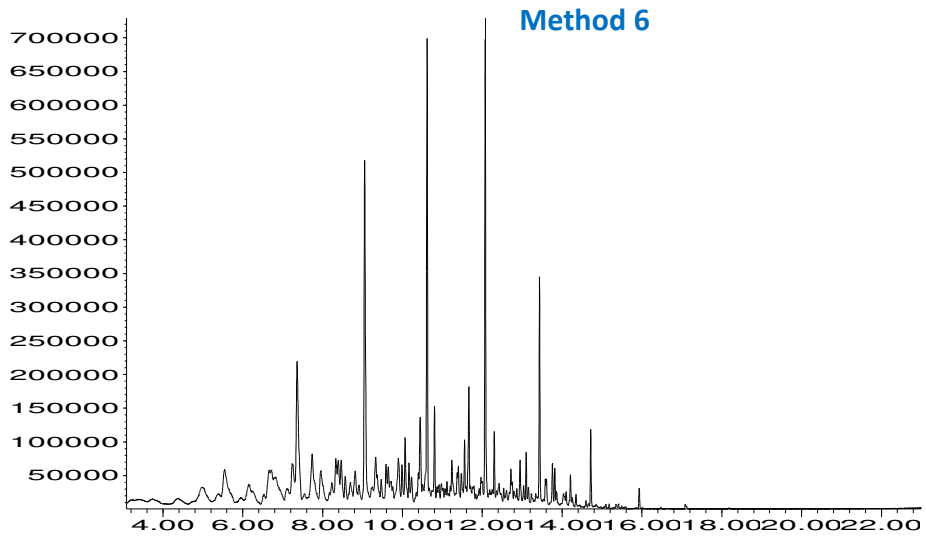


Figure 2.5: *Chromatograms obtained for a petrol standard using the GC-MS Methods 6 to 10.*


Method 6
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650000
600000
550000
500000
450000
400000
350000
300000
250000
200000
150000
100000
50000
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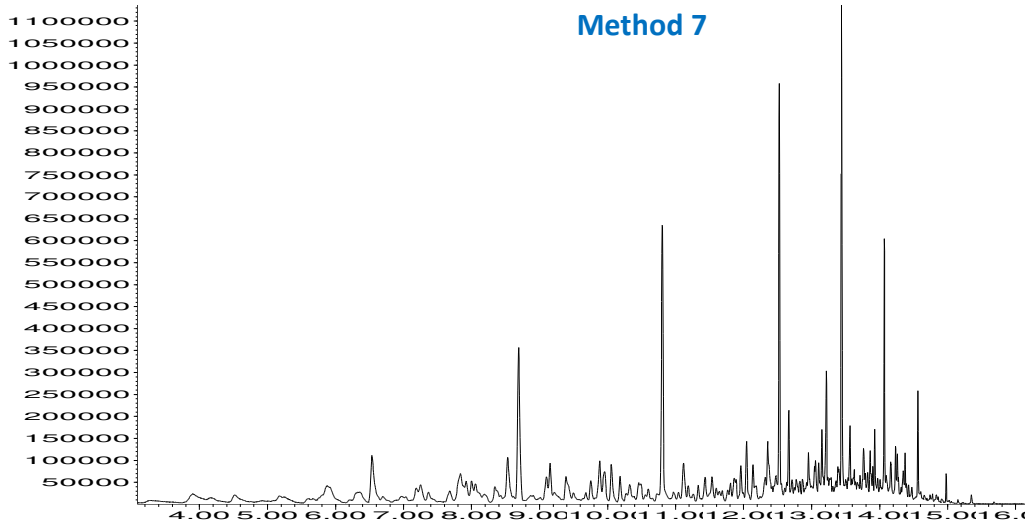
Method 7
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1050000
1000000
950000
900000
850000
800000
750000
700000
650000
600000
550000
500000
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400000
350000
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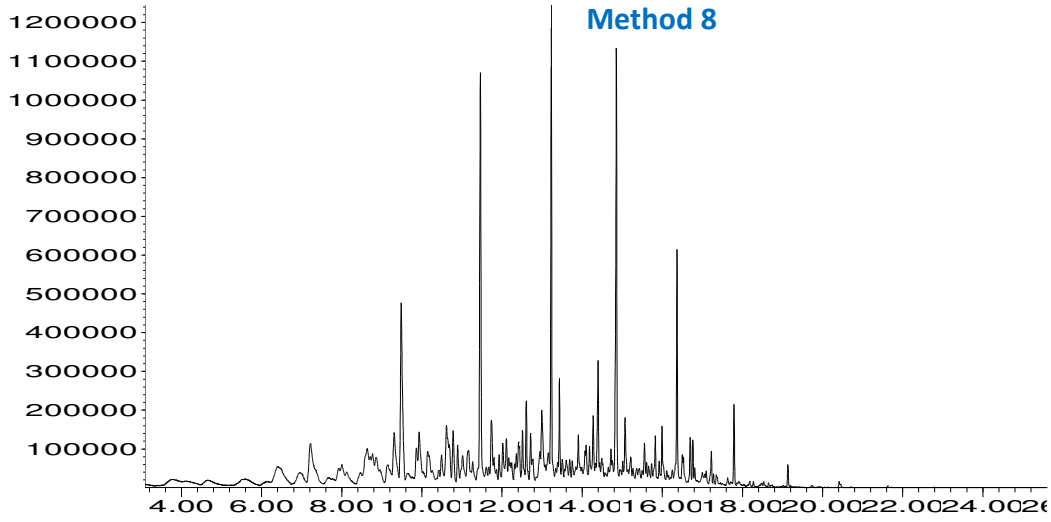

Method 8
1200000
1100000
1000000
900000
800000
700000
600000
500000
400000
300000
200000
100000
4.00 6.00 8.00 10.00 12.00 14.00 16.00 18.00 20.00 22.00 24.00

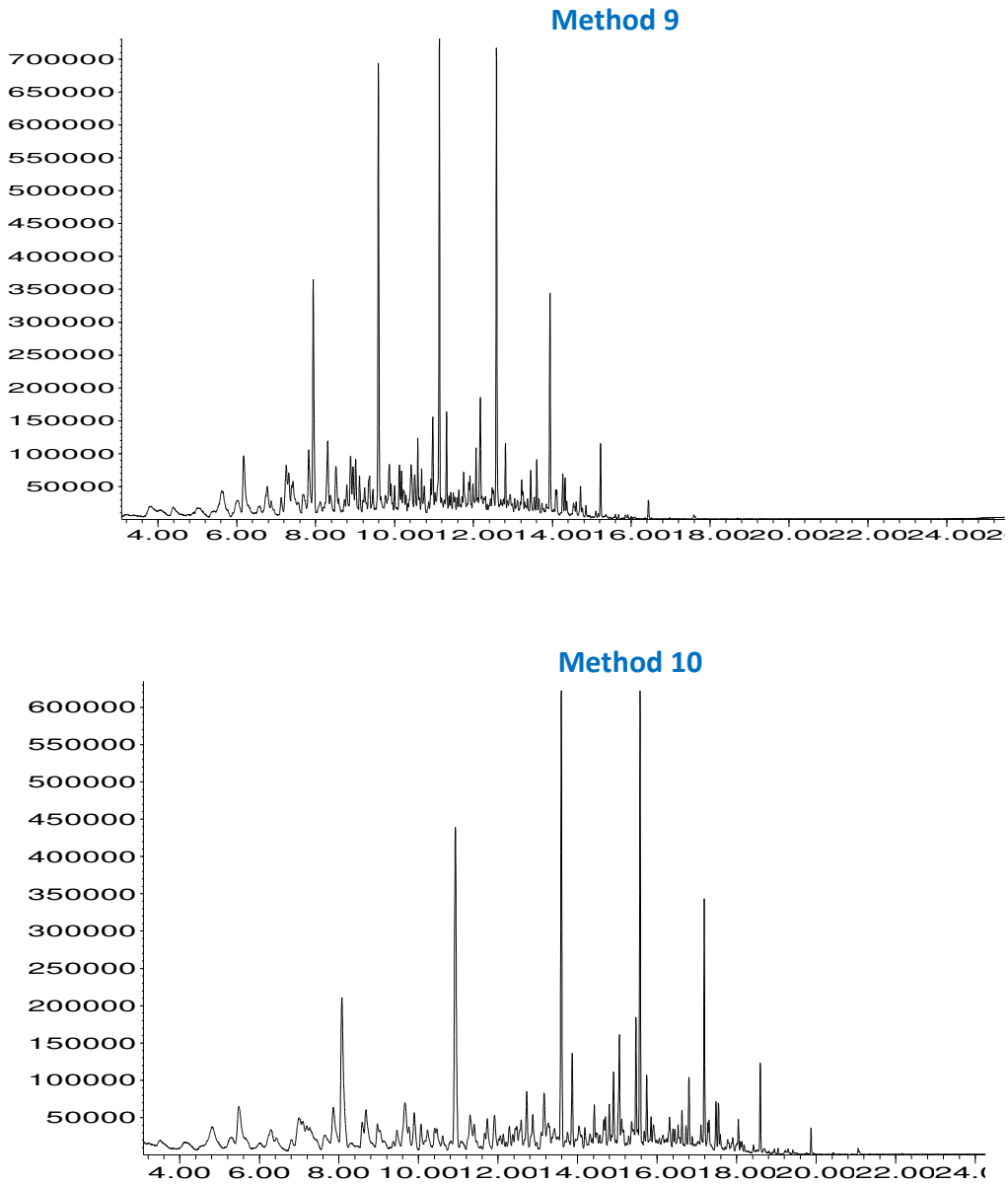
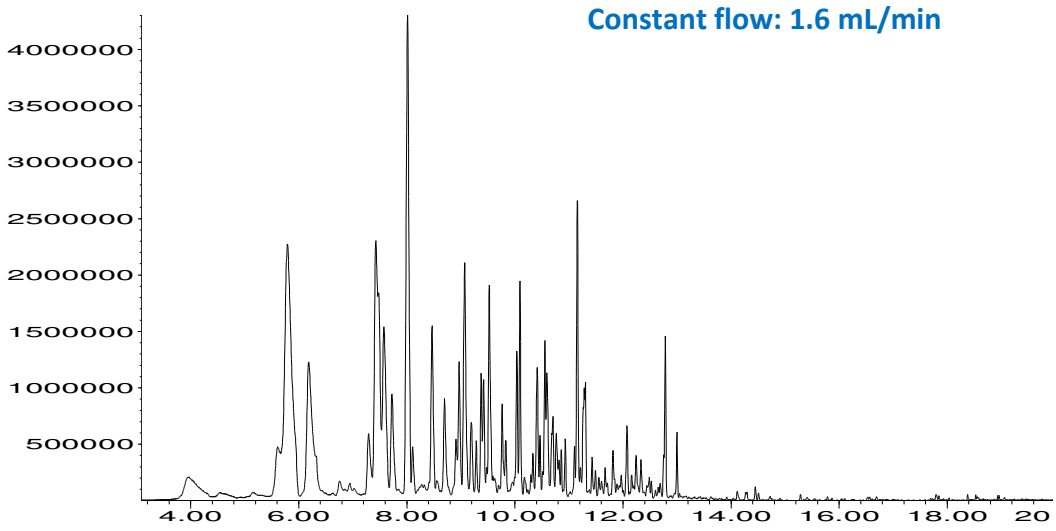


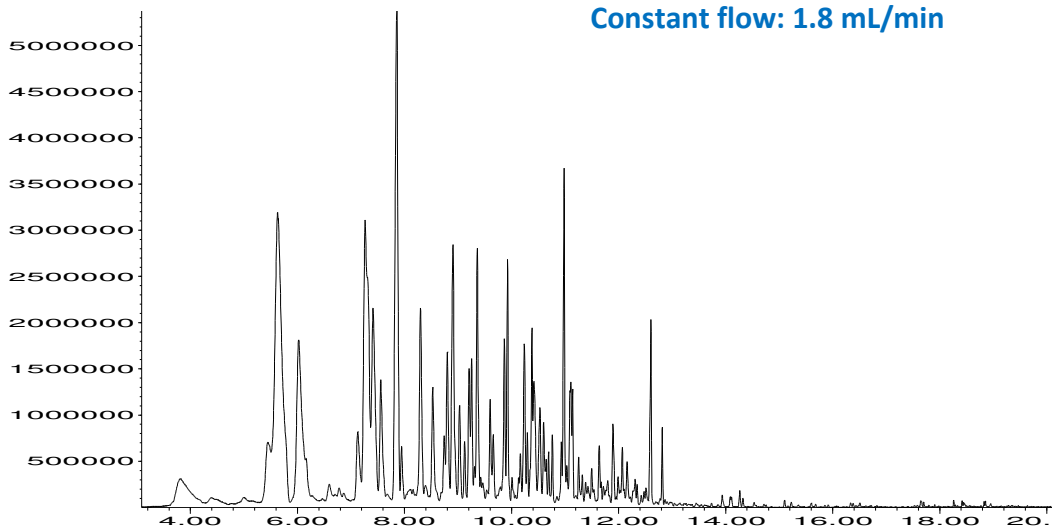
Figure 2.6: *Chromatograms obtained for a kerosene standard using the GC-MS Methods 6 to 10.*

2.3.4 Flow Rates and Pressure

Petrol (10 µL) and kerosene (1 µL) standards were used to test two flow rates (1.6 mL/min and 1.8 mL/min) using Method 5 operated in constant flow mode. In addition, the same standards were also used to test a variety of front inlet pressures (85.4 kPa, 74.7 kPa, 85.4 kPa, 95.5 kPa, 100 kPa and 105.1 kPa) using Method 5 operated in constant pressure mode (as opposed to constant flow where the pressure changes

throughout the runtime). The chromatograms obtained (see Figures 2.7 and 2.8) were evaluated in the same manner as in previous experiments. These results were difficult to differentiate as there was little difference noted between each of the conditions. There was a slight improvement in the separation of peaks noted in the sample run using a constant pressure of 85.4 kPa. This parameter was therefore applied to Method 5.





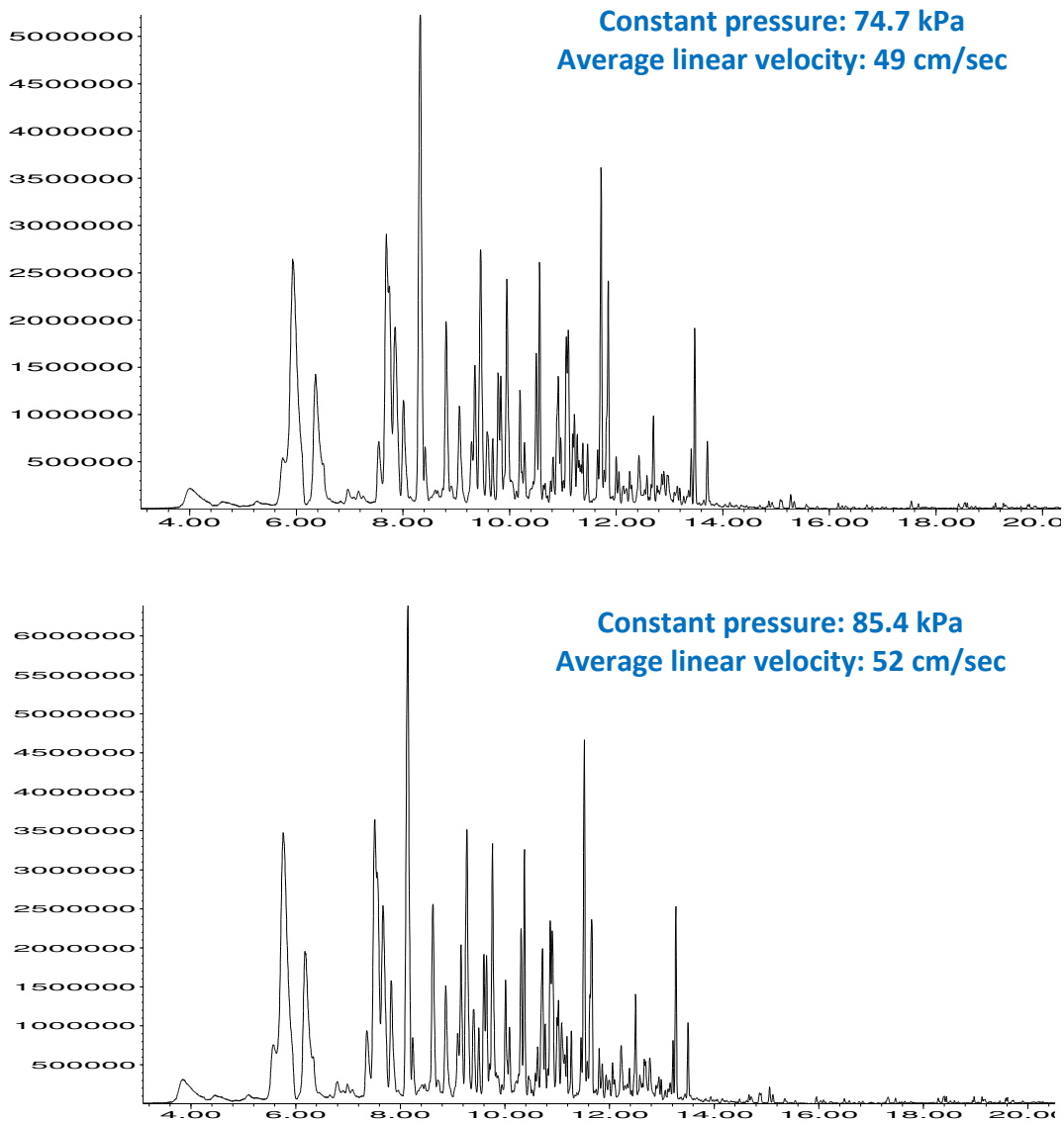
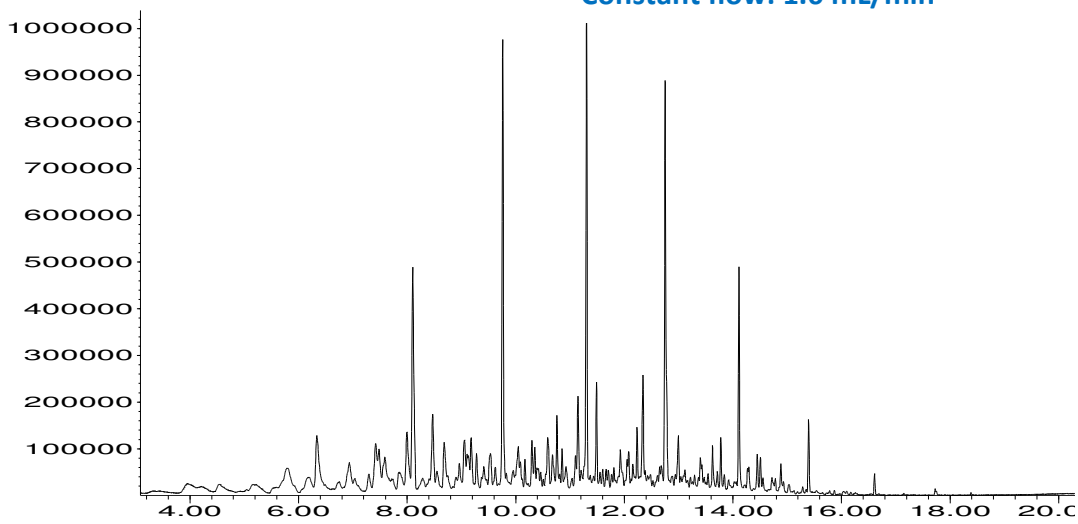
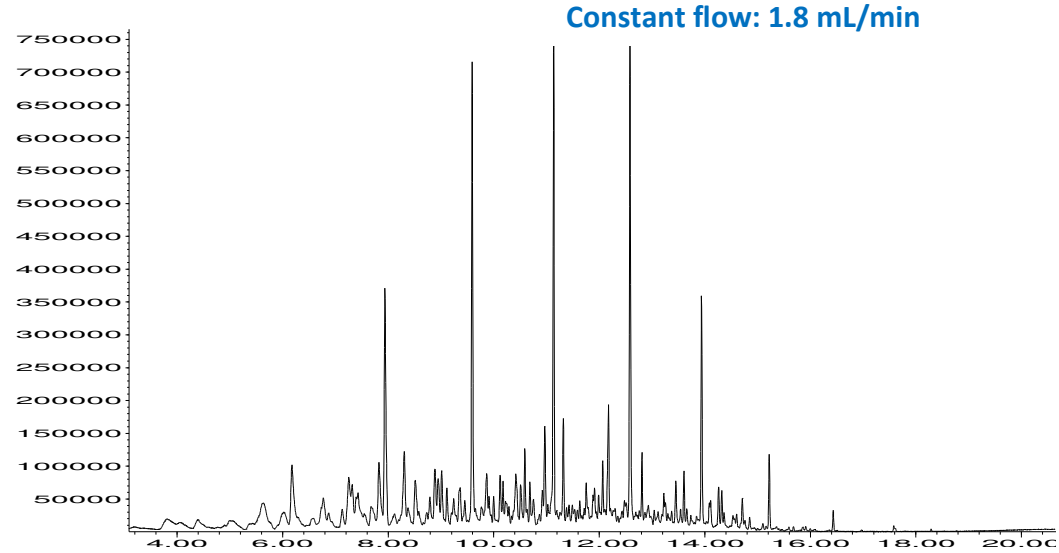


Figure 2.7: *Chromatograms obtained for petrol standards using Method 5 and a variety of flow rates and pressures.*

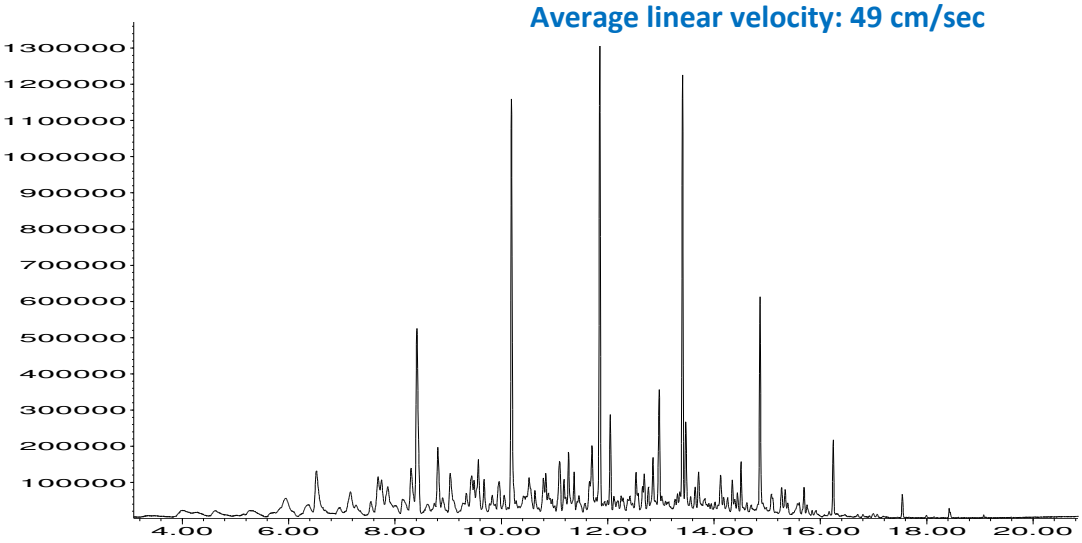
Constant flow: 1.6 mL/min



Constant flow: 1.8 mL/min



Constant pressure: 74.7 kPa
Average linear velocity: 49 cm/sec



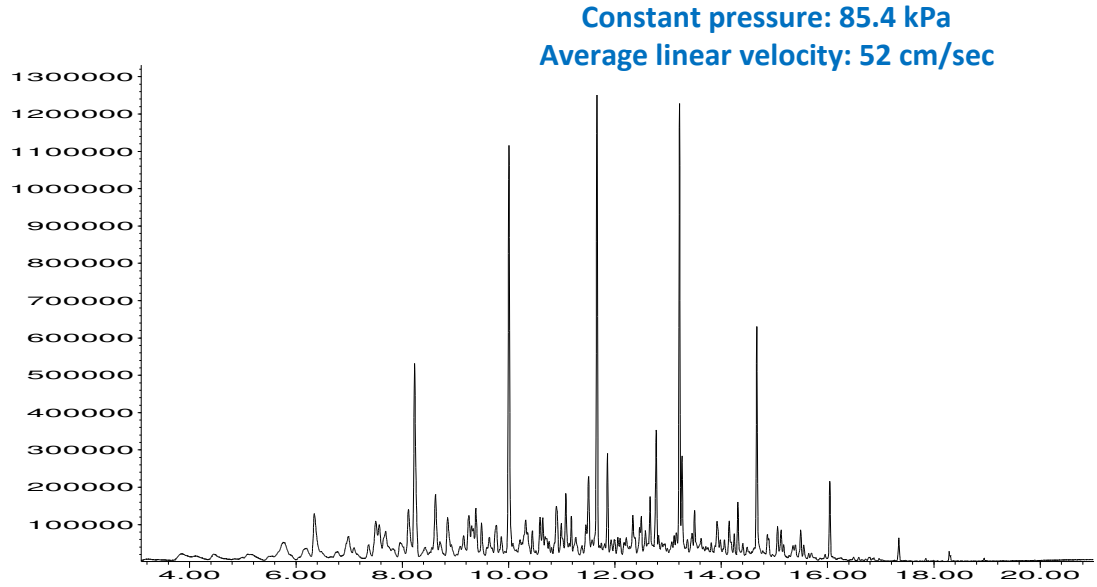


Figure 2.8: *Chromatograms obtained for kerosene standards using Method 5 and a variety of flow rates and pressures.*

2.3.5 Mass Range

In GC-MS, a mass range of 40-400 amu is routinely employed to extract all the ions of interest. However, it was noted during the method development stage that the samples produced elevated baselines in the chromatograms. As a result, the mass range was altered to 40.5-400 amu in order to exclude the mass of 40. This reduced the noise and produced a more consistent and flatter baseline.

2.4 Sample Preparation Technique

Sample preparation techniques are utilised in order to physically isolate an adequate amount of material that is representative of the volatile components absorbed into the substrate [109]. Several sample preparation techniques have been used to extract ignitable liquids from fire debris. These were briefly discussed in **Chapter 1** and

include: steam distillation; solvent extraction; and headspace analysis techniques. Burning is known to begin the evaporation (weathering) process. Therefore the concentration of the ignitable liquids that persist following burning is known to be low and the concentration will only be reduced further as the ignitable liquids continue to be exposed to the ambient environment. Therefore, distillation techniques such as steam distillation could not be used in this work, as this technique requires large amounts of the ignitable liquid to be present. Solvent extraction was also not suitable for this study as this technique does not share the level of sensitivity offered by the headspace analysis techniques [67]. Moreover, samples extracted through the headspace analysis techniques show less interference from the sample matrix compared with traditional techniques, such as solvent extraction.

Headspace analysis can be achieved through direct, dynamic or passive headspace analysis, as discussed in **Chapter 1**. The direct and dynamic headspace analysis techniques are not widely used as they are rather labour intensive and are prone to contamination events [89]. Conversely, passive headspace analysis is an appealing technique given the advantages it offers. That is, it is a simple technique; and only a fraction of the residues are extracted at any one time. The original sample can therefore be tested multiple times, and due to the small volumes that are handled, the occurrence of possible contamination events is reduced.

There are a number of sorbents available for the headspace analysis process. The most widely used is the activated carbon strip (ACS) [110], although Tenax GC is also popular [80]. The most recent advancement in sorbent technology is solid-phase microextraction (SPME). This utilises the same simple technique as the ACS method, but integrates the sampling, extraction, and concentration steps into one [103]. Due to this integration, SPME eliminates the use of toxic solvents, which in turn reduces the overall cost to the laboratory [111]. Furthermore, each extraction removes only very minute amounts of the material, so multiple extractions can be performed without altering the concentration of the volatiles in the sample [107]. This is a significant advantage as it can be difficult to work with small volumes. In fact, ASTM has

recommended the use of SPME in situations where a high level of sensitivity is required due to low concentrations of ignitable liquids in the sample [112]. However, to date ASTM only recommends this technique as a screening test for ignitable liquids [112]. Despite this, the level of sensitivity of this technique makes it appealing for the purposes of this study.

A study conducted by Steffen *et al.* (1996) demonstrated that SPME was capable of detecting petrol in fire debris even when it was present only at trace levels [113]. Conversely, conventional extraction methods, such as static headspace, lacked the sensitivity to detect this ignitable liquid.

SPME and ACS have been compared experimentally and it has been reported that SPME is a viable alternative, providing outcomes comparable to those of the ACS technique, yet with the benefits of eliminating toxic solvents and reducing sample extraction times considerably, from 16 hours to 10 minutes [102,103,107,111,114]. Moreover, SPME has been shown to be a useful tool in the extraction of ignitable liquids from real fire debris samples. In a number of studies, positive results were obtained using this technique where other traditional passive headspace analysis techniques lacked the sensitivity to obtain the same result [101,102,111,114].

Furthermore, SPME has been used to extract volatiles from a diverse range of substrates, not merely traditional fire debris samples. These include (but are not limited to): environmental pollutants in soil, air and water [115]; cocaine and cocaethylene in human hair [116]; ethanol, acetone and isoprene in human breath [117]; petrol on human skin [104] and volatile organic compounds in post mortem blood and viscera samples [118], as well as in decomposing human tissue [119]. Therefore, given the variety of sample matrices from which the SPME technique has been successful in extracting volatiles, and the sensitivity this technique boasts for ignitable liquids, it appears to be ideal for the recovery of ignitable liquids in fly larvae. However, it is important first to gain further understanding of how this technique works.

SPME was first reported in 1989 as a new sample preparation technique that was simple to use [120], fast and did not require the use of solvents [121]. Following this, it was applied to the analysis of ignitable liquids in 1995 [103]. The SPME fibre set-up is manufactured as two pieces of tubing: an outer piece of 24-gauge tubing that acts as a septum-piercing needle and an inner 26-gauge piece of tubing that contains the fibre. The fibre has a 'Z' slot to lock the fibre in position for sampling and desorption purposes (see Figure 2.9). A thin, fused silica rod coated with an adsorbent polymer is used in this technique and termed the *fibre* [90]. This fibre is protected by the needle, which acts as a metal covering when the fibre is not in use.

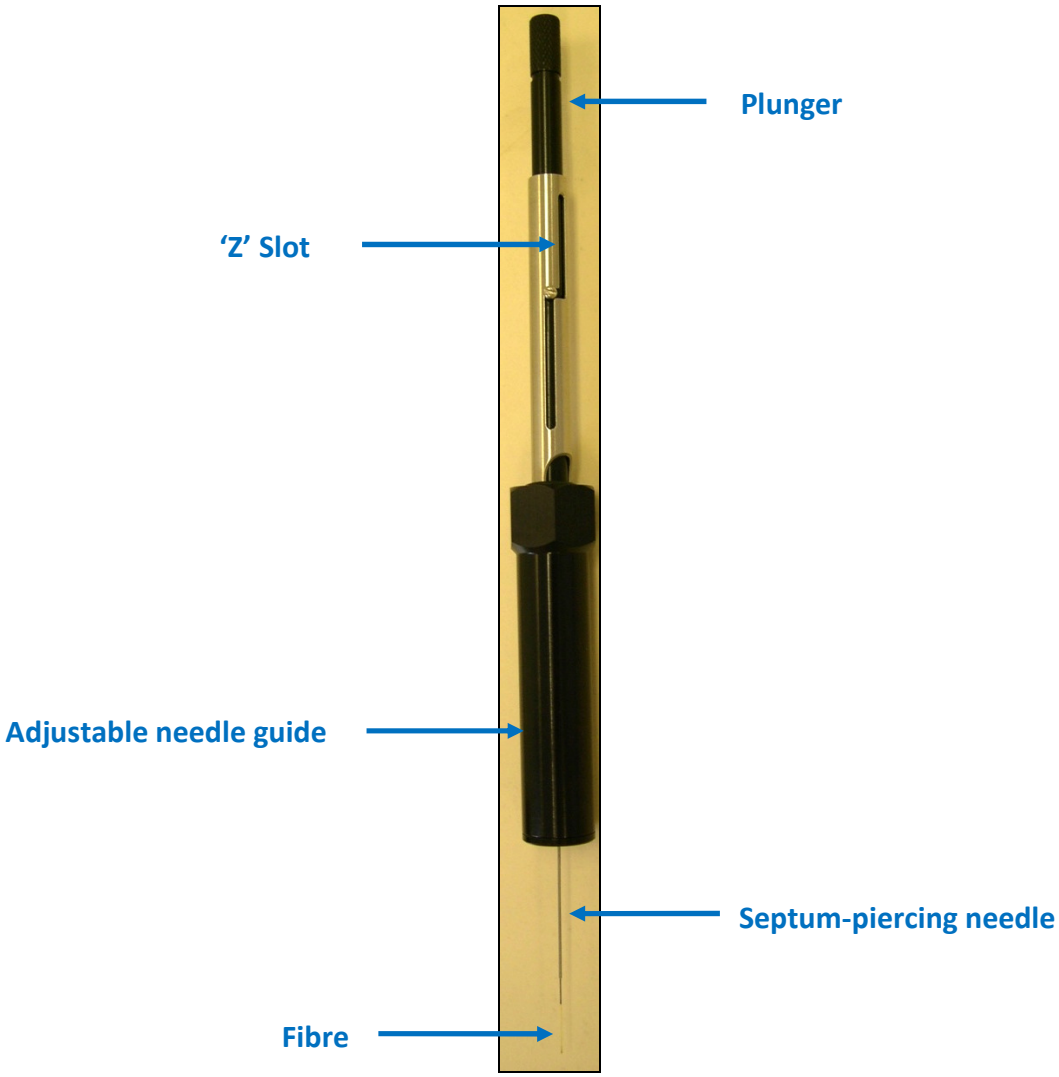


Figure 2.9: *The SPME fibre used throughout these experiments with the important components required for manual applications labelled.*

The extraction process using SPME can be carried out in three different modes. The first, direct extraction mode, involves the fibre being placed in a liquid or air sample from which the analytes are adsorbed onto or absorbed into the fibre coating [80,120]. The second is the headspace extraction mode, which is a two-step process, wherein the sample is placed in a closed vial and the fibre is exposed to the gas phase above the sample for a period. As equilibrium is reached, the analytes in the sample partition between the sample and fibre coating [122,123]. Following a pre-determined time,

the analytes on the fibre coating are desorbed in the hot injection port of a GC [90,124]. This occurs as the fibre coating is exposed to hot temperatures in the injector and can no longer retain the analytes on the surface. This, together with the constant flow of the mobile phase, helps remove the analytes from the surface of the fibre and focus them onto the GC column. Typically, two minutes is sufficient for this process to occur. To assist with this process the manufacturer recommends the use of a fibre holder to secure the fibre in the inlet and to avoid fibre breakage (see Figure 2.10). The third and final extraction mode uses a membrane to separate the fibre from the sample; this allows the analytes to cross the membrane barrier while limiting interference. This also protects the fibre when very dirty samples are analysed [120,125]. This technique typically requires longer extraction times.

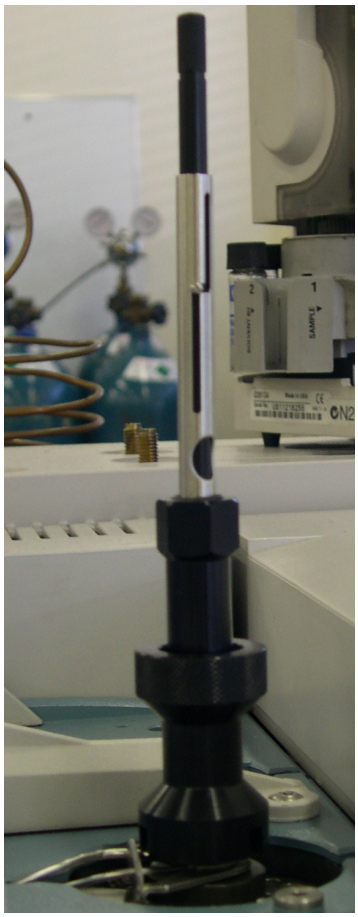


Figure 2.10: *The SPME fibre and fibre holder in the GC-MS inlet.*

There are a number of different fibre coatings available; these can be classified by polarity, extraction type (absorbent or adsorbent) and size exclusivity. Absorbent-type fibres extract through the partitioning of analytes into a liquid-like coating. With this type of extraction, the thickness of the fibre and size of the analyte contribute to the retention of the analyte onto the fibre [98]. Adsorbent-type fibres extract by trapping analytes in pores which can be on the surface or within the fibre [98]. The selection of a fibre coating is dependent on the type of analyte that is involved [90]. The fibre coatings routinely utilised for ignitable liquids are polydimethylsiloxane (PDMS) [114,123], polydimethylsiloxane-divinylbenzene (PDMS-DVB) [103], carboxen-polydimethylsiloxane (Carboxen-PDMS) and Carbowax [111]. These fibre coatings all operate as adsorbent-type fibres.

Although SPME offers many advantages, it does also have some disadvantages that should be considered. As the SPME fibre coating contains only a limited number of adsorption sites, displacement of components can occur. In addition, this technique is labour-intensive and requires all extractions and injections into the GC to be performed manually. Thermal desorption systems that automate the GC analysis are available, but they are costly and were therefore not a viable option for this study. Furthermore, it has been reported that the fibre coating has only a limited number of uses [80]. However, this is dependent on the conditions to which the fibre has been exposed. Harris *et al.* (2003) have reported that the fibre coating they utilised throughout experimental work could be used for 50 or more injections before it required changing [107].

A number of SPME parameters were tested in order to attain the highest sensitivity without compromising chromatographic resolution prior to introducing the fly larvae to this study. SPME parameters were tested using 1 µL standards of petrol and kerosene. This was achieved by spotting separate standards of petrol or kerosene onto laboratory tissues (KimWipes™) and placing them into 1 L metal cans, which were later sealed. These cans were chosen because they are commonly used in this field,

especially in casework in New South Wales, Australia [101,102,107], and they have excellent sealing capabilities [80].

2.4.1 Fibre Coating

It was discussed previously (Section 2.4) that there are a number of fibre coatings available for the extraction of ignitable liquids from sample matrices. For the purposes of the experiments described in this thesis, the polydimethylsiloxane-divinylbenzene (PDMS-DVB) fibre coating was selected. This fibre was chosen due to the findings of a research project conducted at the University of Technology, Sydney [126]. This project evaluated the performance of the PDMS, Carboxen-PDMS, Carbowax and PDMS-DVB fibre coatings using petrol and kerosene standards. The findings revealed that the PDMS-DVB fibre coating successfully detected hydrocarbons from the C_9 to C_{17} range for petrol and from the C_9 to C_{19} range for kerosene, whilst the remaining fibre coatings were only able to detect hydrocarbons up to C_{13} (for petrol) and up to C_{17} (for kerosene). Given that there are a number of compounds of interest that elute after these hydrocarbons that are important for confirming the presence of petrol and kerosene, these fibre coatings were deemed unsuitable. In addition, the PDMS-DVB fibre coating contains pores, which enables it to adsorb and retain analytes (that fit into its pores). This is particularly important for analytes that are only present at low levels.

2.4.2 Inlet Liner

The manufacturers of the SPME fibre recommend that the GC used be fitted with a 0.75 mm inlet liner instead of the standard 2 mm splitless liner when analysing samples from the SPME fibre [127]. This claim was investigated (see Figure 2.11) through the analysis of petrol standards using each of these liners.

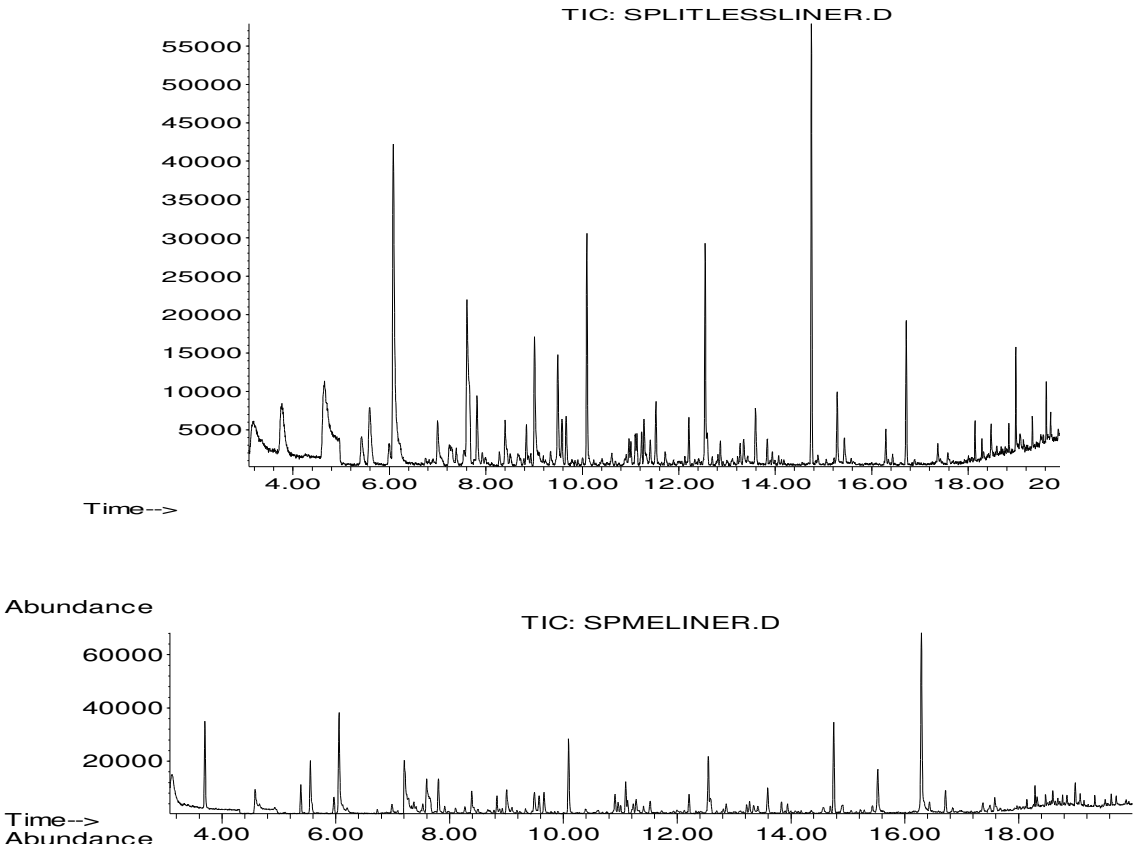


Figure 2.11: *Chromatograms obtained from petrol standards run using a splitless liner (top) and a SPME liner (bottom).*

The early-eluting peaks that were visible in the chromatogram obtained from the sample that used the SPME liner (second chromatogram in Figure 2.11) were sharper than those obtained from the sample that used the splitless liner (first chromatogram in Figure 2.11). In fact, the initial peaks obtained from the sample that used the splitless liner exhibited peak broadening. These observations have been rationalised in the literature, where it is noted that the SPME liner has a narrower bore, which increases the linear velocity through the liner and therefore introduces the analyte onto the GC column in a narrow band, thus in turn resulting in sharper peaks [90,127].

2.4.3 Fibre Blanks

Fibre blanks (where, prior to extraction, the fibre undergoes desorption in the injection port of a GC) are important as they ensure that the SPME fibre is free of all extraneous compounds, so that no unnecessary contamination is introduced to the sample [112,124]. The literature recommends that a fibre blank be run after each sample injection. This recommendation was investigated further in the current project since (given the manual nature of this technique and the number of future samples to be analysed) the fibre blanks would significantly reduce the number of samples that could be run each day. As a result, a petrol standard was run and the fibre was left in the injection port of the GC for three minutes. The same fibre was analysed immediately afterwards to determine if it was free of foreign compounds following desorption and if the desorption time was sufficient to achieve this (see Figure 2.12).

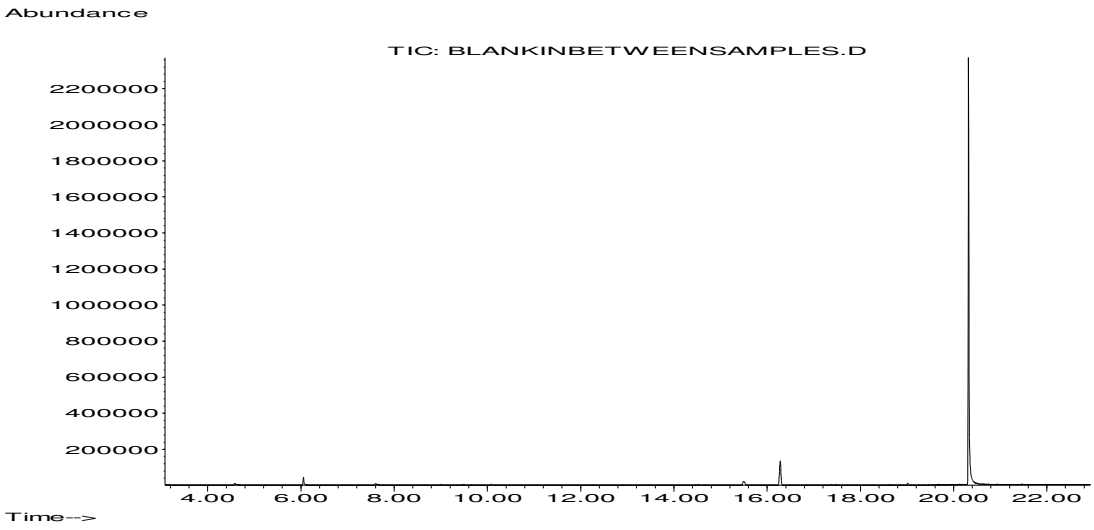


Figure 2.12: *Chromatograms obtained from the fibre that was run following a 3 minute desorption in the injection port of GC.*

The chromatogram obtained from the fibre that was analysed immediately following desorption contained several peaks. However, after closer examination, none of these

peaks could be attributed to the ignitable liquid that was previously analysed. The peaks can be identified as predominantly large polysiloxane compounds which are most likely derived from the breakdown of the fibre coating and thus would be present in every run. It was concluded that if the fibre remained in the injection port of the GC for three minutes, this desorption time was sufficient to remove any compounds of interest. Therefore, it was deemed unnecessary to run a fibre blank between each sample. However, if a sample was particularly concentrated, it would be necessary for a fibre blank to be run in between samples so as to avoid carryover to subsequent samples.

Furthermore, prior to analysing any samples, two fibre blanks should be run and the fibre left in the injection port of the GC for the entire duration of the run. The reason for this is that the first fibre to be run on a given day needed to be run twice to remove all foreign compounds. This is most likely due to the time between uses; even if the fibre had been used one day earlier, at least 14 hours would have elapsed. This would provide ample time for the fibre to equilibrate with any volatiles in the air, and thus they would need to be removed.

It was noted while conducting these experiments that it was important to monitor the head pressure of the column when the fibre was injected and removed from the injector port of the GC. On a number of occasions leaks were noted when the septum was not installed tightly enough, and if this problem was not rectified, the pressure in the system was affected.

2.4.4 Metal Cans

Prior to extraction, a small hole was punched in the lid of each metal can to accommodate the insertion of the SPME fibre. However, there was some concern that the size of this hole, albeit small, would allow volatiles to escape from the container. As a result, a piece of adhesive tape was placed over it to minimise the escape of volatiles and this was only pierced when the fibre was inserted for extraction purposes

(see Figure 2.13). A blank sample of the tape indicated that no additional contaminating peaks were introduced due to the adhesive. The addition of the tape improved the overall abundance produced by the sample present in the metal can.

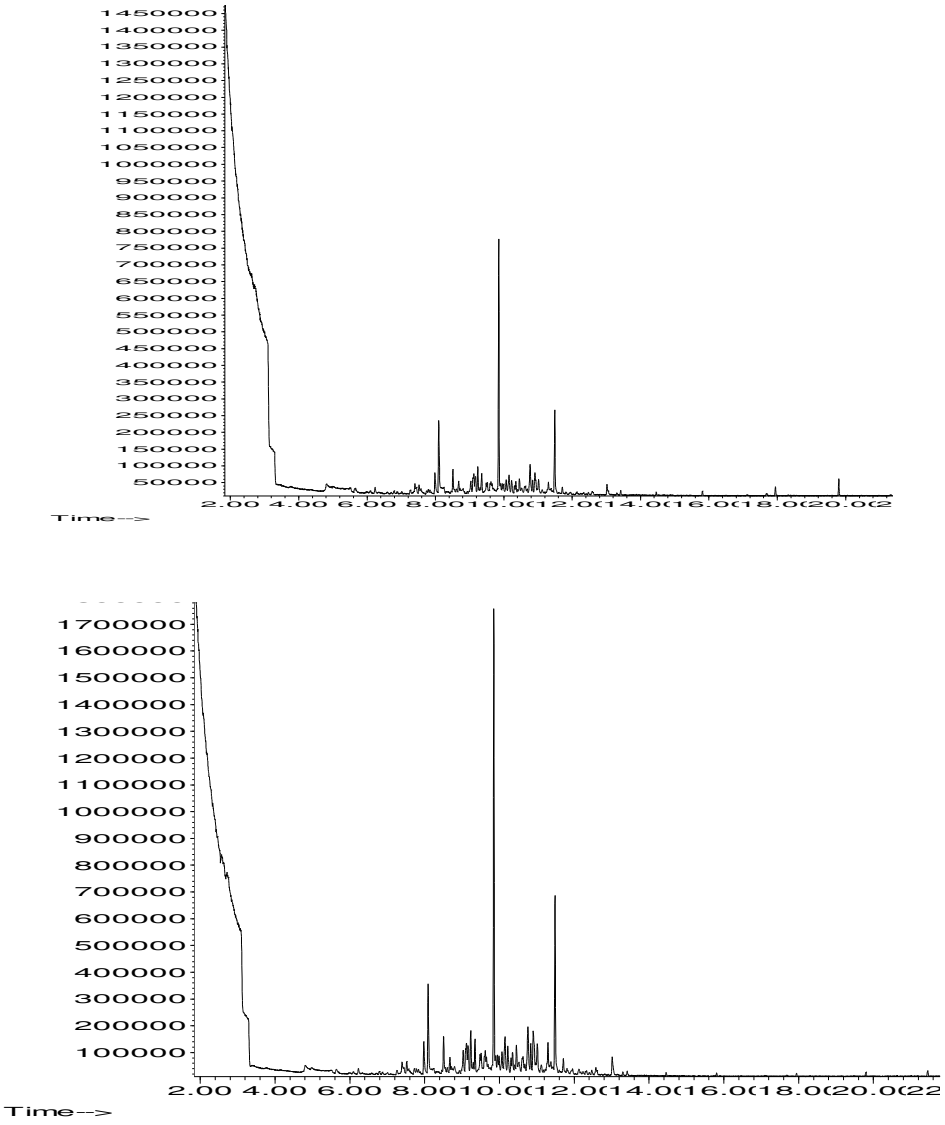


Figure 2.13: *Chromatograms obtained from kerosene standards run without adhesive tape on the metal can (top) and with adhesive tape on the metal can (bottom).*

2.4.5 Headspace Conditions

Petrol and kerosene standards were analysed using the previously optimised GC-MS method to evaluate the optimum headspace temperature and exposure time that would produce chromatograms that contained all of the target compounds (see Table 2.4 and 2.5). It was challenging to develop conditions that were suitable for both petrol and kerosene, given the differences in the range of components each ignitable liquid comprises.

It was noted that when the headspace temperature was increased, the sensitivity of the higher boiling point components also increased, but this resulted in a decrease in the sensitivity of the lower boiling point components. Furthermore, an increase in headspace temperature resulted in higher headspace concentrations, which in turn resulted in shorter extraction times [90]. It was therefore important to select a temperature and a sampling time that would effectively recover the higher boiling *n*-alkanes, but would not result in lower yields of the more volatile components [80]. That is, it was important that the conditions were suitable for both ignitable liquids.

Target Compounds Detected at Different Conditions for Petrol															
Condition		**Target compounds**													
		1,3,5-tri methyl benzene	**1,2,4-tri methyl benzene**	**1,2,3-tri methyl benzene**	**indane**	**1,2,4,5-tetra methyl benzene**	**1,2,3,5-tetra methyl benzene**	**5-methyl indane**	**4-methyl indane**	**C_{12}**	**4,7-dimethyl indane**	**2-methyl naphthalene**	**1-methyl naphthalene**	**1,3-dimethyl naphthalene**	**2,3-dimethyl naphthalene**
60 (°C)	**1 min**	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	N
	2 min	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	N
	3 min	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	N
70 (°C)	**1 min**	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	N
	2 min	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
	3 min	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
80 (°C)	**1 min**	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	N
	2 min	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	N
	3 min	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
	5 min	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
	10 min	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
90 (°C)	**3 min**	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
	5 min	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
	10 min	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y

Table 2.4: *Target compounds detected for petrol standards run using a variety of headspace temperatures (°C) and exposure times (min) where (Y) indicates the target compound was present and (N) indicates the target compound was absent.*

Target Compounds Detected at Different Conditions for Kerosene																								
Condition		**Target compounds**																						
		C_{10}	***n*-butyl cyclo hex-ane**	**trans dec-alin**	C_{11}	**1,2,3,5-tetra methyl benzene**	***n*-pentyl cyclo hexane**	C_{12}	***n*-hexyl cyclo hexane**	**2-methyl naph-thalene**	**1-methyl naph-thalene**	C_{13}	***n*-heptyl cyclo hexane**	**1,3-dimethyl naph-thalene**	C_{14}	***n*-octyl cyclo hexane**	C_{15}	C_{16}	C_{17}	**pristane**	C_{18}	**phytane**	C_{19}	**eico-sane**	**Hene ico-sane**
70 (°C)	**3 min**	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	N	N	N	N	N	N	N	N	N	N	N
	5 min	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	N	N	N	N	N	N	N	N
	10 min	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	N	N	N	N	N
80 (°C)	**5 min**	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	N
90 (°C)	**3 min**	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
	5 min	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
	10 min	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y

Table 2.5: *Target compounds detected for kerosene standards run using a variety of headspace temperatures (°C) and exposure times (min) where (Y) indicates the target compound was present and (N) indicates the target compound was absent.*

It was found that all the target compounds were present for both petrol and kerosene when the headspace was heated to 90 ^{o}C. The exposure time of three minutes was selected to maximise the abundance of the target peaks, particularly for the petrol target compounds. Although this exposure time is prior to full equilibrium (that is, when the amount of extracted analyte remains constant), the SPME technique can be used both before and after equilibrium is achieved [120]. Since an exposure time prior to equilibrium is recommended, it is imperative that there is consistency in the sampling time, otherwise there will be a considerable effect on reproducibility [127].

2.5 Conclusion

This chapter describes the testing of a number of parameters that relate specifically to the analysis and sample preparation techniques required for this project. As a result, the testing of ignitable liquids was limited to petrol and kerosene, and GC-MS was selected as the instrument of choice, with an initial GC oven temperature of 35 ^{o}C, which was held for 2 min, then increased at a rate of 10 ^{o}C/min to 190 ^{o}C, followed by a second temperature ramp of 70 ^{o}C/min to 290 ^{o}C, held for 4 min. This method was operated with a constant pressure of 85.4 kPa and a mass range of 40.5 to 400 amu. Furthermore, the sample preparation technique selected was SPME, utilising the extraction mode with a PDMS-DVB fibre coating. This sample preparation technique was carried out with an oven temperature of 90 ^{o}C and an extraction time of three minutes. These parameters will be used to design a framework for the small-scale experiment that will be discussed further in **Chapter 3**.

Chapter 3:

Small-Scale Experiments

Chapter 3: Small-Scale Experiments

3.1 Overview

This chapter encompasses a number of topics that were referred to in **Chapter 1** but which will now be described in greater detail. These include fire debris analysis and the major challenges associated with the confirmation of these residues and entomotoxicology. Further to this, specific studies that link both fire investigation and forensic entomology are described. Finally, small-scale experiments are described and the results discussed to evaluate the hypothesis that ignitable liquids can be detected in fly larvae and thus are a viable alternative to fire debris when a scene contains burnt human remains.

3.2 Introduction

The investigation of a fire involves the determination of whether it was an accident, or ignited under suspicious circumstances [66]. An important aspect of fire investigation is the collection and chemical analysis of fire debris to establish whether materials present in this debris may have assisted in starting or accelerating the fire. The confirmation of ignitable liquids may indicate a deliberately lit fire. However, it is important to note that there may be legitimate explanations for the presence of components of certain ignitable liquids in some sample matrices [83]. Therefore we cannot assume that the presence of such compounds will always be indicative of a suspicious fire. In addition, the results from the chemical analysis of fire debris are only one aspect of a fire investigation that also incorporates physical evidence and eye witness accounts [64].

The detection of ignitable liquids in fire debris is a challenging task for a number of reasons. Firstly, ignitable liquids are extremely volatile and will therefore begin to

evaporate as soon as they are exposed to the elements. Typical fire scene conditions (high temperatures) will cause ignitable liquids (if they are indeed present) to evaporate more rapidly [84]. Subsequent exposure of fire debris to the elements (post-suppression) will cause further evaporation [83,93] as the evaporation of ignitable liquids is predominantly controlled by temperature and the duration of exposure [99]. The weathering of ignitable liquids modifies their GC profile such that the lighter, more volatile components (which diffuse into the air first) will be absent and the heavier, less volatile components will dominate the profile (see Figure 3.1). Therefore, weathering and matrix interferences can make the identification of ignitable liquids in fire debris difficult.

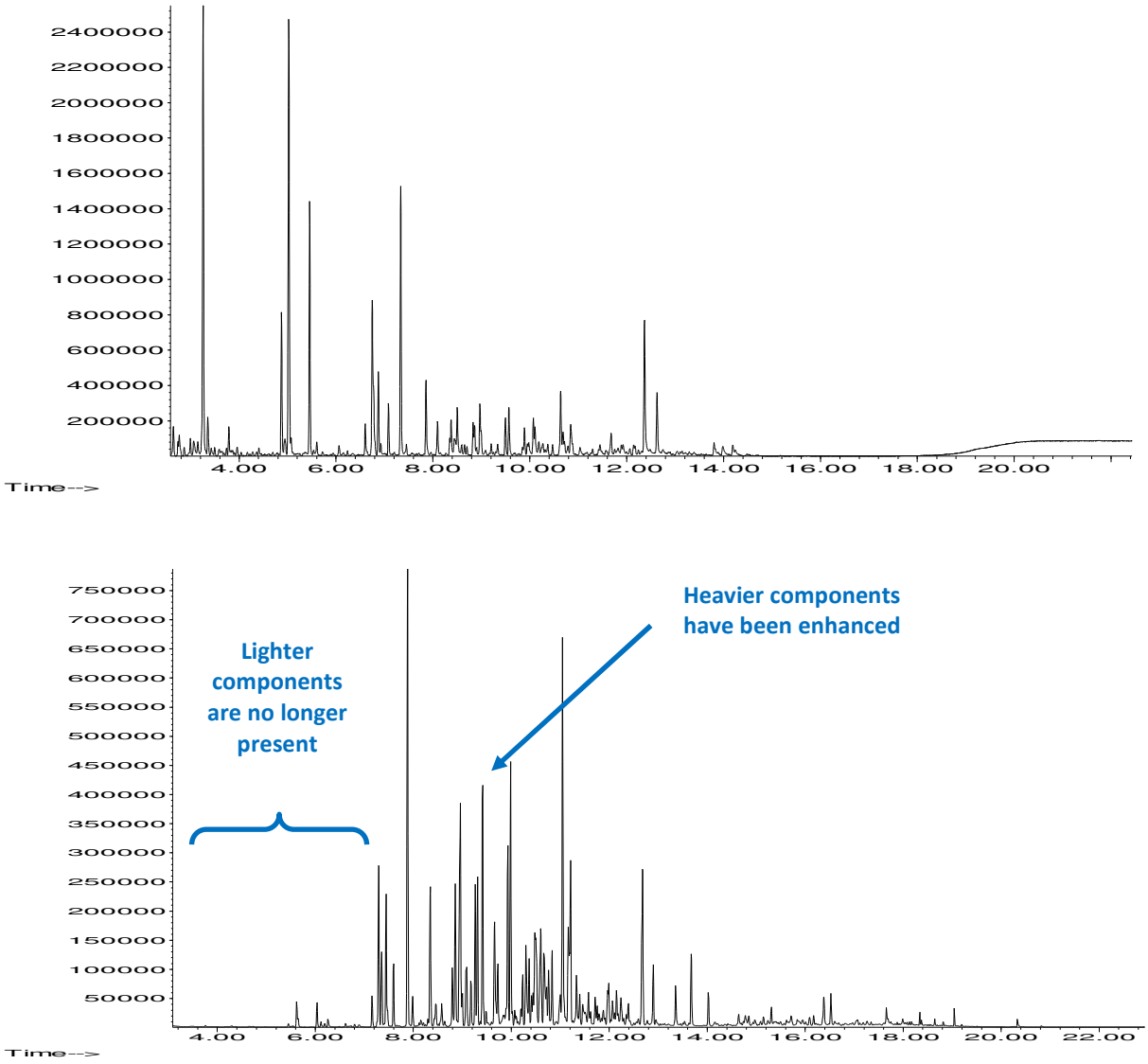


Figure 3.1: *Example of a fresh petrol profile (top) and an example of an evaporated petrol profile (bottom).*

There are a number of factors that will affect the concentration of the ignitable liquids that remain at a fire scene. These include the quantity and type of ignitable liquid initially used, the location of the debris, whether the debris is buried, protected in some way or has been exposed to heat, the nature of the material it is present on, the time that has elapsed between the fire and collection of the fire debris, and the severity of the fire itself [128]. Despite this, there does not appear to be any published

research that provides an estimate of the maximum time that fire debris can be exposed to the external environment before compounds derived from a particular ignitable liquid can no longer be detected. There has, however, been a study that investigated the detection of petrol on car carpets between 24 hours and four weeks after the ignitable liquid had been added. It was found that the sample could no longer be classified as petrol one week after the initial sample was added. However, these findings are not meaningful in the context of fire debris analysis as only fresh petrol was used and the samples were not tested at regular intervals (i.e. testing conducted 24 h after the addition of the petrol and then at 1 week). This does not provide information on the persistence of ignitable liquids on material once they are burnt. The authors, however, make vague comments that higher temperatures increase the rate of evaporation of the volatile components of petrol, thus shortening the time in which it can be detected [83]. Anecdotally, it has been suggested that reportable traces of ignitable liquids may evaporate in as little as a few hours [129].

Notwithstanding the major challenges in confirming the presence of ignitable liquids, numerous methods have been proposed to analyse fire debris, but none of these has succeeded in overcoming these issues [80]. In fact, the current American Society for Testing and Materials (ASTM) test method (for ignitable liquid residues in extracts from fire debris) states that the essential requirement for making a classification using this procedure is the matching of the sample chromatogram with a reference chromatogram, noting points of correlation [82]. That is, despite technological advances, standard methods still rely upon the correlation of numerous volatile components to indicate the presence of an ignitable liquid. Unfortunately, these highly volatile components are more likely to diffuse into the air rather than remain at a fire scene [68,93].

It is clear that time remains the critical factor when attempting to recover ignitable liquids from burnt matrices. However, a quick recovery may not always be possible if, for example, the fire scene is located in a remote area, because it may take the investigator considerable time to arrive at the scene and recover evidence. Moreover,

many homicide cases involve bodies in remote areas. This may result in a delay of days, months or even years until recovery [18]. According to the New South Wales Fire Brigade (Australia), of the 319 fires investigated in 2008/09, the cause of 90 remains undetermined [130]. This figure has remained relatively constant over the last five years [130-134]. Although there are many factors that contribute to the number of undetermined fires, devising alternative ways to analyse fire debris may help identify the cause of a fire and thus contribute to the reduction of this figure. For fire scenes involving burnt human remains, one such alternative is the analysis of fly larvae that may infest the remains. Furthermore, fly larvae may be analysed in conjunction with traditional fire debris samples to strengthen findings.

As discussed in **Chapter 1**, insects can be utilised as alternative specimens for qualitative toxicological analyses when a body is in an advanced state of decomposition, and conventional post-mortem samples, such as blood or urine, may not be available or do not provide reliable results [52,53]. In these situations, fly larvae are typically abundant [12,27,39,47-51,54,55,59,61,62,135].

Drugs and toxins that have been identified in this way include (but are not limited to) barbiturates [51,54,59], analgesics [54,59,136], benzodiazepines [19,47], cocaine and its metabolites [48], opiates [46,55,60], organophosphates [52], antidepressants [62], and most recently, gunshot residue [137]. Methyl mercury [40,41,43], copper, iron, zinc and calcium have also been detected in adult flies [44,45]. These studies have focused on substances associated with the cause of death. The potential of utilising larvae in the detection of chemicals associated with the destruction of a cadaver (to conceal the cause of death or identity of the deceased) has been largely overlooked. Of particular interest here are chemicals used to accelerate the spread of fire.

Although the analysis of insects in casework is a relatively new application, extraction techniques specific to fly larvae are not required as they can be analysed in the same manner as other samples [12,54]. In addition, it has been reported that results obtained from fly larvae exhibit less interference from decomposition by-products

compared with other traditional specimens [47,48,50]. Furthermore, perhaps the most significant advantage of using insects as alternative toxicological specimens is they have the potential to extend the sampling period. In particular, puparia cannot only withstand changing climatic conditions, but unlike the larvae and adults, they are immobile, and hence can be found close to human remains for extended periods [12,55,56]. In addition, when the foreign substances present in tissues are volatile (that is, evaporate quickly), bioaccumulation of these substances in fly larvae would also assist in extending the sampling period. Moreover, fly larvae often feed in protected areas and hence the tissues in which they are feeding may be less vulnerable to the external environment, which will be of importance if the foreign substance is volatile.

Although the proposal to use fly larvae as an alternative to traditional fire debris evidence is a novel application, there are several studies that relate to the research presented in this thesis, or indeed have incorporated the two major subject areas of interest. One such study noted the lack of confirmation methods available in order to report the presence of ante-mortem burns, as previously this conclusion replied upon physical observations alone. As a result, they presented a method for the qualitative and quantitative detection and identification of fuel components in blood from animals exposed to petrol and kerosene vapour, using headspace and solvent extraction techniques combined with GC-MS [138]. As in this thesis, these authors were thus proposing a different type of sample that can be utilised to confirm the presence of petrol and kerosene.

The second study involved the investigation of succession patterns on burnt carrion [139]. This study was the first of its kind to be conducted. In this experiment, the authors placed domestic pigs, *Sus scrofa,* one burnt with petrol and the other unburnt, in two contrasting environments, namely a xerophytic habitat and a rainforest habitat. No major differences were observed in the insects present on the burnt and unburnt pigs, or in the duration of the individual stages of decomposition. However, it was noted that major oviposition occurred one day earlier in the xerophytic habitat,

compared with the control, and four days earlier in the rainforest habitat, also compared with the control.

This was a significant study, as the findings suggested that adult blow flies are not deterred by burnt carrion, but will readily oviposition on such a food source. In addition, it was observed that the burning process caused the flesh to crack and thus create outlets from which body fluids could seep. The authors postulated that this was one of the major cues that attracted blow flies to these additional oviposition sites and might also explain the earlier oviposition on the burnt carrion compared with the unburnt [139].

A subsequent study, conducted in Taiwan, confirmed these findings. Once again a pig was used as a human substitute and burnt using petrol. Adult blow flies were observed on the pig within five minutes of placement and first-instar larvae were noted within 17 hours [15]. These results were comparable to another study conducted in the area using an unburnt pig. While this conclusion would be more significant if the burnt and unburnt pigs were exposed at the same time, it suggests that burning a pig with petrol does not delay oviposition. Interestingly, these authors state that soon after this study was completed, a case involving a burnt body was reported. The physical evidence obtained and eye witness accounts, together with the size of the larvae, indicated a PMI of two days. This finding was consistent with the experimental data obtained and reinforced the finding that there is no delay in oviposition when a body has been burnt.

Prior to the landmark study that was performed in order to compare the colonisation of insects on burnt and unburnt pigs [139] (described above), gross generalisations were being made based on unsubstantiated findings alone. For example, it was stated that burnt remains located in the trunk of a vehicle will delay insect colonisation by three days, with no comprehensive study to qualify this statement [3]. Further to this, other authors have stated that forensic cases involving charred remains are not infrequent, and based on their observations, when a human body is not completely

combusted and the internal organs are exposed, blow flies will skilfully seek suitable oviposition sites [14]. However, oviposition at these sites could occur only after the flames have been extinguished and when the decline in body temperature has reached favourable conditions for larval development. They also state that such cases provide no useful data in which to calculate a PMI. These findings contradict those of Avila *et al.* (1998) [139], who found that oviposition was observed on burnt remains earlier than the unburnt. However, the cases discussed by Introna *et al.* (1998) [14] involved burnt out vehicles, and the colonisation of remains in a car would differ in comparison with those exposed to the external environment. Therefore, it is difficult to comment on delays experienced due to a body being burnt under such circumstances.

The final study investigated the ability to recover and calculate the PMI from entomological evidence collected after a series of house fires [22]. To test this, three pigs were placed in a house and allowed to decompose to the active decay stage. As a result, these pigs contained an abundance of entomological evidence. A total of four fires were set, one for each pig (two of these three involved the use of petrol to accelerate the fire), and a final fire that involved setting the entire house alight using a mixture of petrol and diesel. Another experiment was also run simultaneously where the colonisation of the pigs contained in the house was compared with the pigs that had been placed outdoors. Five days elapsed before the pigs in the house became infested with insects. However, this delay (as was suggested for the burnt out vehicle) may have been due to the enclosed nature of the structure. The findings from this study revealed that entomological evidence could be successfully recovered after each of the four fires. Therefore, even the intense heat from the fire could not destroy the entomological evidence, and enough evidence was able to be recovered to permit a PMI calculation [22].

3.3 Experimental Design

3.3.1 Study Site

The small-scale experiments were conducted at the University of Technology, Sydney (UTS), within a ventilated laboratory. Fly larvae were stored under temperature-controlled laboratory conditions monitored with small data loggers (iButtons® Maxim Integrated Products Sunnyvale, CA) every 30 minutes.

3.3.2 Food Source

A point to consider when designing entomological experiments is the type of food source selected, and in particular, maintaining uniformity of that food source. This consideration was motivated by studies that have revealed that fly larvae develop at different rates depending on the type of tissue on which they are reared [140]. Larvae moulted later and produced smaller pupae when fed on sheep liver, as compared with sheep meat and brain tissue. Conversely, larvae developed two days faster on pig lung, kidney, heart and brain tissue compared with pig liver [141]. Therefore, irrespective of the number of replicates used for the small-scale experiments, it was important to maintain a uniform feeding substrate for the larvae.

Superficial sheep's meat with associated adipose tissue (lamb neck chops weighing between 80 and 120 g) was used, as the presence of adipose tissue increased and prolonged burning (see Figure 3.2).

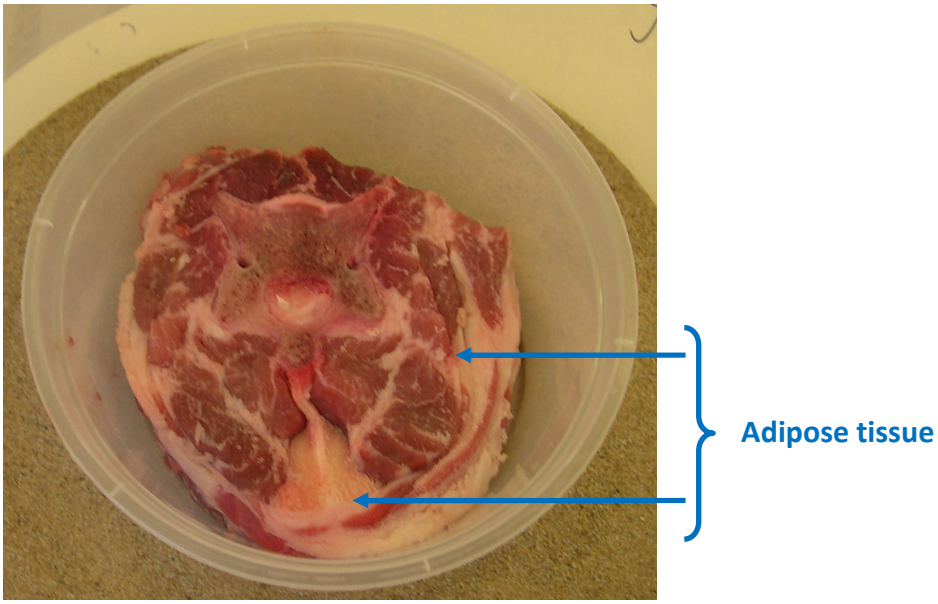


Figure 3.2: *Food source (lamp neck chops) used throughout the small-scale experiments (with adipose tissue that prolonged burning highlighted).*

Meat was stored in 500 mL plastic containers, within a 5 L container (see Figure 3.3). The larger container was sealed with cotton material and the base was covered in sand (the smaller container rested on the surface of the sand). The sand acted as a medium in which the larvae could pupate and simplified their retrieval at the conclusion of the experiment, as it could be sifted. The sand was replaced after each experiment in order to remove any contaminants.

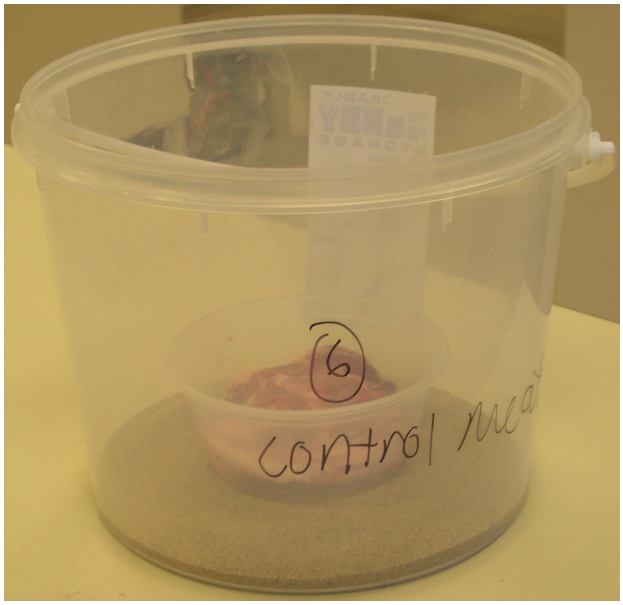


Figure 3.3: *500 mL plastic containers within a 5 L container, containing the meat used in the small-scale experiments.*

3.3.3 Insect Samples

Adults (flies) of *Lucilia cuprina* (Wiedemann) (Diptera: Calliphoridae) (see Figure 3.4) were used in these experiments as these blow flies are commonly encountered in forensic cases in eastern Australia and there are established protocols for their rearing [23,142]. The specimens used throughout these small-scale experiments were derived from a culture maintained by the Department of Medical Entomology, Westmead Hospital, Australia.

Lucilia cuprina flies are commonly known as green bottles due to their metallic-green abdomen. This species is commonly termed the Australian sheep blow fly, as it is most often responsible for 'flystrike' in Australia [143]. 'Flystrike' occurs as flies lay their eggs in damp, protected areas of the sheep soiled with urine and faeces (under the tail) and in areas that have become drenched (backs and stomach) [23,143]. The

larvae then feed and thus lacerate the skin in this area, which results in festering sores, which, if left untreated, cause bacterial infection and eventually death [143].

Figure 3.4: *The Australian sheep blow fly,* Lucilia cuprina *(Photo: [144]).*

3.3.4 Ignitable Liquids

As discussed previously (Section 2.2), the ignitable liquids tested in this experiment were petrol and kerosene. In order to simulate burnt remains in this simple experiment, the lamp neck chops used as the food source required burning. As a result, there were a number of variables that had to be considered that involved the meat itself.

First, the time that elapsed between the pouring of the ignitable liquid onto the meat and ignition was considered to ensure repeatability among experiments. It was found that one minute provided sufficient time for the ignitable liquid to absorb into the meat, while ensuring that it did not completely burn when ignited. It was evident that

the ignitable liquid had absorbed into the meat when it was observed to be seeping out from the outer surface of the meat.

It was also necessary for the meat to be ventilated following burning as in a 'real life' situation; first-instar larvae (first growth stage after hatching) would not be present on the food source immediately after it had been set alight. Furthermore, ventilation was an important aspect of the method design, as preliminary experiments with no ventilation resulted in high mortality rates among the fly larvae. It was also noted that the fly larvae that survived moved away from the unventilated meat. It was therefore concluded that burnt meat that is not ventilated is not an attractive food source for fly larvae. Therefore a ventilation period, where the meat remained undisturbed in the laboratory for six hours prior to the addition of first-instar larval samples, was selected to best represent the time required for larvae to develop from the egg stage.

The volume of ignitable liquid to be added to the meat was also considered. For example, selecting too large a volume could pose a safety threat in the laboratory where the ignitable liquids were ignited, while selecting too small a volume might not accurately depict a 'real life' situation and the ignitable liquid may fail to be detected following ventilation and subsequent ingestion by the fly larvae. Therefore, 5 mL and 10 mL volumes of each of the ignitable liquids of interest were added to the meat samples. Once these were burnt and ventilated, the larvae were allowed to feed on the meat. Larval samples were then collected and analysed (see Figure 3.5). The greatest abundance of the target compounds was obtained from the sample prepared using 10 mL of kerosene. Therefore, a volume of 10 mL was adopted.

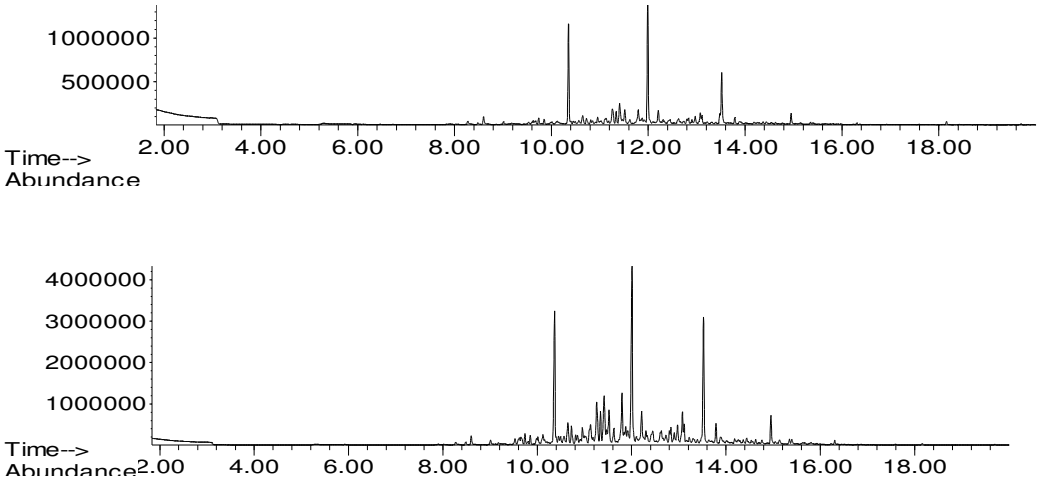


Figure 3.5: *Chromatogram obtained from fly larvae feeding on meat that was burnt with (5 mL) of kerosene (top) and (10 mL) of kerosene (bottom).*

3.3.5 Sample Size

Several tests were performed to investigate the optimum number of larvae to be collected and the frequency of this collection. Varying numbers of larvae were trialled to determine how many larvae would need to be sampled to give reproducible results. It was concluded that individual larvae varied in their feeding habits, as the analysis of three larvae often did not give a relative abundance three times greater than that of one particular larva. Therefore, a total of six larvae were collected on a daily basis to ensure more representative sampling of the population. Although some studies have collected samples more frequently than once a day, due to the controlled nature of the experiments described here, a collection frequency of once a day was considered sufficient [145,146].

A sampling window of five days was selected as this was approximately the time normally taken before larvae of *Lucilia cuprina* began to pupate under these conditions.

3.3.6 Larval Sampling Conditions

The conditions for sampling the fly larvae were also investigated. Firstly, homogenised and intact larvae were analysed (see Figure 3.6). The chromatogram produced from the homogenised larvae resulted in a reduced response compared with the unaltered larvae. This was most likely due to the volatiles escaping into the air when the larvae were homogenised. Therefore, it was concluded that it was not necessary to homogenise the fly larvae when a headspace extraction mode was used.

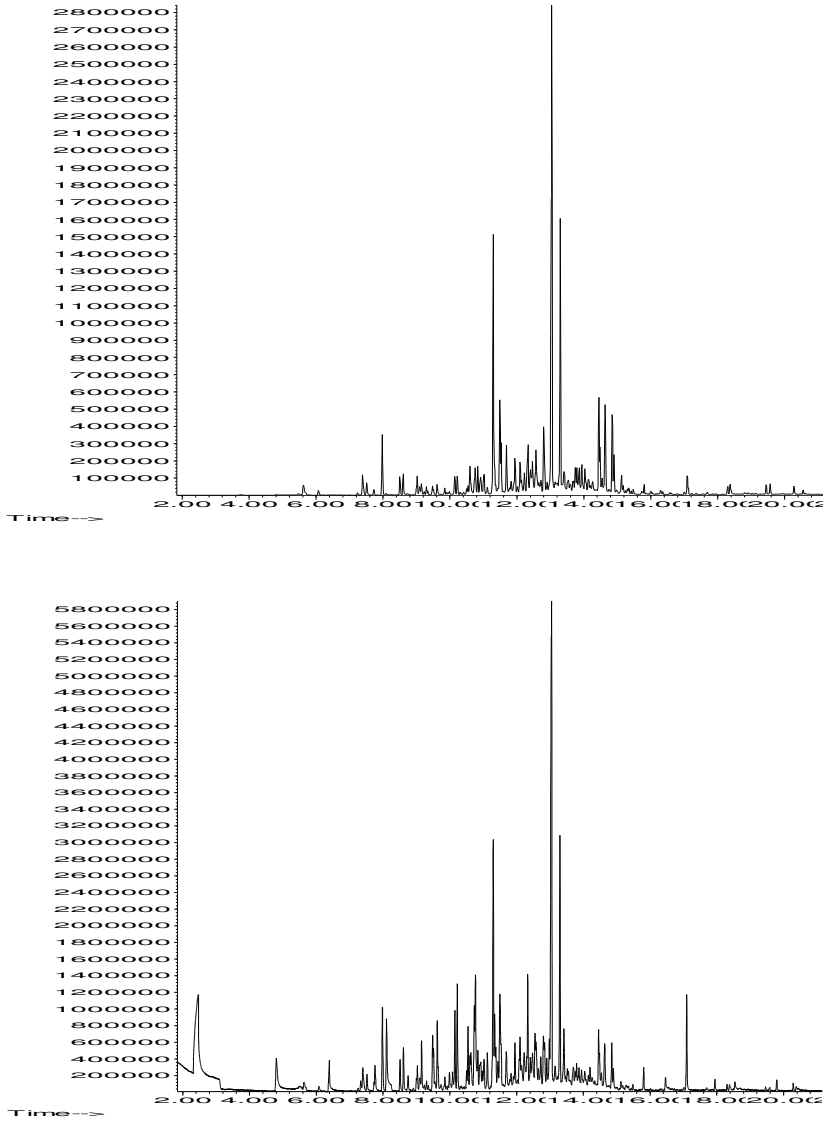


Figure 3.6: *Chromatogram obtained from fly larvae that were homogenised (top) prior to analysis and fly larvae that were unaltered (bottom) prior to analysis.*

It has been common practice in many entomotoxicology studies [47,54,59,61,135] to wash larval samples prior to the extraction step; therefore, this was trialled in the current study. It was noted that washing the larvae, irrespective of the liquid used, reduced the overall response of the ignitable liquid. This is a significant disadvantage, since for the purposes of these experiments, the presence or absence of an ignitable liquid is significant, not its concentration. Therefore, washing was not incorporated

into the methodological design. Furthermore, these experiments are aimed at treating fly larvae as an alternative to fire debris. Since washing and homogenisation are not common practice in fire debris analysis, they were not considered necessary for the larvae in this project.

3.4 Material and Methods

3.4.1 Experimental Design

The small-scale experiments comprised four sample sets of meat, each with six replicates. This was the maximum number of replicates that the facilities within the laboratory could manage without increasing the risk of cross-contamination. The first set consisted of meat burnt with petrol, the second was burnt with kerosene, and the third was burnt without an ignitable liquid. The final sample set was unburnt. The third and fourth sample sets acted as burnt and unburnt control groups, respectively.

The ignitable liquid sample sets were prepared by adding 10 mL of the relevant ignitable liquid to the surface of the meat. Following this addition, the ignitable liquid was allowed to absorb for one minute prior to ignition. The meat was ignited using a match and allowed to burn until it self-extinguished. It was then left in a laboratory to ventilate for six hours. A Bunsen burner was used to achieve the same approximate degree of burning in the samples of meat that did not have an ignitable liquid present. This sample set, together with the unburnt samples, was also ventilated for six hours. Following ventilation, approximately 100 first-instar larvae of *Lucilia cuprina* were transferred to each of the replicates and allowed to feed undisturbed for a minimum of 14 hours.

Six larvae were collected from each of the 24 meat samples on a daily basis for five days. The remaining larvae feeding on the meat samples were allowed to pupate. Once adults had emerged, six adults and six empty puparia from each meat sample

were collected separately into 1 L metal cans. Prior to and following their use, the metal cans were placed into an oven for at least 24 hours at 110 ^{o}C. Preliminary testing indicated that this was sufficient to remove any volatile contaminants.

3.4.2 Sample Preparation

Solid-phase microextraction (SPME) was used for the extraction process. Prior to extraction, a small hole was punched in the lid of each metal can to accommodate the insertion of the SPME fibre. A piece of adhesive tape was placed over this hole to minimise the escape of volatiles. The SPME fibre coating selected for these experiments was the polydimethylsiloxane-divinylbenzene (PDMS-DVB) coating. Prior to extraction with SPME, the metal can (housing the entomological samples of interest) was preheated in an oven to achieve a headspace temperature of 90 ^{o}C. Once this temperature had been achieved, the SPME fibre was inserted into the headspace and fastened in place with a retort stand (to ensure the fibre holder remained stationary, reducing the chance of the fibre breaking) and the tin was placed in an oven at 90 ^{o}C for three minutes.

Following adsorption, the fibre was inserted directly into the GC-MS injection port for three minutes to thermally desorb. It was important that the time between extraction and analysis was kept to a minimum, as after the fibre is withdrawn from the sample it begins to equilibrate with air. This process is decelerated when the fibre is withdrawn into the needle [124]

3.4.3 Sample Analysis

The analysis was carried out on an Agilent 6890 series GC system, coupled to an Agilent 5973 network mass selective (MS) detector using a Zebron ZB – 5 ms 30 m × 0.25 mm ID × 0.25 µm 5% polysiloxene 95% polydimethylsiloxane column (see Table 3.1 for a list of the parameters used).

GC-MS System Parameters	
Carrier gas	Helium
Injector temperature	250 °C
Initial flow rate (as operating at constant pressure)	1.8 mL/min
Pressure	85.4 kPa
Injection mode	Splitless
Purge time	1 minute
Oven program Initial Ramp 1 Ramp 2	 35 °C, held 2 minutes 10 °C/min to 190 °C 70 °C/min to 290 °C, held 4 minutes
Solvent delay	3 minutes
Scan range	40.5 - 400 amu

Table 3.1: *GC-MS instrument parameters used throughout the small-scale experiment.*

3.4.4 Data Analysis

Total ion chromatograms (TIC) and extracted ion chromatograms (see Section 1.6) were produced for all fly larvae, puparia and adults tested. These chromatograms were evaluated as being positive for either petrol (P) or kerosene (K) through visual pattern matching against known reference chromatograms, according to the presence of target compounds and guidelines set out by the current ASTM method [82]. In the case of petrol, the three C_2 alkyl benzene peaks and the five C_3 alkyl benzenes had to be present and in the case of kerosene, a series of successive *n*-alkanes had to be present producing a bell-shaped curve in order to report a positive result for these ignitable liquids. However, if a sample differed from the reference chromatogram (for example an unexplainable absence of known components in the centre of the chromatogram), the sample was classified as negative for an ignitable liquid (N).

Profiles obtained from petroleum distillates are quite complex and thus can be difficult to interpret and this process is quite subjective. This can be complicated further as the volatile components of ignitable liquids are lost during the burning process and hence the chromatogram produced is visibly different from a fresh (neat) sample. It has been stated that this is the weakest aspect of the analysis process [91,147]. One approach used in a number of studies to overcome the subjective nature of this interpretation is through the use of a pattern recognition technique, such as principal component analysis (PCA) [79,91,99,148,149]. PCA reduces the dimensionality of a dataset whilst retaining as much of the variability in the original data as possible [79]. This reduction takes place through linear transformations of the data to yield principal components (loadings), the first few of which encompass the majority of the variability in the data. The weightings or scores for these first few components can be plotted against each other to give *scores* plots, in which similar samples, each represented by a single plot, will tend to cluster together. These plots allow for easy visualisation of the clusters that result from the dataset. Therefore, this technique was utilised for the chromatographic data produced from the small-scale experiment. It was anticipated that the clustering observed from this analysis would concur with the designations made by the visual inspection of the chromatograms produced.

Initially the peaks corresponding to the 34 target compounds (corresponding to petrol and kerosene) were integrated manually for each chromatogram produced from the small-scale data. This was a very time-consuming process. A more convenient alternative involved using an in-house program (a Visual Basic macro in Microsoft Excel) to perform batch integration on the chromatographic data, in order to avoid manual integration of every chromatogram. This program performed a simple fixed-width integration of the peaks corresponding to the 34 target compounds in Table 2.1 (see Appendix B for details of the macro used). An integration width of less than 5 points (the width chosen did not appear to make a great difference in the separation of sample sets until the width was quite large) on each side of the peak of interest was selected. These peak areas were used to construct a 34-variable profile for each sample; these profiles were then used to construct a dataset for PCA.

Pre-processing of both the manually integrated data and the macro-generated data was required prior to the multivariate analysis. This included removing the effects of absolute intensity differences between samples by vector normalisation of the dataset, and using mean-centring to remove unwanted variability and to ensure that the scores plot would be centred about the origin. PCA was performed on this dataset using Pirouette® 4.0 software.

It was found that PCA results obtained from the data that had been integrated manually exhibited closer association (tighter clustering) within each of the kerosene, petrol and control sample sets, compared with the data produced from the in-house program (see Figure 3.7). However, both methods successfully separated the two ignitable liquid sample sets and the control sample set from each other. It was therefore concluded that, the additional time spent on the manual integration process was unwarranted.

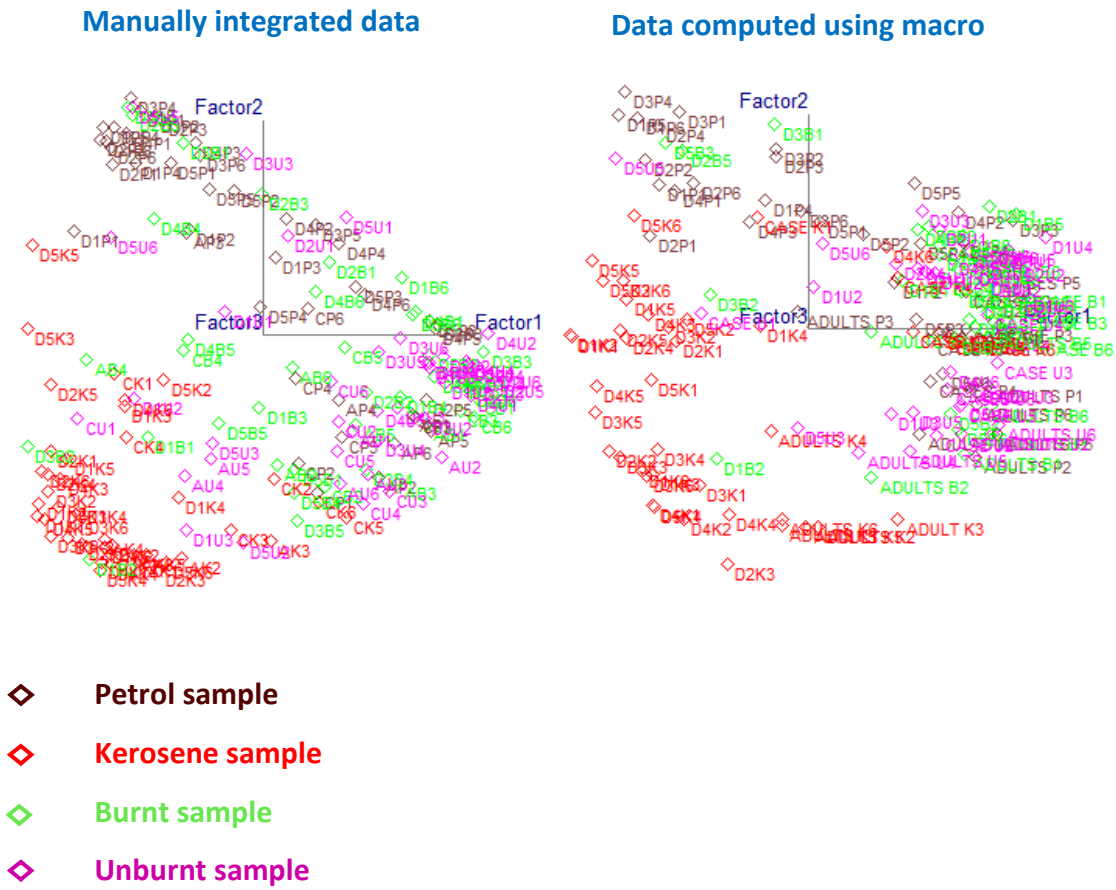


Figure 3.7: *Scores plot generated from manual integration and data computed using a Visual Basic macro.*

Further analysis of the differences between the four sample sets was performed using a one-way multivariate analysis of variance (MANOVA), using SPSS®, on selected principal components scores that had been calculated by Pirouette®. MANOVA is an extension of the analysis of variance for use when there is more than one dependent variable. Post-hoc analysis was also carried out to further investigate the differences found and to determine which principal component was responsible for these differences.

3.5 Results and Discussion

3.5.1 Study Site

Temperature readings were taken for each of the four sample sets every 30 minutes. The maximum, minimum and mean daily temperatures were plotted (see Figure 3.8, 3.9 and 3.10). These graphs indicate that generally over the duration of the experiment, the sample sets could not be separated by their maximum, minimum and mean daily temperatures. In order to investigate this further, a two-way analysis of variance (ANOVA without replication) calculation was performed using Microsoft Excel. In this case, the calculation was made in order to determine if there were significant differences between the maximum, minimum and mean daily temperatures of the four sample sets. The results obtained indicated that the four sample sets were not statistically different based on these readings (maximum: $F_{3,40} = 0.119$, $p>0.05$, minimum: $F_{3,40} = 1.558$, $p>0.05$ and mean: $F_{3,40} = 1.644$, $p>0.05$).

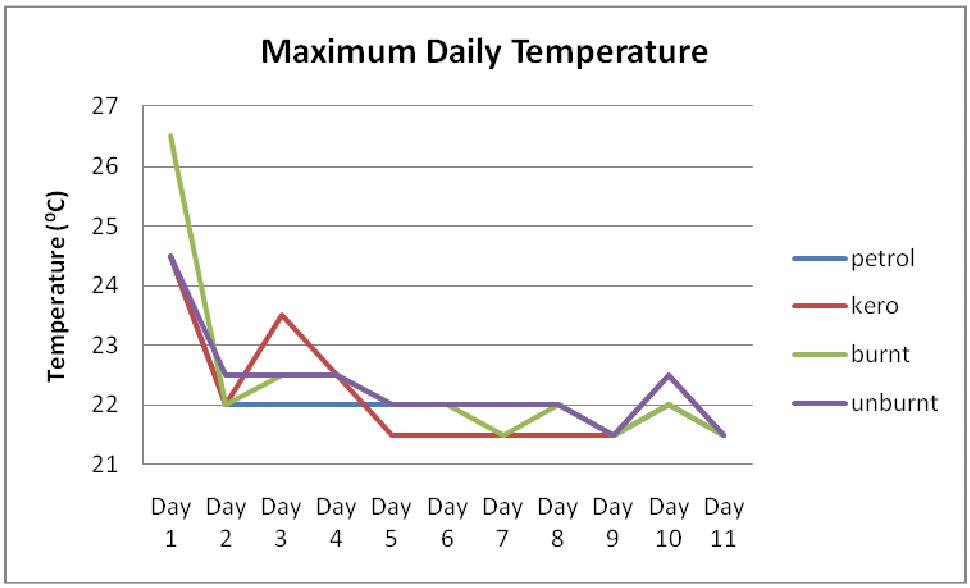


Figure 3.8: *Maximum daily temperatures recorded for the four datasets over the duration of the laboratory controlled small-scale experiments.*

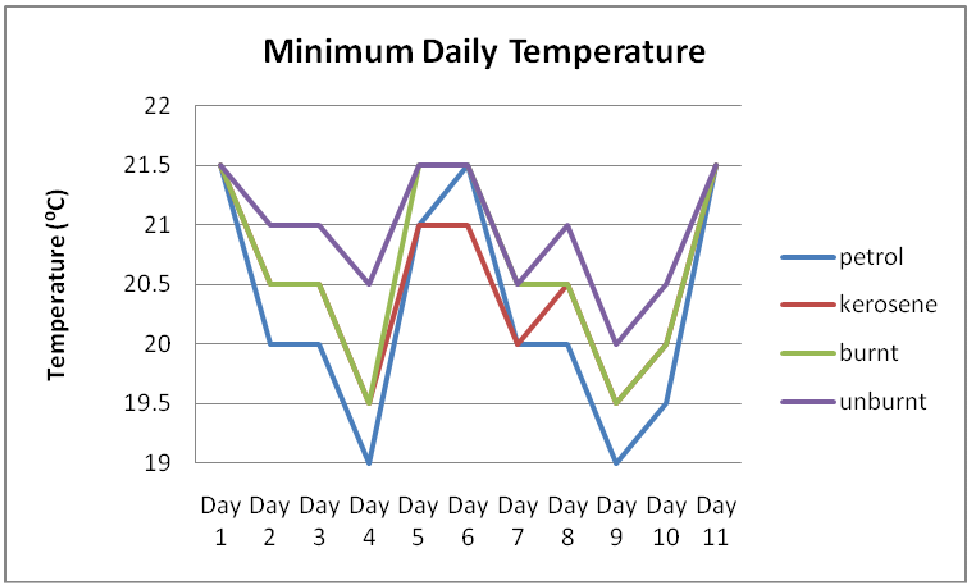


Figure 3.9: *Minimum daily temperatures recorded for the four datasets over the duration of the laboratory controlled small-scale experiments.*

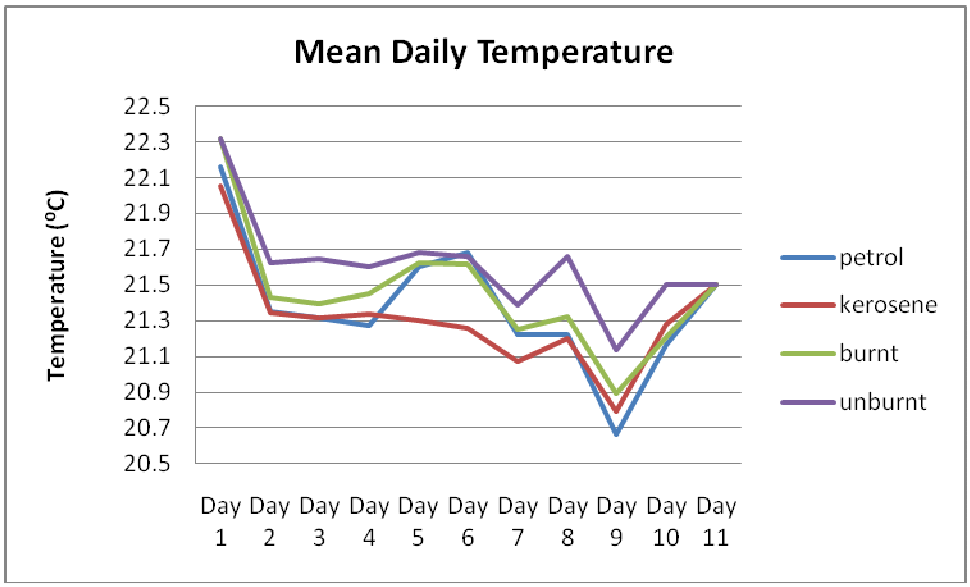


Figure 3.10: *Mean daily temperatures recorded for the four datasets over the duration of the laboratory controlled small-scale experiments.*

3.5.2 Food Source

The burning process (using 10 mL of the ignitable liquid of choice) appeared to cook the surface of the meat slightly. The area that exhibited the most damage was the edge of the meat; however, the remaining area of the meat showed little damage (see in Figure 3.11).

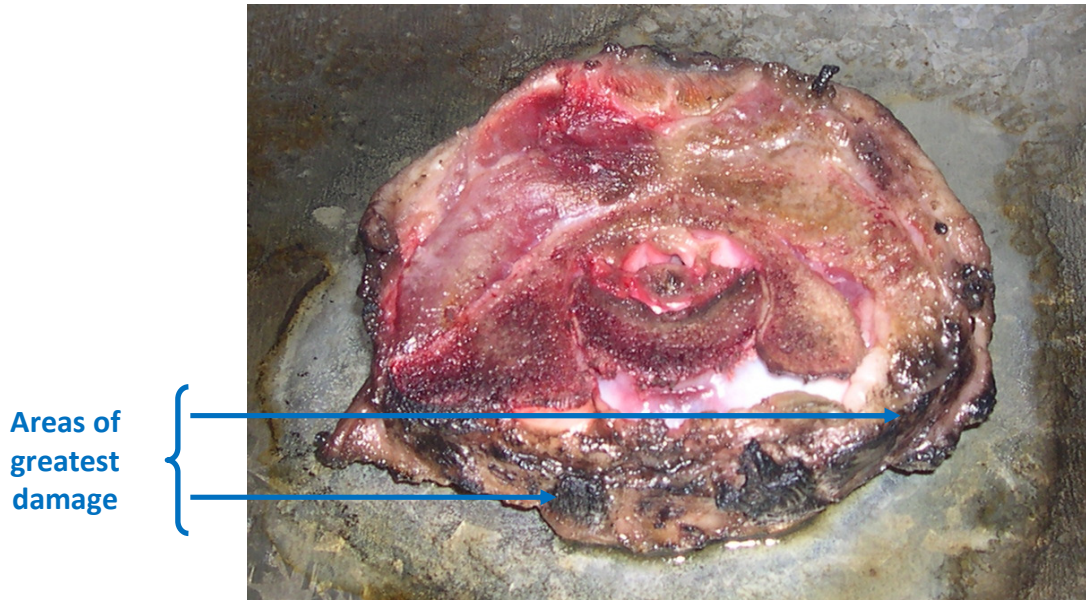


Figure 3.11: *The appearance of the food source post-burn using an ignitable liquid with the areas that experienced the greatest damage highlighted.*

3.5.3 Insect Samples

No differences were observed between the sample sets in terms of the larval feeding habits, the duration of the larval, pupal or adult stages, or the number of adults that emerged.

Previously (Section 3.2) it was stated that fly larvae prefer to feed in protected areas and thus are less vulnerable to the external environment. Although it is difficult to draw conclusions based on observations made from such small food sources, the fly larvae were noted to be burrowing within the meat and this may have provided them some protection from the surroundings.

3.5.4 Chemical Analysis

The results obtained from the samples reared on the meat burnt with petrol (see Table 3.2) indicate that 76.7% of larvae, 33.3% of adults and 33.3% of pupae contained traces of petrol. The results obtained from the samples reared on the meat burnt with kerosene indicate that 100% of larvae, 66.7% of adults and 66.7% of pupae contained traces of kerosene. It is important to note that all of the petrol and kerosene replicates that were recorded as negative for an ignitable liquid still contained peaks corresponding to components of the respective ignitable liquid, either petrol or kerosene. Since an insufficient number of components were detected, these samples were categorised as negative for an ignitable liquid.

It was of some concern that the ignitable liquid used to accelerate the burning of these samples was known to the experimenter, as this may have influenced the designation of positive or negative for an ignitable liquid, particularly when some of the peaks of interest were present. To counteract this bias, in order for the presence of an ignitable liquid to be reported, the majority of target compounds needed to be present at acceptable levels. In addition, it is recommended that any future experiments are conducted so that the interpretation is made without any knowledge of the sample's origin.

The results obtained from the meat burnt with petrol indicate that the profiles from 73.3% of larval samples still exhibited the distinctive petrol pattern. Given the volatile nature of petrol, these positive findings are particularly noteworthy. Meanwhile, kerosene persisted and was detected in *all* of the larval samples, probably because of its higher boiling point. Thus, even after five days of exposure, petrol and kerosene could both be identified in many of the larval samples.

Positive results for petrol and kerosene were also obtained from the adult and puparial samples. However, there were differences between the ignitable liquids here. Only one of the six adult samples analysed from the petrol sample set tested positive, while

four of the adult samples analysed from the kerosene sample set tested positive. Meanwhile, only one of the six puparia samples analysed from the petrol sample set tested positive, while four of the six puparia analysed from the kerosene sample set tested positive. It has been documented that post-feeding larvae expel all remaining food, begin to wander and finally pupate [16,23]. Given this fact, lower levels of ignitable liquids would be expected in the adult and puparial samples. However, it is unknown if the differences observed between the two ignitable liquids are merely due to their volatilities or arise from some other unknown factor. In addition, it is not known whether the mechanism of metamorphosis may affect the level of ignitable liquids present in the puparium and whether this may also contribute to the lower levels observed in the adult and puparium samples - further investigation is required.

It has been highlighted previously that both petrol and kerosene comprise volatile components that evaporate rapidly rather than remain in fire debris. With this in mind, the positive results obtained highlight the potential value in collecting and analysing entomological samples for casework applications, especially puparia. This will allow for potential analysis of fire debris long after the fire occurred. In addition, these findings demonstrate an additional use for SPME in the field of fire investigation that has not previously been examined.

The replicates analysed from the petrol and kerosene sample sets were compared and although all samples were prepared, stored and treated in the same way, the results obtained were not always comparable i.e. some were negative for an ignitable liquid and others were positive (see Table 3.2). This would suggest sample-to-sample variations exist between larvae. That is, competition may exist among a group of larvae resulting in certain larvae feeding more than others. Since a representative sample of six larvae was collected at random on a daily basis, there was no way to collect those that had fed to the same extent. Whilst studying the accumulation of amitriptyline, other authors have observed that when the concentrations in 45 separate larval rearings were recorded, considerable variations were apparent between larvae, even though they had been fed on the same food source with the

same concentration of amitriptyline. They attributed these findings to biological variability in the larvae themselves [135]. This variability may also explain the observed differences in these small-scale experiments.

Another factor which may contribute to this variation is the use of unwashed larvae. For the purposes of this experiment, the presence or absence of an ignitable liquid was considered significant, not its concentration (see Section 3.3.6). Hence, the washing of larvae was not considered necessary, as this would reduce the overall response due to the ignitable liquid. However, not washing the larvae has potentially added some variability to the results which cannot be controlled. These findings highlight the importance of collecting multiple larvae to incorporate as many replicates as possible, and thus improve precision.

The replicates from the control sample sets were compared and it was noted that on Day 1, a larval sample derived from each of the burnt and unburnt control groups and a puparium sample derived from the unburnt group all contained traces of kerosene. The remaining replicate samples all tested negative for any traces of the ignitable liquids tested. It is important to note that the larval control samples that tested positive for kerosene did so only on the first day of analysis. This would indicate that the entire population was not contaminated, but that a contamination event most likely occurred post collection. There are a number of factors that may have contributed to this result, including improperly cleaned tools, insufficient desorption from the fibre, minor traces of kerosene remaining in the metal cans, or carryover in the GC-MS. All the possibilities listed, except for the tools, were tested during the method development stage and therefore it is unlikely that they are the source of contamination; however, they cannot be ruled out altogether. Although these results raise potential issues with the experiment, it must be considered that a total of 24 meat samples were prepared and stored in a small area. Efforts were made to store the four treatment groups separately, but the confined laboratory environment could not be altered.

It is important to note that if this type of analysis were to be adopted for casework purposes, the experiment described here would not be performed. That is, the larval samples would be collected *in situ* and sent to a laboratory for testing in appropriately sealed sample containers, with only the extraction and analysis steps to be performed. Therefore, over a hundred samples would not be analysed as was the case in this experiment and hence there would not be such a strain on resources. As a result, to be extra cautious, blanks could be run in between sample injections in the GC-MS. The potential significance of this experiment should therefore not be overlooked because of these three samples that had been contaminated with traces of kerosene. However, it is recommended that, in an attempt to remove any source of contamination, a larger experiment that mirrors casework more closely be carried out in order to develop a protocol for the appropriate collection, handling and storage of this type of evidence.

Another issue that complicated the results obtained was that the control groups "naturally" contained some peaks corresponding to components of the ignitable liquids. The most prevalent of these were the aromatic compounds including 1,3,5-trimethylbenzene, 1,2,4-trimethylbenzene, 1,2,3-trimethylbenzene, 2,3-dihydro-1H-indene (indane) and eicosane. These compounds have also been reported in studies that examined the volatile organic components that evolve from decaying remains [150,151]. It is also possible that these volatile organic compounds were derived from the breakdown of the soft tissue in the meat samples [151].

Despite the presence of these compounds, a negative result for the ignitable liquids of interest could still be concluded as the profiles obtained for the control groups did not exhibit the same distinctive patterns as petrol or kerosene. However, this may be an important point to consider, especially if the experiment is continued for longer periods when these compounds may become more pronounced. These findings do stress the importance of complete correlation between reference and sample profiles, as noting merely a few common components as an indication of a positive result could lead to misidentification. They also reinforce the caution from the ASTM test method

that the C_3 alkyl benzene group (comprising 1-methyl-3-ethylbenzene, 1-methyl-4-ethylbenzene, 1,3,5-trimethylbenzene, 1-methyl-2-ethylbenzene and 1,2,4-trimethylbenzene) must be present in the same relative concentrations as observed in known samples. Therefore, this group of compounds must not be relied upon alone to designate a sample positive for petrol [81].

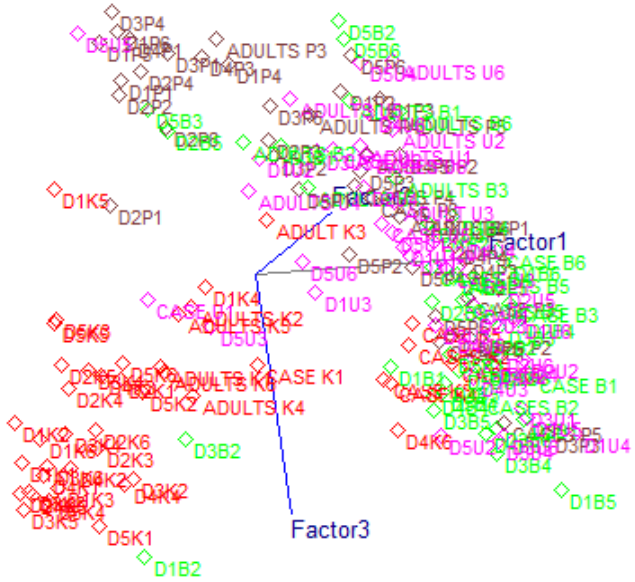
Additionally, the ASTM method states that common fire debris, that has been exposed to fire conditions, will contain these compounds as well as high levels of other non-related compounds that may indicate pyrolysis of the sample matrix [100]. This was noted in the burnt samples and to a lesser extent the unburnt samples. This issue will be discussed further in **Chapter 5**.

Results for Small-Scale Experiments							
Replicate	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Adults**	**Puparia**
Petrol 1	P	P	P	P	N	N	N
Petrol 2	P	P	P	P	N	N	N
Petrol 3	P	P	P	P	N	N	N
Petrol 4	P	P	P	P	N	N	N
Petrol 5	P	P	N	N	N	N	N
Petrol 6	P	P	P	P	P	P	P
Kerosene 1	K	K	K	K	K	K	K
Kerosene 2	K	K	K	K	K	N	N
Kerosene 3	K	K	K	K	K	K	K
Kerosene 4	K	K	K	K	K	K	K
Kerosene 5	K	K	K	K	K	N	N
Kerosene 6	K	K	K	K	K	K	K
Burnt 1	N	N	N	N	N	N	N
Burnt 2	N	N	N	N	N	N	N
Burnt 3	K	N	N	N	N	N	N
Burnt 4	N	N	N	N	N	N	N
Burnt 5	N	N	N	N	N	N	N
Burnt 6	N	N	N	N	N	N	N
Unburnt 1	N	N	N	N	N	N	K
Unburnt 2	K	N	N	N	N	N	N
Unburnt 3	N	N	N	N	N	N	N
Unburnt 4	N	N	N	N	N	N	N
Unburnt 5	N	N	N	N	N	N	N
Unburnt 6	N	N	N	N	N	N	N

Table 3.2: *Summary of the results obtained for all replicates for the duration of the small-scale experiments. Samples are indicated as positive for petrol (P) or kerosene (K) or negative for an ignitable liquid (N).*

3.5.5 Data Analysis

One of the challenges arising from examining multiple chromatograms is that the results are subject to interpretation by the operator. Performing PCA removes this bias as peak areas for all target compounds from each sample are used in the analysis to produce a scores plot. PCA is used in this experiment to highlight the differences among sample sets. By incorporating the control groups in this exploratory analysis, PCA identified the differences introduced by burning as well as the differences among the two ignitable liquids. As a result, negative or contaminated samples were easily identified as they did not cluster with their designated group in the scores plot (Figure 3.12). It is also clear from the spread of data in this plot that sample-to-sample variation exists, supporting the conclusions made from the chromatographic results.



◇ **Petrol sample**

◇ **Kerosene sample**

◇ **Burnt sample**

◇ **Unburnt sample**

Figure 3.12: *Scores plot generated for the small-scale experiments.*

The graphical representation (scores plot) of the first three principal components (representing the greatest variability) in the data demonstrates that the petrol, kerosene and control groups can be distinguished from each other (see Figure 3.12). It is not surprising that the control groups clustered together since they contained only a few, if any, target compounds that corresponded to the ignitable liquids of interest.

These control groups are fairly diffuse, and there is some overlap with the ignitable liquid groups (particularly petrol). This overlap can be attributed to results where an insufficient number of target compounds were detected from the petrol and kerosene samples (most likely due to evaporation) and thus the samples were categorised as negative. The samples derived from the control groups that were positive for kerosene are also included in this group. Since these 'false positives' can most likely be attributed to a contamination that would likely be absent for samples taken from the field, the overall results still suggest that the method used was sufficient for correctly identifying petrol and kerosene in the majority of samples using *Lucilia cuprina* larvae, adults and puparia. These results are particularly significant in that they highlight the potential value in collecting and analysing such samples for casework applications, especially puparia. This type of sample, unlike the larvae and adults, is not mobile, and does not readily degrade; hence it is postulated that the puparia would remain close to the remains for extended periods. In addition, these findings demonstrate an additional use for SPME in the field of fire investigation that has not previously been examined.

A one-way multivariate analysis of variance (MANOVA) was performed to further investigate the differences between the sample sets. The scores for the first seven principal components (PC1-PC7) obtained from the principal component analysis were used as the dependent variables, as these represented the greatest variability in the data. In fact, these components together explained 90% of the variability in the data. The independent variable, *sample set,* comprised four groups: petrol, kerosene, burnt and unburnt. (Preliminary assumption testing was conducted to check normality, linearity, univariate and multivariate outliers, homogeneity of variance-covariance

matrices, and multicollinearity, with no serious violations noted.) A statistically significant difference was found between the four sample sets on the combined dependent variables: $F_{21,480}$ =10.36, p<0.05, Pillai's Trace=0.936, partial eta squared=0.312.

When the results for the dependent variables (PC1-PC7) were considered separately, PC1, PC2 and PC3 were the only components to reach statistical significance (PC1: $F_{3,164}$ = 40.98, p<0.05, partial eta squared = 0.428; PC2: $F_{3,\ 164}$ = 16.17, p<0.05, partial eta squared = 0.228; PC3: $F_{3,164}$ = 17.51, p<0.05, partial eta squared = 0.243). This indicates that although seven principal components were chosen, only the first three were responsible for the differences between the sample sets.

Post-hoc analysis for PC1, PC2 and PC3 consisted of conducting pair-wise comparisons. This revealed that, based on PC1 (corresponding to petrol), PC2 (corresponding to kerosene) and PC3 (corresponding to petrol) the means of the petrol and kerosene groups demonstrated significant differences (plotted in the scores plot). This indicates that these principal components correspond to the ignitable liquids. By comparison, the burnt and unburnt groups were the only groups found to have the same means based on PC1, PC2 and PC3, which explains why these groups clustered together in the scores plot.

Based on PC2, the petrol and burnt groups were not found to be significantly different. This indicates that burning the meat samples, even without the use of an ignitable liquid, creates characteristics similar to the petrol group. This finding can be explained based on the presence of the volatile organic compounds (from decomposition) in the burnt (control) samples that are also present in the petrol samples. However, the petrol and burnt groups can still be separated based on PC1 and PC3. If we consider the chromatographic results, all the burnt samples (except for those classified as false positives) could be differentiated from the petrol samples.

Thus, statistical differences were noted between the petrol and kerosene sample sets using principal component analysis (PCA) and multivariate analysis of variance (MANOVA). This is important because it indicates that even though these ignitable liquids have several common components within their distinctive profiles, they can still be identified as separate sample sets. Conversely, the PCA results from the burnt and unburnt control groups are fairly diffuse and there is some overlap with the ignitable liquid groups. However, according to the MANOVA results, these control groups were considered statistically different from both the ignitable liquid groups. The diffuse nature of these groups in the PCA results may be attributed to two factors: (i) volatile organic compounds that are common to both the control and ignitable liquid groups; and (ii) some of these common compounds are present at low and/or inconsistent levels in the control samples.

3.6 Conclusion

The method designed and tested in these small-scale experiments were successful in detecting petrol and kerosene in *Lucilia cuprina* larvae for five days, and in adults and puparia approximately four weeks after the initial burn (period from initial exposure to when these samples were collected). This is the first experiment to have investigated the detection of ignitable liquids in entomological samples. These findings are significant as they have potential implications for casework. That is, they demonstrate that entomological evidence may be a good alternative to traditional fire debris analysis when burnt human remains are involved, particularly when they are recovered well after the fire. However, it is important that these findings are further tested in an experiment that mirrors a realistic scenario more closely before this method can be recommended for casework purposes. Therefore, further development of the experimental design underlying this technique is required.

Chapter 4:

Further Method Development

Chapter 4: Further Method Development

4.1 Overview

This chapter discusses a series of experiments designed to further optimise and ultimately increase the sensitivity of the sample preparation and analysis techniques described in **Chapter 3**. Achieving this would allow the proposed method to be tested in a setting that more closely mirrors a realistic casework scenario. Furthermore, method validation experiments were carried out in order to verify that the proposed method was indeed appropriate for its intended use.

4.2 Chemical Analysis

The GC-MS method utilised throughout the small-scale experiments provided a level of sensitivity that enabled ignitable liquids to be detected even in puparia and adult samples. However, this result was obtained from a highly controlled laboratory experiment. It was anticipated that fieldwork experiments would introduce numerous variables, many of which could not be controlled. In addition, the fieldwork experiments were anticipated to be of a longer duration than the small-scale experiments. Therefore the concentration of ignitable liquids ingested by arthropods was expected to be lower than in the small-scale experiments. Hence it was important that a highly sensitive analytical method was designed and validated that would detect low levels of the compounds of interest.

4.2.1 Acquisition Mode

The GC-MS method described in **Chapters 2 and 3** utilised a full scan acquisition mode, that is, the resultant Total Ion Chromatogram (TIC) contained all the masses in a

designated range of mass-to-charge ratios (i.e. 40.5 to 400 atomic mass units). The disadvantage of this acquisition mode is that because a full range of masses is scanned, the time taken for each scan is diminished and therefore the number of scans over a chromatographic peak is limited.

The sensitivity of this technique can be increased by reducing the number of ions scanned. This is achieved using a different acquisition mode, Selective Ion Monitoring (SIM). SIM scanning technique enables specific mass-to-charge ratios to be scanned, allowing the mass spectrometer to dwell longer on ions of interest (which provide the most information). This increases the sensitivity of the method (as the chromatograms produced contain fewer interfering peaks), it increases the signal-to-noise ratio and increases abundance (see Figure 4.1). Previous studies have also employed this technique for the purposes of obtaining higher sensitivity and to minimise the interference of pyrolysis products, as was the case in this project [99,116].

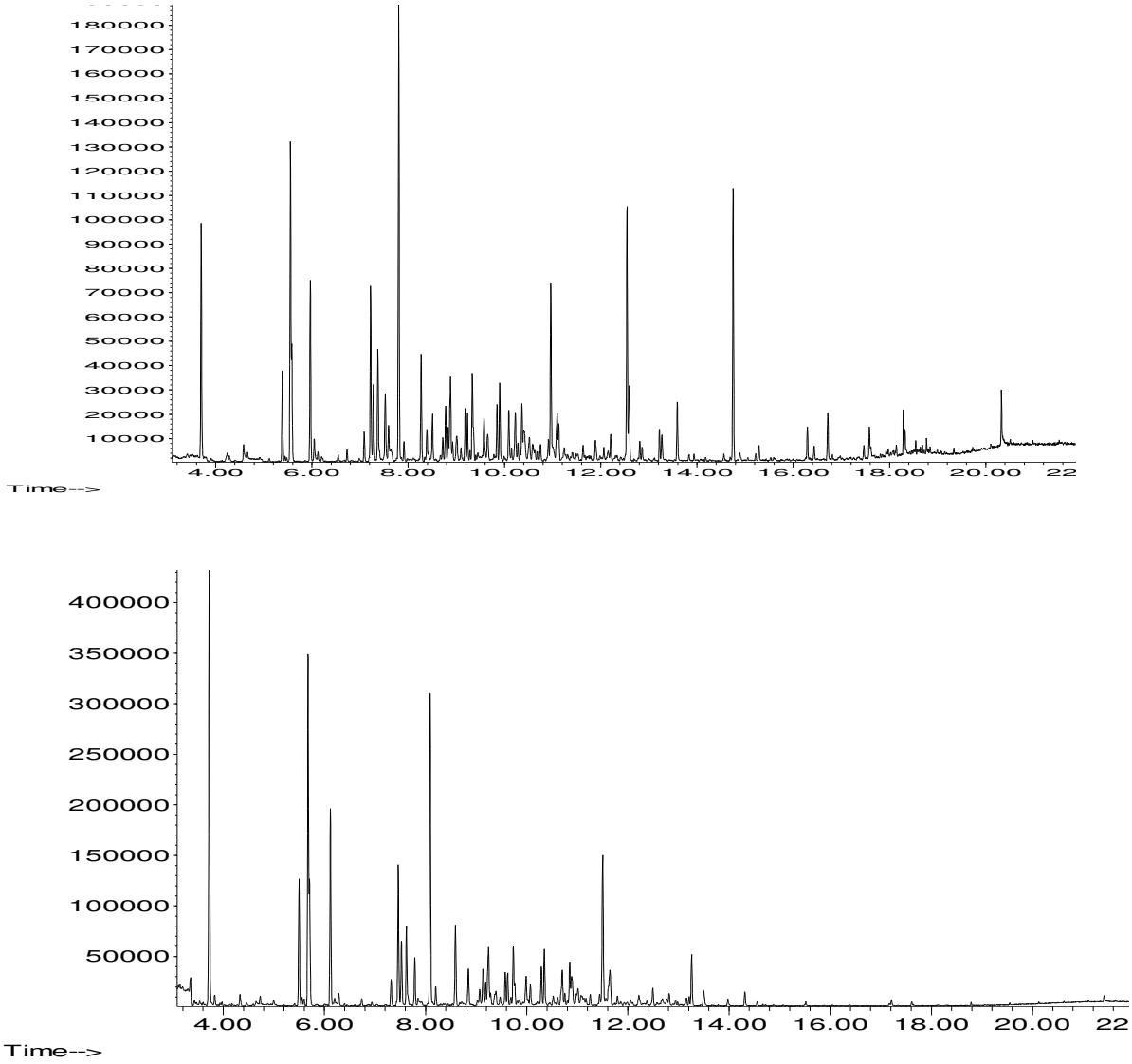


Figure 4.1: *The chromatograms obtained using the full scan mode (top) and the selective ion monitoring acquisition mode (bottom).*

In **Chapter 3**, the presence of petrol and kerosene residues was confirmed by monitoring the presence of a number of target compounds. Confirmation was achieved using this same method in these experiments. Therefore, the mass-to-charge ratios selected for the SIM method corresponded to the major ions produced by the target compounds (see Table 4.1).

Ions for Specific Classes of Compounds	
Class of compound	**Mass-to-charge ratio (m/z)**
Alkanes	43, 57, 71, 85, 99
Aromatics	91, 105, 119
Naphthalenes	128, 133, 142

Table 4.1: *The mass-to-charge ratios used for the selective ion monitoring acquisition mode representing the major ions produced from the target compounds selected.*

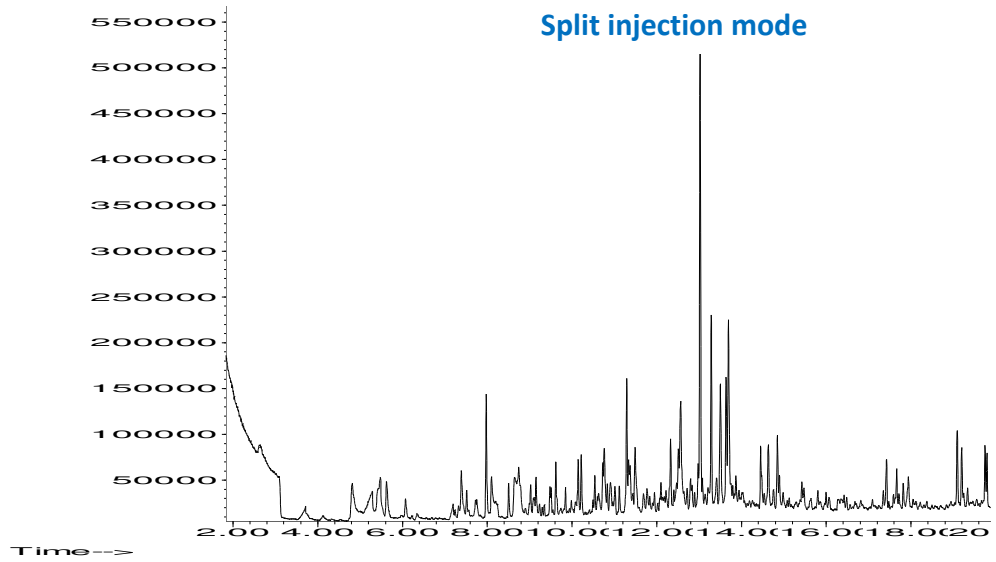
4.2.2 Injection Mode

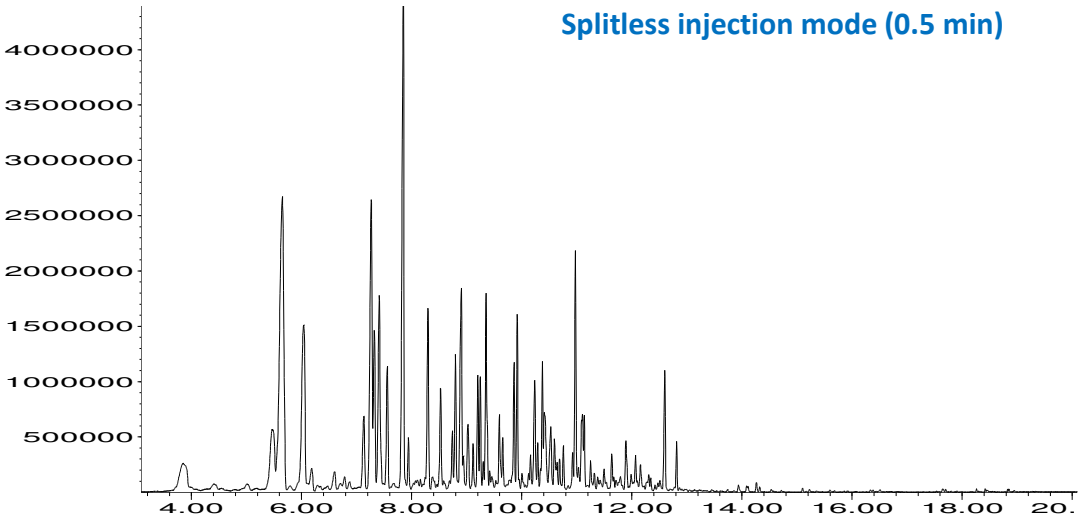
It is important when a sample is injected, vaporised and subsequently swept onto the column, that this is performed quickly. This will ensure that the sample is introduced in one narrow band, which in turn improves the column efficiency and resolution. To achieve this there are a number of injection modes available for use with capillary GC columns, including: split, splitless and pulsed splitless modes.

The split injection mode involves only a fraction of the sample entering the column, with the remainder vented as waste. The advantages of using such a system are that the sample will not overload the column, the sample band will be narrow and the injections using this mode often result in good chromatographic results. However, when a high level of sensitivity is required, this technique is not ideal as the majority of the sample is not introduced onto the column. In such a situation, the splitless injection mode is superior. This involves the whole sample being deposited onto the column. However, after a specified time, the split vent is opened and the injector is purged, and thus the system reverts to a split system. The splitless injection system results in higher responses and greater sensitivity, although this may be at the cost of chromatographic resolution. The third type of sample injection is the pulsed splitless mode. This involves a high initial carrier gas flow rate, which is lowered after a specified sampling period to the normal value determined for correct GC analysis. As a

result, the level of resolution offered matches that from the split mode whilst maintaining the level of sensitivity given by the splitless mode.

The three injection modes described above were tested using petrol standards. For the split mode, a split ratio of 50:1 was selected [95]. For the splitless and pulsed splitless modes, the split vent was opened to purge after thirty seconds. The chromatograms obtained (see Figure 4.2) were inspected to determine which system provided the best result.





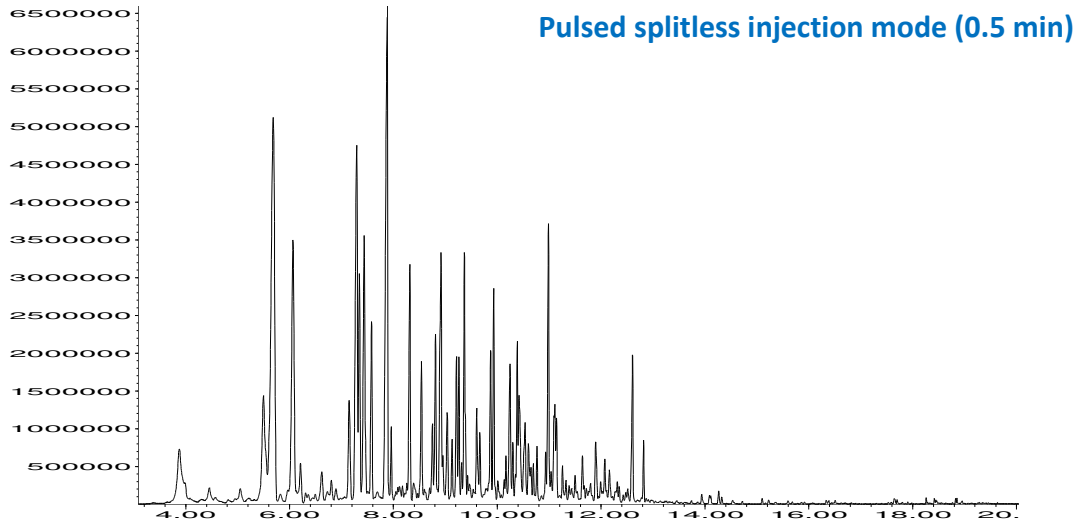


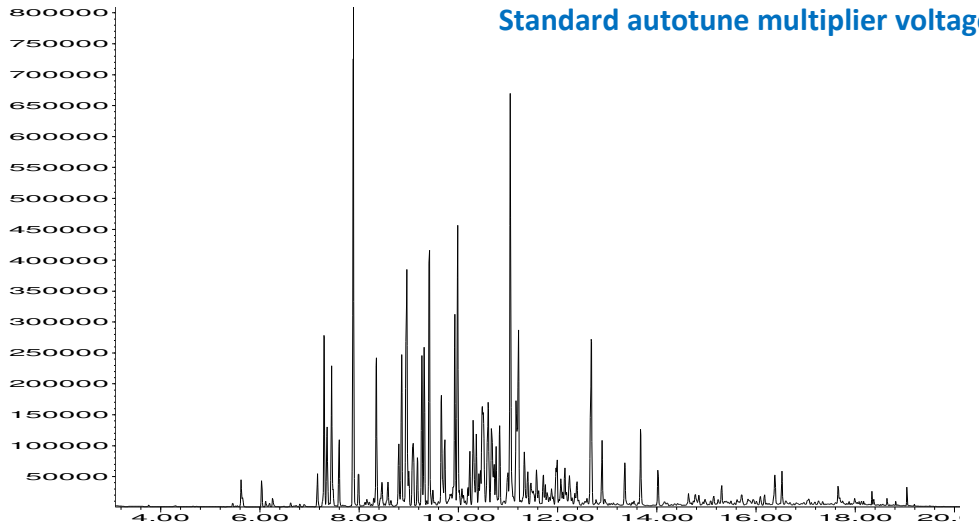
Figure 4.2: *The chromatograms obtained using the split, splitless and pulsed splitless injection modes.*

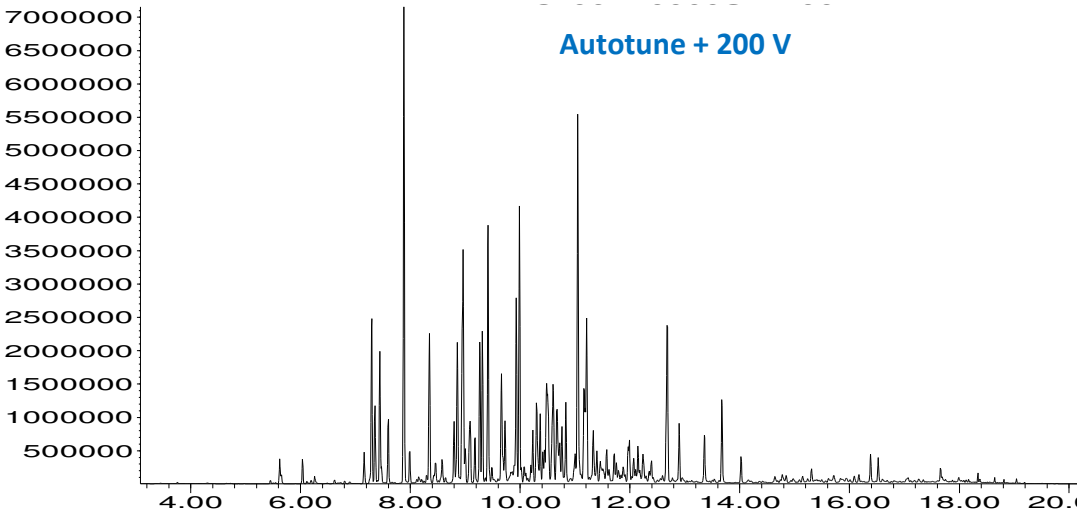
The chromatograms obtained were inspected and it was concluded that the split injection mode resulted in the lowest abundances; therefore, this mode was not adopted. Conversely, the pulsed splitless injection mode resulted in the greatest response compared with all the other modes that were trialled. The pulsed injection system allows for higher initial flow rates and transfers more of the sample to the column. It also allows for higher initial inlet pressures, which reduces the likelihood of highly volatile components escaping via the injection port. Higher inlet pressures also reduce the volume of the initial sample, which enables the entire volume to move onto the column [152].

4.2.3 Detector Sensitivity

A chromatogram was produced using the standard autotune multiplier voltage. This chromatogram was compared against petrol standards analysed using different multiplier voltages. This was performed to determine the effect that increasing the detector sensitivity would have on the chromatograms produced (see Figure 4.3). The chromatograms obtained indicate that the greatest abundance was achieved when the

multiplier voltage was increased by 400 volts, and therefore this value was adopted for this parameter.





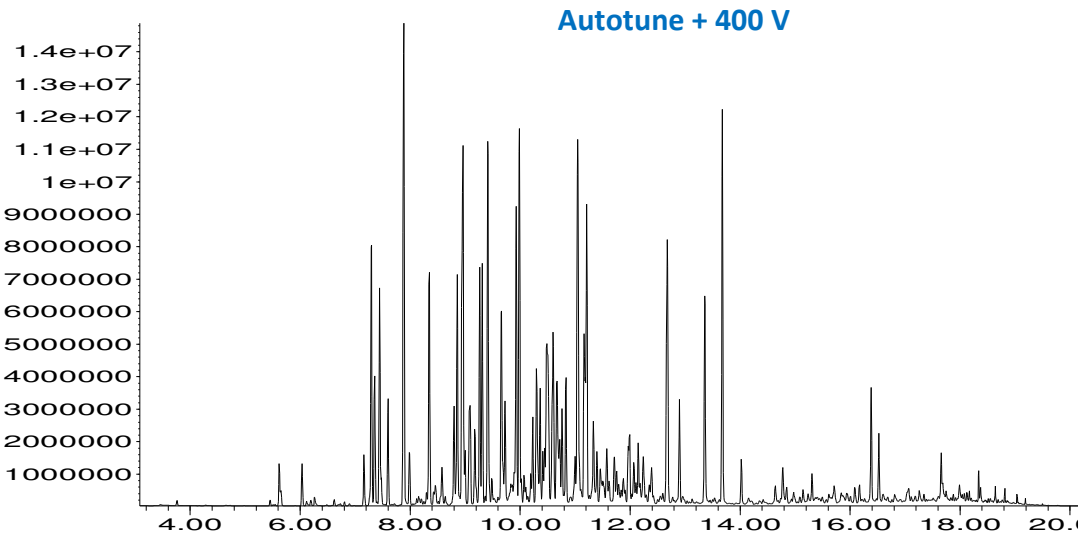
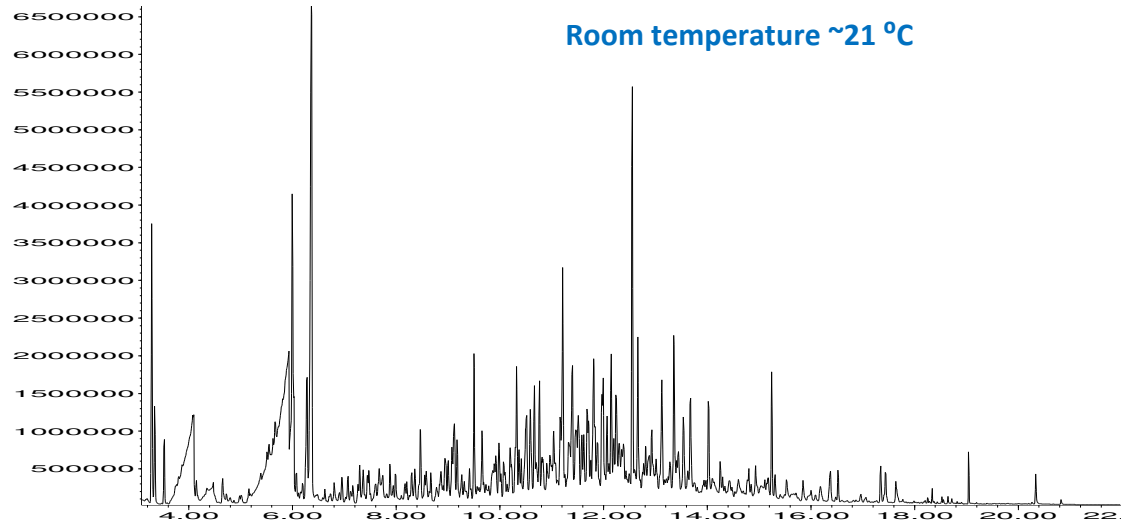


Figure 4.3: *The chromatograms obtained from applying the tune multiplier voltage and increasing this voltage by 200 and 400 V.*

4.3 Sample Preparation Technique

4.3.1 Storage

An experiment was designed to investigate a number of storage conditions. This was achieved by placing six third-instar larvae (selected for ease of comparison as generally this growth stage contained the highest abundance of the ignitable liquids) of *Lucilia cuprina* in scintillation vials and storing them under varied conditions in the laboratory for a total of five days. These included ambient temperatures of approximately 21 ^{o}C, in a refrigerator at approximately 3 ^{o}C and in a freezer at approximately –16 ^{o}C. These larval samples were collected and analysed using SPME-GC-MS. The resulting chromatograms were assessed to determine which storage condition provided the greatest response (see Figure 4.4).


Room temperature ~21 °C
6500000
6000000
5500000
5000000
4500000
4000000
3500000
3000000
2500000
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10.00
12.00
14.00
16.00
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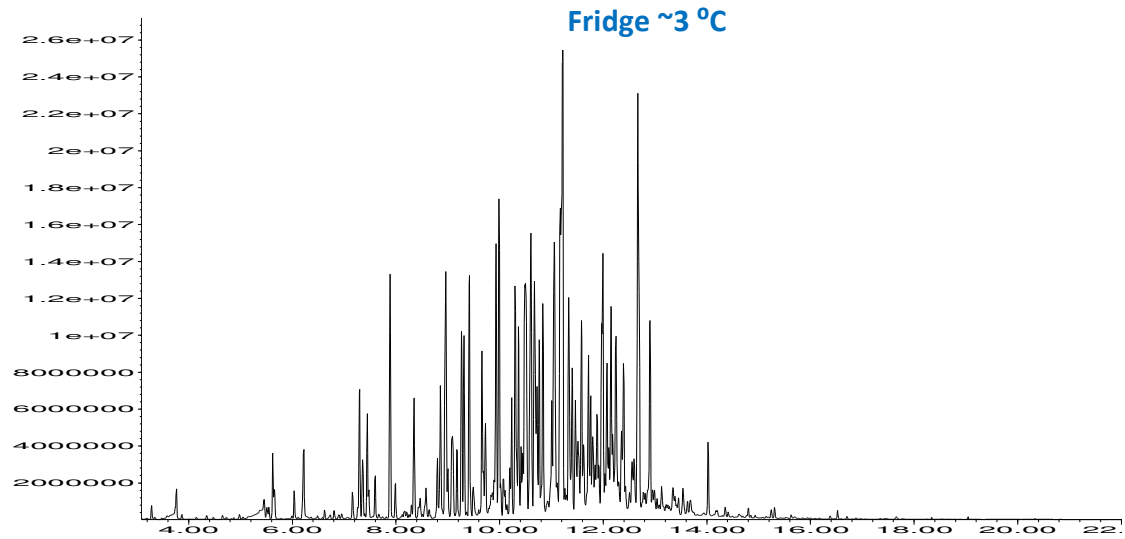
Fridge ~3 °C
2.6e+07
2.4e+07
2.2e+07
2e+07
1.8e+07
1.6e+07
1.4e+07
1.2e+07
1e+07
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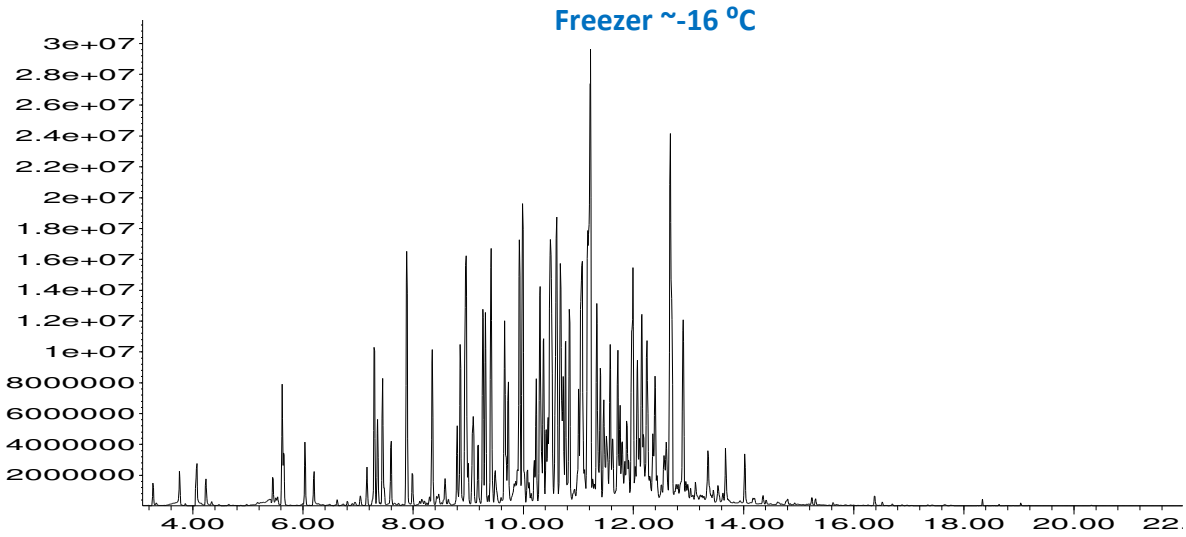


Figure 4.4: *Chromatograms obtained from petrol standards that were stored at room temperature, in the fridge and in the freezer for five days and then analysed using SPME-GC-MS.*

The chromatograms obtained from the storage experiment were examined and it was found that the sample stored at room temperature gave the lowest response, whereas the sample that was stored in the freezer gave the highest. Given these results, the freezer offered the optimum storage conditions maximising the chances of detecting the components of the ignitable liquids of interest. However, this meant that for the SPME step, it was important that sufficient time be given for the headspace of the container to reach a temperature of 90 °C. A failure to do so would affect the extraction efficiency, as the optimal extraction temperature found during method development experiments was 90 °C.

4.3.2 Sampling Containers

During the method development stage, a number of different-sized sampling containers were tested. It is important for the SPME extraction technique employed that the headspace volume be consistent, as this affects the recovery and thus the reproducibility of the extracted volatiles [19,124,127]. In particular, for this sampling

technique, the headspace volume selected should be as small as the sample allows, as smaller headspaces volumes result in higher sensitivities [123].

The sampling container used throughout the small-scale experiment was a 1 L metal can. A number of smaller metal cans were subsequently tested; namely, 500 mL, 250 mL and 100 mL. In addition, a new type of container was trialled. This container was a gas-tight, screw-top glass vial with a 20 mL volume, and was fitted with a PTFE/silicone septum (see Figure 4.5). The septum, which is designed specifically for headspace extraction, was easy to pierce with the SPME fibre, and therefore adhesive tape was no longer required. As an extra precaution, the lid of the SPME vial was also wrapped in paraffin film. These sampling containers were tested using petrol and kerosene standards and the resulting chromatograms were assessed to determine which resulted in the greatest response (abundance) (see Figure 4.6 and 4.7). The exception to this was the 100 mL sampling container. This container could not be incorporated into this experiment as it resulted in fibre breakage due to the shallow height of the container.

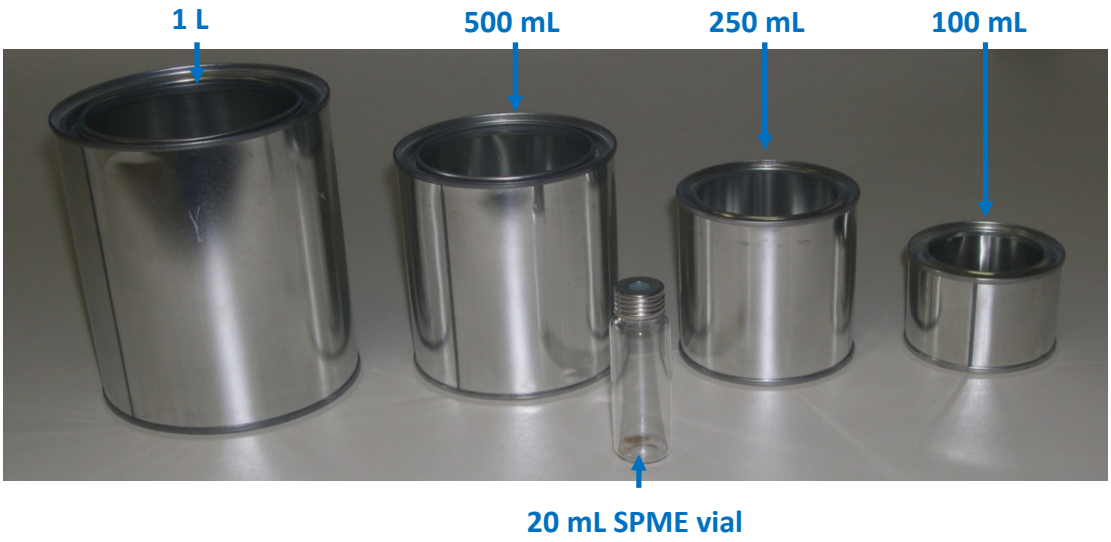
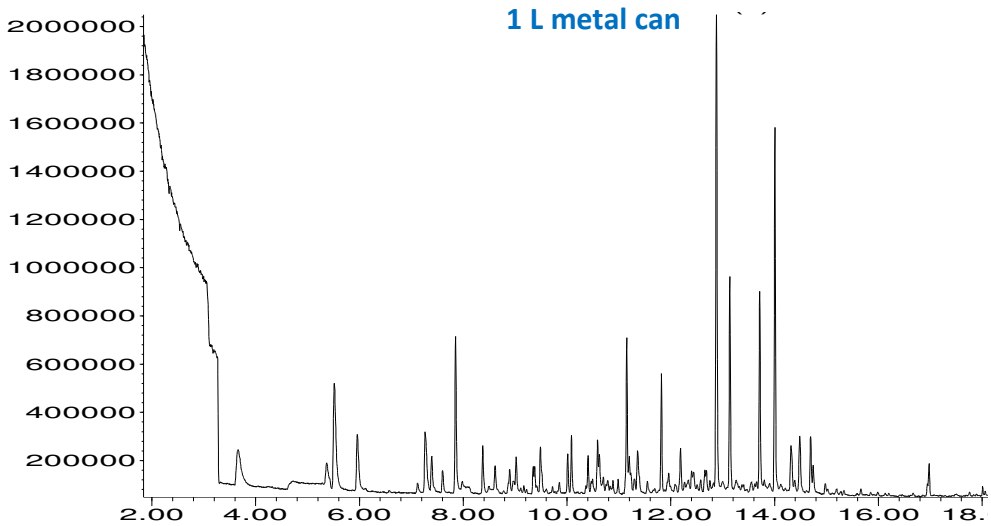
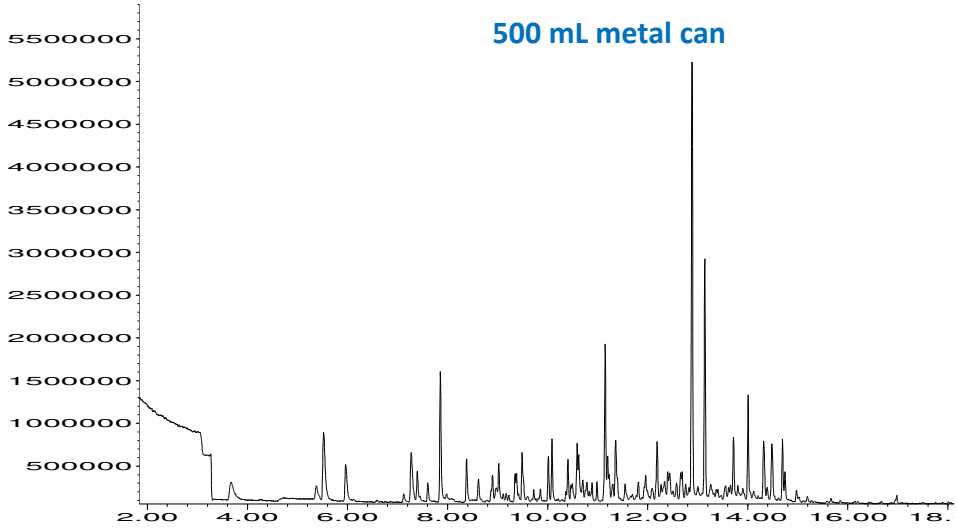


Figure 4.5: *The 1L, 500 mL, 250 mL and 100 mL metal cans and 20 mL SPME glass vial that were selected for headspace sampling.*




500 mL metal can
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8.00
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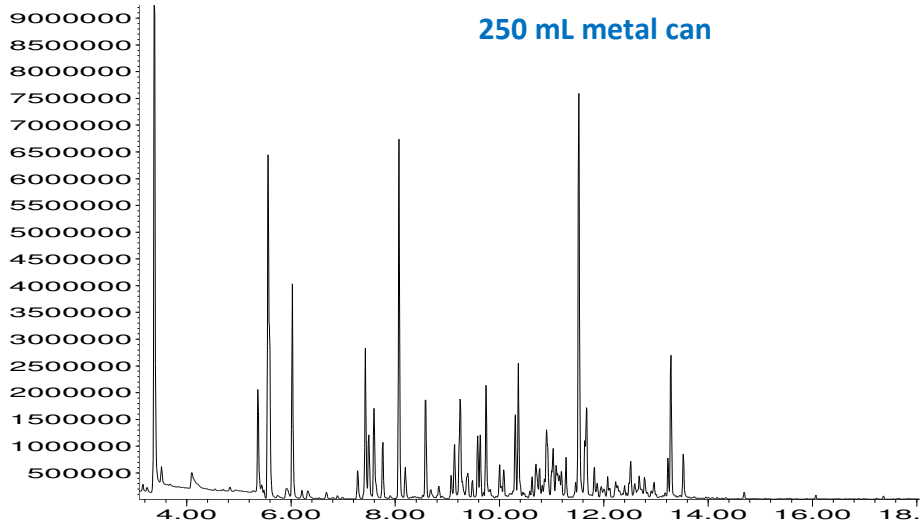
18.


250 mL metal can
9000000
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8000000
7500000
7000000
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6000000
5500000
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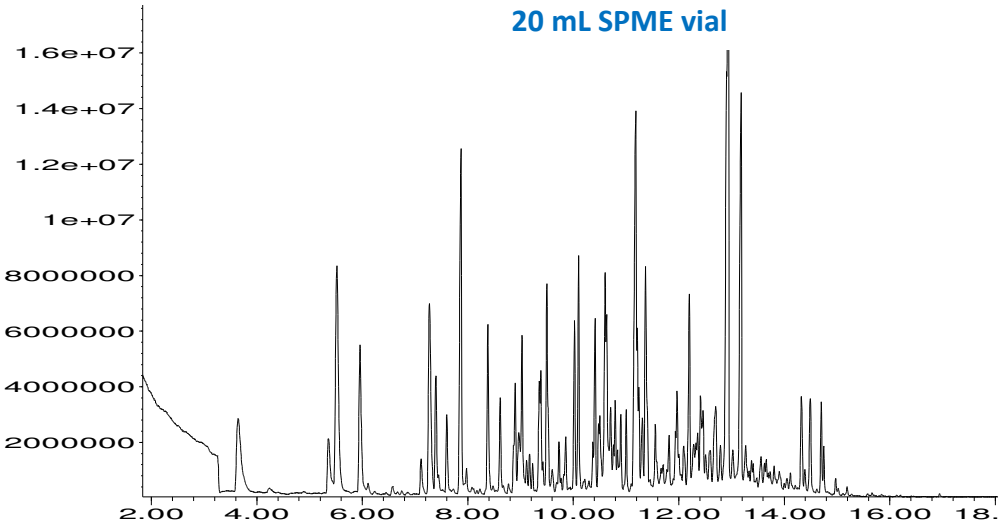
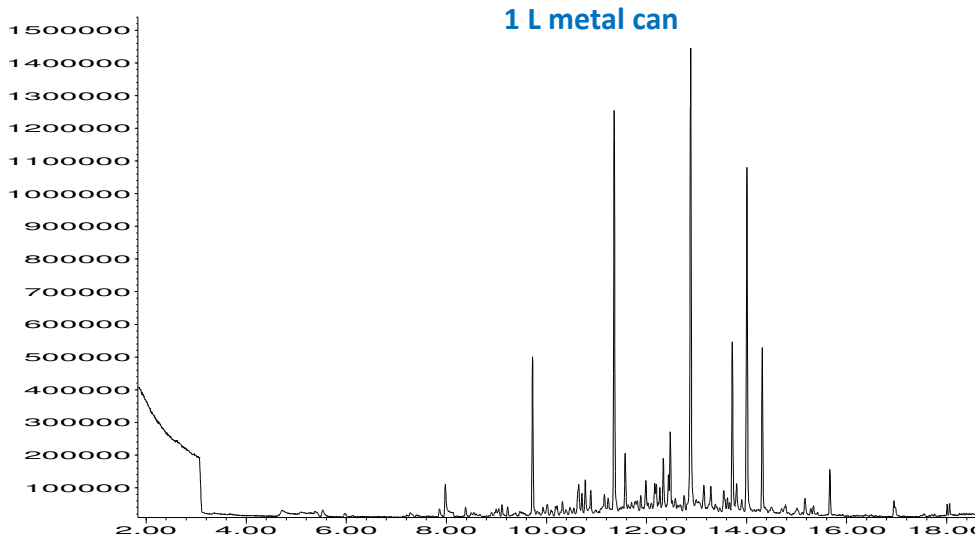
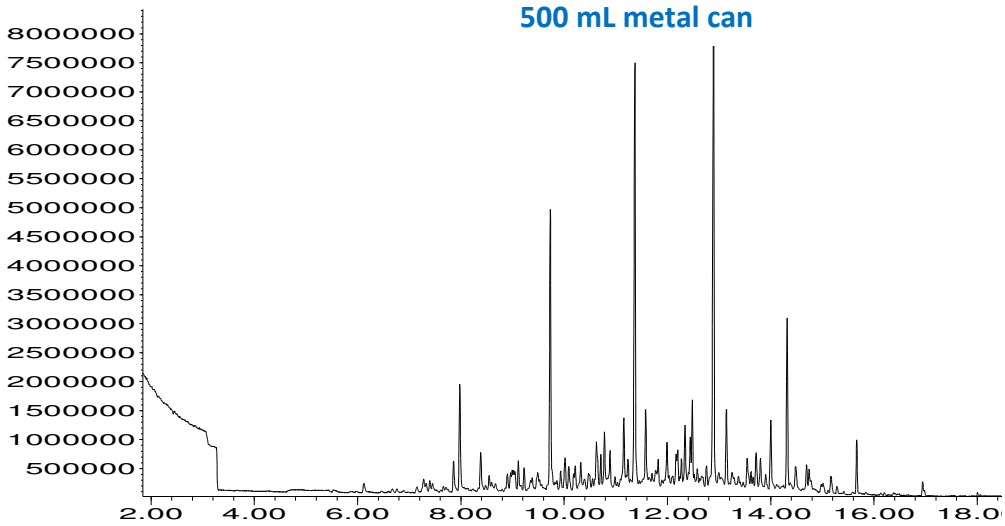


Figure 4.6: *Chromatograms obtained for petrol standards using a variety of headspace sampling containers.*




500 mL metal can
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7500000
7000000
6500000
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4500000
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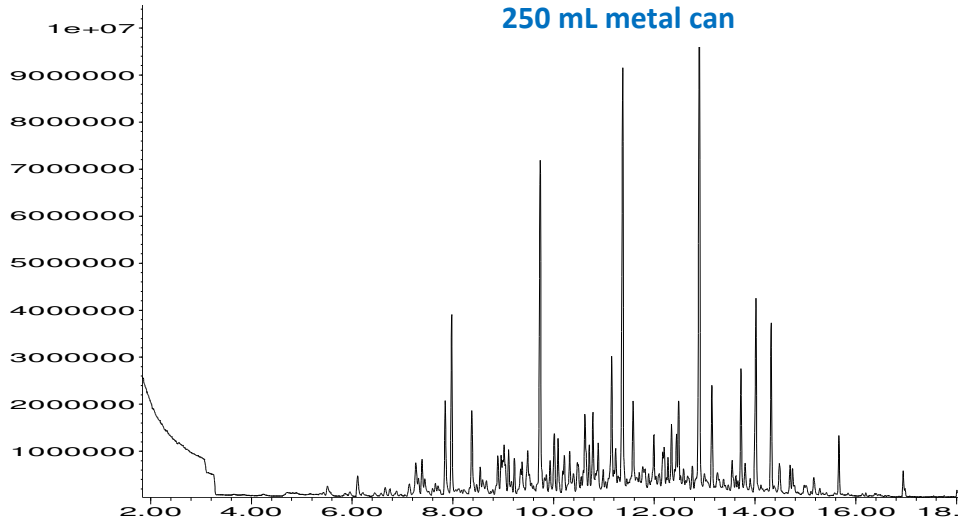
14.00
16.00
18.0


250 mL metal can
1e+07
9000000
8000000
7000000
6000000
5000000
4000000
3000000
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6.00
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18.

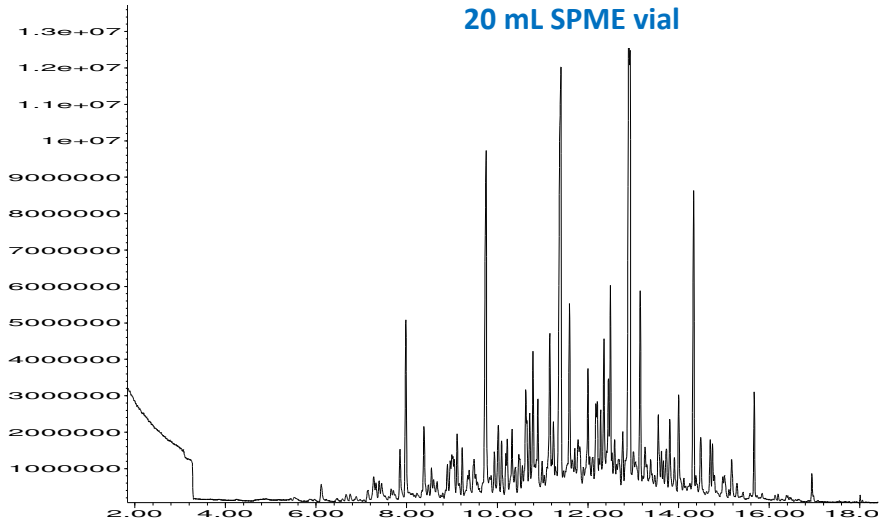


Figure 4.7: *Chromatograms obtained for kerosene standards using a variety of headspace sampling containers.*

As expected, the chromatogram with the lowest response was associated with the 1 L metal can; that is, the container with the greatest volume, and therefore the lowest analyte pressure for a fixed quantity of analyte. Conversely, the SPME vial (which represented the smallest sampling container) produced the chromatogram with the greatest response. The general trend observed in these results was that the response increased with a decrease in the headspace volume. This finding is supported by the manufacturer's recommendations to use as small a volume as practicable, as this results in higher sensitivities [123]. Therefore the 20 mL SPME vial was adopted.

4.4 Method Validation

A series of method validation experiments were carried out in order to ensure that the proposed analytical method was acceptable for its intended use. Due to the qualitative nature of this study, full method validation was not required. Relevant validation parameters were specificity, precision, robustness and limit of detection.

4.4.1 Specificity

The purpose of demonstrating specificity in a method is to illustrate that it has the ability to measure the analyte response in the presence of other sample components. This aspect of the method validation was tested by analysing standards of diesel and mineral turpentine. Diesel and mineral turpentine contain some of the same target compounds as petrol and kerosene. Thus the method needed to be able to differentiate these ignitable liquids from those of interest.

Diesel is a heavy petroleum distillate which contains an *n*-alkane distribution much like kerosene, but in the range of C_2 to C_{20+}. A 0.1 µL standard of diesel was run using the SPME-GC-MS method presented (see Figure 4.8). This ignitable liquid was found to contain all of the target compounds (albeit highly saturated) except for the naphthalene compounds and 4,7-dimethylindane. However, diesel was differentiated from the target ignitable liquids due to the additional compounds present and the distinctive pattern it produced.

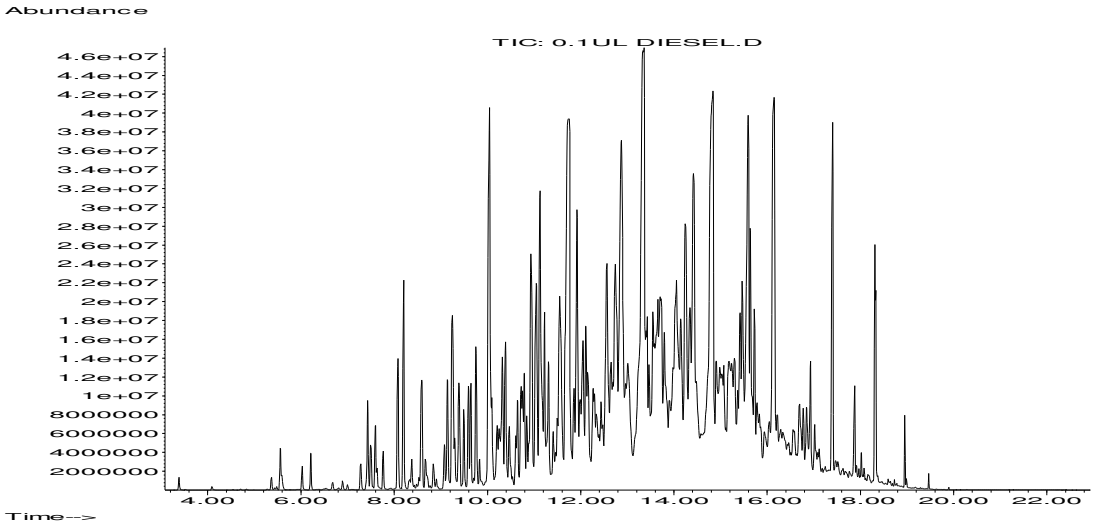


Figure 4.8: *The chromatogram obtained for a highly saturated sample of diesel using the SPME-GC-MS method presented in the small-scale experiments.*

Mineral turpentine is a medium petroleum distillate which contains an *n*-alkane distribution in the range of C_9 to C_{12}. A 1 µL standard of mineral turpentine (albeit highly saturated) was also analysed in the same way (see Figure 4.9). Many of the target compounds were also detected, including the alkanes and the C_3 and C_4 alkyl benzenes. Irrespective of this, the method could successfully differentiate this ignitable liquid from the ignitable liquids of interest based on the target compounds that were detected and the pattern produced. Therefore, these findings indicate that the proposed method has the ability to distinguish between similar ignitable liquids.

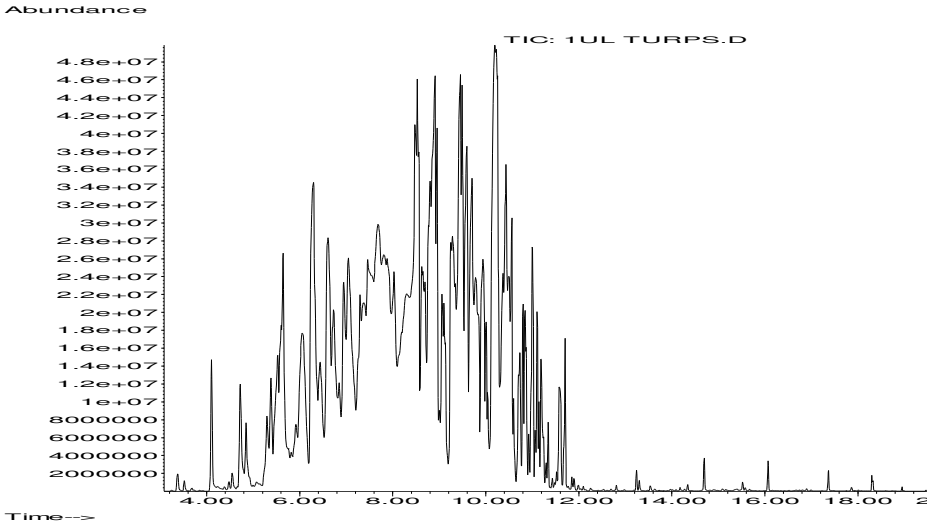


Figure 4.9: *The chromatogram obtained for a highly saturated sample of mineral turpentine using the SPME-GC-MS method presented in the small-scale experiments.*

In order to demonstrate specificity in the context of the experiment described in this thesis, a third-instar maggot was analysed that had been reared on meat that did not contain any ignitable liquids (see Figure 4.10). The prominent peaks that were detected did not correspond to any of the target compounds.

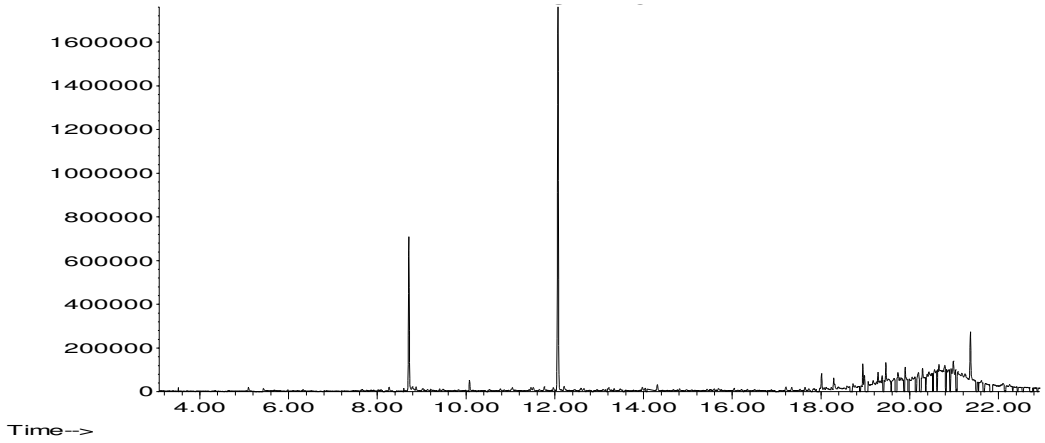


Figure 4.10: *Chromatogram obtained from a larva (single maggot) that had been reared on unburnt meat.*

4.4.2 Precision

The precision is a measure of the agreement between multiple analyses of a sample without any knowledge of what the true value should be. This was tested using instrument precision and repeatability studies. The instrument precision was tested using a 10 µL standard of petrol which was injected 10 times, and the ratio of 1,2,4-trimethylbenzene to 1,2,3-trimethylbenzene was calculated from each sample and compared (see Table 4.2). The relative standard deviation was then calculated for these ratios to determine the closeness of the values. The repeatability studies were carried out by analysing five 0.1 µL and five 10 µL standards of petrol using the SPME-GC-MS method and by calculating the same ratios, as was done previously (see Table 4.2). Only a single ignitable liquid was selected for this study as it was not important which one was being tested, but rather how well the instrumentation and methods performed.

Results from Precision Experiments			
Ratio obtained for instrument precision		**Ratio obtained from 0.1 µL standard**	**Ratio obtained from 1 µL standard**
0.300901		0.212491	0.280655
0.303793		0.207666	0.286792
0.302398		0.199794	0.281342
0.289508		0.208795	0.276153
0.309935		0.208653	0.29283
0.302497			
0.299676			
0.297699			
0.296338			
0.294820			
% RSD	1.86414	2.252021	2.262323

Table 4.2: *Ratios of 1,2,4-trimethylbenzene:1,2,3-trimethylbenzene found for a petrol standard computed through multiple direct injections using an autosampler (Column 1) and the SPME-GC-MS method (Columns 2 and 3).*

The values obtained for the relative standard error in the precision experiments were less than 1.9 % for the instrument precision and less than 2.3 % for the repeatability study. These values indicate that the method has the ability to measure a value several times over and produce values that have an acceptable degree of closeness. Therefore, these results have shown that the method exhibits adequate precision.

Over the course of method development, preliminary testing and validation, intermediate precision was demonstrated. No changes to results or anomalies were observed over different months or different columns. Therefore intermediate precision was inadvertently demonstrated.

4.4.3 Robustness

Experiments involving slight variations to oven temperature, temperature ramp, flow rate, pressure, injection system and scanning mode were performed. Changes in sensitivity were observed, but the compounds of interest were still detected. Given these findings, the GC-MS portion of the method was deemed to exhibit acceptable robustness.

Similarly, with respect to the sample preparation technique, slight variations to the extraction conditions were made, wherein non-optimal oven temperatures and exposure times were used, and extractions were also carried out using a different fibre coating to the one that had been utilised thus far (see Figures 4.11 and 4.12).

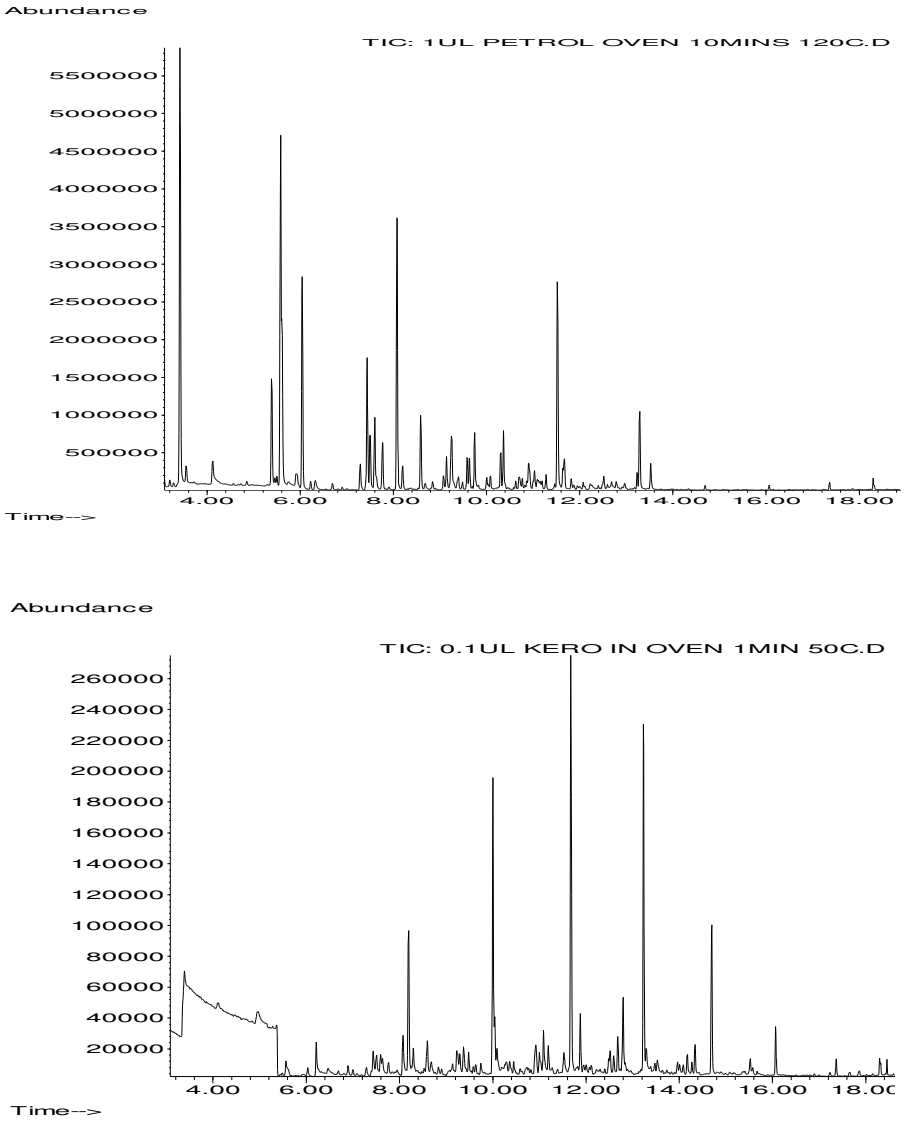


Figure 4.11: *The chromatograms obtained from standards of petrol (top) and kerosene (bottom) that were extracted under less than optimum conditions.*

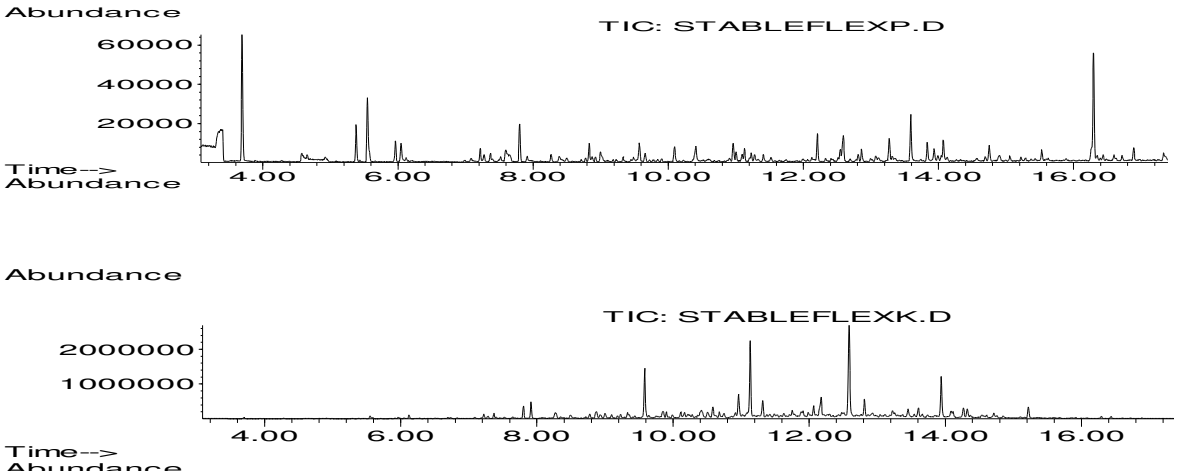


Figure 4.12: *The chromatograms obtained from standards of petrol (top) and kerosene (bottom) extracted using a different fibre coating (stable flex) to the one that had been used thus far (PDMS-DVB).*

The robustness of the method was tested further by exposing samples of petrol and kerosene to less than ideal conditions. This involved placing the uncovered standards in a fume hood (with an average wind speed of 0.92 m s^{-1}) for three hours and then analysing them to determine if the target compounds could still be detected (see Figure 4.13).

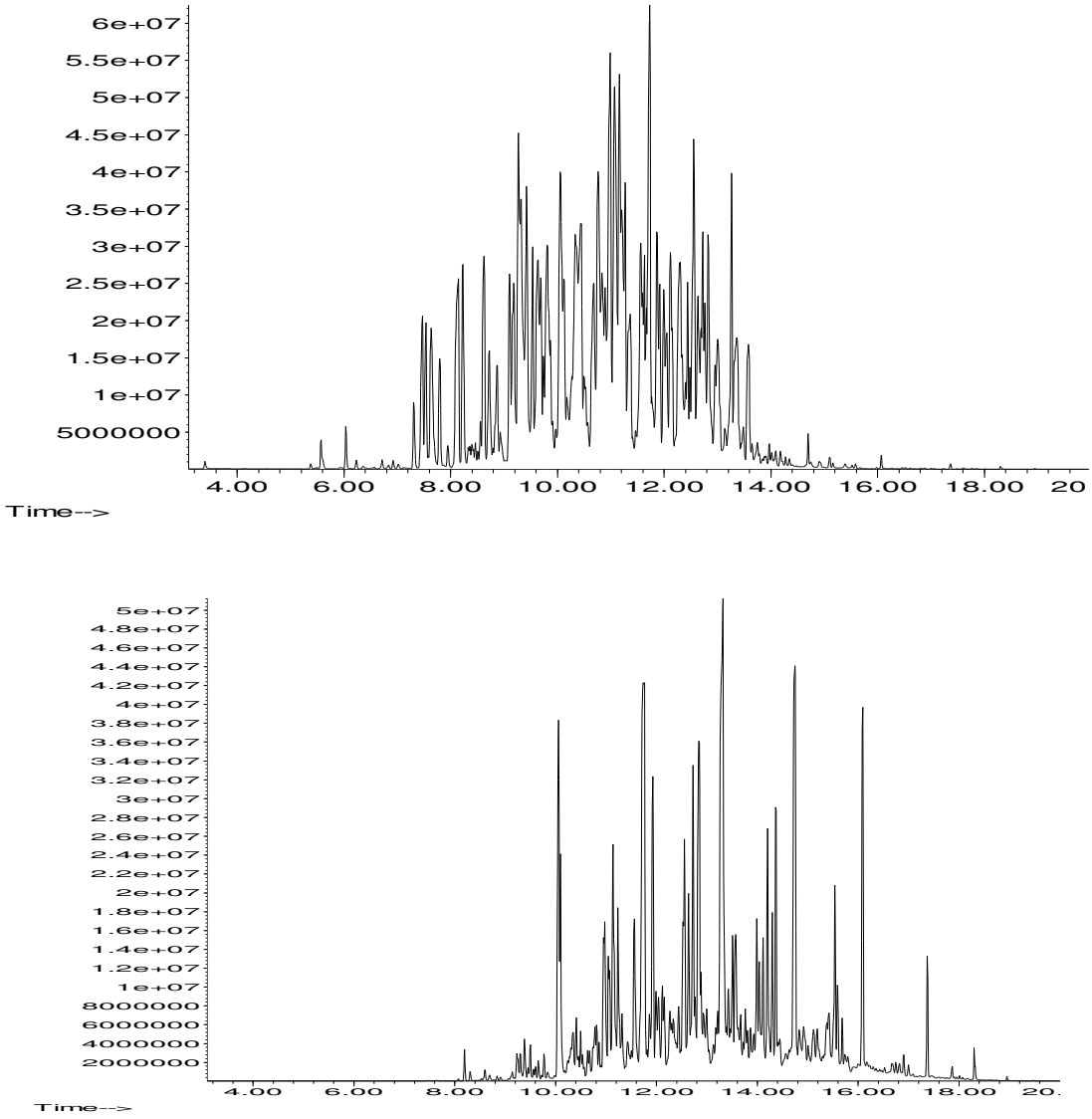


Figure 4.13: *The chromatograms obtained for petrol (top) and kerosene (bottom) that were stored uncovered in a fume hood for three hours.*

Although the chromatograms obtained from the samples that were stored uncovered in the fume hood for three hours did not exhibit optimum chromatographic resolution, and showed elevated baselines, all the target compounds were still present. This demonstrated that even when samples are stored under less than ideal conditions the method is still able to detect the analytes of interest.

The results obtained from each of the experiments designed to test the robustness of the method were successful in detecting the target compounds, and in each case, despite the changes in extraction parameters, the petrol and kerosene could still be identified. These results indicate that the method is unaffected by small deliberate changes and is therefore sufficiently robust. However, care must be taken to retain the level of sensitivity to reduce the frequency of false negatives.

4.4.4 Limit of Detection

The limit of detection is a measure of the lowest analyte concentration that produces a response that is three times greater than the noise level of the system. This aspect of the method validation was tested by preparing and running a series of diluted standards of petrol and kerosene to determine the lowest concentration at which the limit of detection is attained for these ignitable liquids. The analyte that was targeted in each ignitable liquid to perform this calculation was 1-methyl-2-ethylbenzene for petrol, and decane for kerosene (see Table 4.3).

Results from Limit of Detection Experiments	
Concentration (µL)	**Signal-to-nose ratio**
Petrol	
1	5:1
0.001	3:1
Kerosene	
0.01	16:1
0.00001	3:1

Table 4.3: *The signal-to-noise ratios obtained for 1-methyl-2-ethylbenzene (petrol) and decane (kerosene) in diluted samples of the ignitable liquids of interest.*

The limit of detection (signal-to-noise ratio of 3:1) was achieved using a 0.001 µL sample of petrol (made up with pentane) and a 0.00001 µL sample of kerosene (made up with pentane). Therefore, samples of the ignitable liquids of interest below these concentrations cannot be relied upon and thus should not be reported.

Obviously, the intended use of the method that has been discussed throughout the validation process is for the analysis of entomological samples, not neat samples of petrol and kerosene as have been tested in these experiments. Therefore, when interpreting a chromatogram, in addition to inspecting the pattern produced by an unknown sample and the presence of various target compounds, the signal-to-noise ratio should also be inspected to ensure that the sample is above the limit of detection and can be reported.

4.5 Conclusion

This chapter described a series of experiments that were designed to present and validate a new method that achieved greater sensitivity than the method presented in **Chapter 3**. As a result, a method was designed that utilised a SIM method to limit the number of ions scanned using a pulsed splitless injection system. The storage conditions for the samples collected were also considered and the most ideal were found to be placing the entomological samples in SPME vials and storing them in a freezer. Validation experiments were also performed on the proposed method. This method was found to demonstrate an acceptable level of specificity, as it could differentiate between different ignitable liquids that contained the same target compounds as petrol and kerosene. Further to this, results obtained from instrument precision, repeatability experiments and intermediate precision resulted in minimal variation between analyses, therefore demonstrating an acceptable level of precision. Moreover, the robustness of the method was successfully demonstrated when the method was unaffected by small, but deliberate changes to the chemical analysis and sample preparation techniques. Finally, experiments investigating the limit of

detection of the method indicated that this was achieved for a 0.001 µL sample of petrol and a 0.00001 µL sample of kerosene. Given that the proposed and validated method had demonstrated a higher degree of sensitivity than the method used for the small-scale experiments, it was concluded to be appropriate for future fieldwork experiments.

Chapter 5:

Fieldwork Experiments

Chapter 5: Fieldwork Experiments

5.1 Overview

This chapter describes the experimental design for fieldwork experiments (two experiments run in duplicate) that more closely mirrors casework scenarios, together with a protocol for the appropriate method to collect and package the specialised evidence involved. The purpose of extending this study to encompass a larger fieldwork design was to determine whether the method has the ability to withstand the greatly increased variability presented by the external environment. This demonstration would be important if the method were to be adopted for casework purposes.

5.2 Introduction

It has been suggested that, in order to improve PMI calculations, alternative methods that complement existing techniques are required [145]. Although this project does not involve PMI calculations, *per se* the same approach is used: to design an alternative method to detect ignitable liquids, which complements existing techniques. The technique presented involves utilising entomological samples in a new way in order to detect ignitable liquids. This new type of sample offers many advantages. First, since there are often a multitude of entomological samples available at scenes involving decomposing remains, there would be many samples available to collect; second, the sampling period may be extended considerably; and third, methods for the analysis of other fire debris could be used.

The small-scale experiments discussed in **Chapter 3** tested the hypothesis in a controlled laboratory setting, providing a valuable preliminary assessment of this novel technique. However, since it was the first time such a study has been carried out in

Australia, and indeed the world, fieldwork experiments were necessary in order to mimic situations in which forensic entomology is utilised [18], and to determine if the technique presented in this chapter could withstand the changing variables that the external environment presents. According to a review of forensic entomology cases in New South Wales between 1984 and 2001, 51% of bodies were located outdoors [142]. Although burnt bodies are not routinely located in the external environment, such scenarios do occur [14]. Therefore it would be beneficial in such circumstances to refer to a method that had been extensively tested. Such a scenario occurred in 2003 in Taiwan, where a homicide involving post mortem burning occurred [15]. In this situation, baseline data from a research project that investigated the PMI on a burnt victim was applied to a case to assist with the determination of the minimum time since death. This demonstrated that this type of experimental data would be potentially useful for casework purposes.

Ideally, decomposition studies carried out for the purposes of creating baseline data should involve human remains [153]. This is not a viable option in Australia, as strict legislation dictates how the body of a deceased person should be handled for research purposes [154]. This prevents the collection of data relating to Australian decomposition and insect succession studies on human remains. The studies reported in Australia often centre around casework. Conversely, in America, studies involving human remains are not infrequent, particularly at research facilities such as the Forensic Anthropology Research Centre at the University of Tennessee, Knoxville. This facility has conducted studies on over 400 human corpses. However, difficulties are still encountered obtaining large numbers of suitable remains and an appropriately sized area in which to conduct the research [31]. Therefore, suitable alternatives to human remains are required.

Several animal models have been utilised in decomposition studies. A particularly notable study involved preliminary experiments that compared and in turn selected the most suitable carrion to act as substitutes for human remains. This experiment investigated the decomposition of frogs, toads, mice, rats, chipmunks, dogs, cats,

squirrels, rabbits, chickens, birds and pigs [21]. The findings from this study highlighted the need for carrion to be uniform and of a relatively large size. It was also noted that there was difficulty collecting and observing insects in feathers. Subsequent studies have involved animals as large as elephants [155] but such an animal was too large to provide adequate baseline data that could be applied to humans. Based on these findings, the carrion that fulfilled the requirements of size and ease of collection was the baby pig, *Sus scrofa*.

It has been proposed that pigs are closely related to humans as they share similarities in terms of their internal features [37], fat distribution and omnivorous diet [3] and therefore could be used as human models. However, the validity of using non-human remains for the purposes of extrapolating findings for cases involving human remains has been challenged in the legal system [3,10]. Although this assumption has been shared by many forensic entomologists, there is little experimental proof to validate it. Therefore, there is an increasing need to confirm this theory. As a result, a number of workers have performed experiments to investigate this further.

The first experiment was carried out at the University of Tennessee, Department of Anthropology. This involved the remains of three human adult males and one adult female. The remains of these subjects were placed in the facility at different times of the year. Based on the insect activity present and the decomposition of these subjects, the authors concluded that there was a close correlation between human decay rates and carrion insect activity [28]. However, there were issues of concern in regards to this experiment. Firstly, the facility where the subjects were housed consisted of a concrete slab. This is problematic as studies have revealed that cadavers decay at a slower rate on concrete compared with on the ground [31]. Moreover, each cadaver was placed in a wire coffin that was suspended above the concrete floor. This was not an ideal experimental design as the conditions that are likely to be encountered in real casework were not replicated and thus the validity of the findings may be questioned [37]. Furthermore, these workers commented on seasonal effects on decomposition and insect activity, but this study involved only one

subject exposed in each season. In order to gain comprehensive information on seasonal variation, it is important to conduct decomposition studies for more than one year [156]. Finally, the insects observed on the subjects in this study were compared with those from two other studies conducted on pig carcasses. Ideally, such comparisons should not have been conducted at different times and in excess of 10 years apart, but rather in the one experiment, such that all the subjects would have been exposed to the same conditions to minimise differences due to external conditions.

Another study validated the use of carrion as a substitute for human remains. This involved an observation of the insect activity on two pigs and an adult human male, which were exposed at the same time in the same research facility on bare soil. The findings from this study confirmed that there was a negligible preference by arthropods for human over pig remains [157]. Therefore, it was concluded that pig carcasses can be substituted for human remains. Although many of the criticisms of the previous study involving human remains were addressed in this study, there were still some issues associated with the experimental design. The experimental scale was relatively small (i.e. two pigs and one adult human male) and the experiment was only conducted once. Despite these shortcomings, the experimental findings instil some confidence in the general practice of using pig carcasses as alternatives to human remains.

Another important aspect of the decomposition process to consider is the chemical cues that attract insects to decomposing remains. This is particularly important in the context of this thesis, as it is speculated that these cues relate to volatile organic compounds which are intermediate products of decomposition. They are produced, in addition to several gases, at the putrefaction stage when soft tissue is broken down by microorganisms [151]. Some of these compounds correspond to the target compounds for the ignitable liquids used throughout this study. These include: 1,2,4-trimethylbenzene, 1,3,5-trimethylbenzene, 1,2,3-trimethylbenzene [151], toluene and *p*-xylene from decaying human remains [119] and eicosane from decaying pig

carcasses [150]. Therefore, it will be of interest to determine if the fieldwork experiments described in this thesis detect notable volatile organic compounds similar to those described above.

5.3 Experimental Design

5.3.1 Study Site

A number of factors had to be considered when selecting an appropriate site at which to perform these fieldwork experiments. It has been suggested that in order for there to be independent olfactory orientation amongst insects (when multiple carcasses are involved), a minimum spacing of 50 m is required between subjects [18,34]. Many field studies have followed this advice, so as to ensure total independence of insects between sites [35,146,150,156,158,159]. Conversely, many other studies have designed experiments where the subjects were much closer, including distances of: 4 m [25]; 6 m [36]; 7-10 m [38], 10 m [139,157]; 12 m [21]; 30 m [160]; and 40 m [161]. Further to this, certain authors have defended their selection of a shorter spacing than 50 m by modelling experimental designs on other studies where smaller spacings were used and by claiming that a 10 m spacing offers arthropods access to all the subjects simultaneously under similar environmental and temperature conditions. However, these same authors also state that total independence would require spacing in excess of several hundred meters [157]. Despite the lack of consistency with regards to the design of fieldwork studies described in the literature, the experimental design for the fieldwork experiments described in this thesis required a site that would enable the subjects used to be placed a minimum of 50 m apart.

This was not an easy task, particularly given the negative public opinion associated with decomposition studies and the area required to achieve this spacing. Fortunately, an appropriate site was located. The site that was used was an operational military site, the Holsworthy Military Area, southwest of Sydney, in New South Wales,

Australia. This site offered ample open space (in excess of a 3 km radius), which ensured that the subjects could be sufficiently separated from each other. Also, due to the restricted access to the site, there was little concern that the subjects would be moved, tampered with or attract any unwanted attention.

The Holsworthy Military Area contains relatively undisturbed vegetation with low weed invasion. It is an open woodland area dominated by *Eucalyptus haemastoma, Angophora hispida, Cyathochaeta diandra, Corymbia gummifera, Eucalyptus oblonga, Leptospermum trivervium* and *Lomandra glauca* (see Figure 5.1).

Figure 5.1: *Southern (top) and eastern (bottom) aspect of the fieldwork site.*

5.3.2 Food Source

An important factor to consider for the fieldwork experiments was the type of food source or carrion that would be used. Since the aim of this study was to test the new

technique in a way that mirrors real life more closely, the food source selected had to reflect this. Ideally, to demonstrate absolute validity for casework purposes, the most suitable food source would be human remains. However, this was not a viable option (see Section 5.2). Therefore, for the purposes of these fieldwork experiments, non-human carrion was used as a substitute. As discussed in Section 5.2, pigs have been recognised to be closely related to humans therefore they were sought for this study.

The pig size recommended for research purposes is 20 to 27 kg [3,34]. The fieldwork experiments presented in this thesis involved four sample sets. In order to perform statistical analyses, a number of replicate subjects were required, and ideally the experiment was to be repeated a number of times. Therefore, it was anticipated that a large number of pigs would be required. This was not only difficult, but also costly to achieve.

Neonatal piglets were considered a more viable option as they could be obtained free from piggeries, and as these animals were not being killed for the purposes of this study, no ethical concerns were raised. However, care needed be taken in extrapolating the findings of this study to casework scenarios, as it has been shown that the size of the carcass has an effect on the rate of decomposition and the number of arthropods present: heavier pig carcasses decompose at a faster rate than lighter ones [160]. Given the small size of neonatal piglets, extrapolation to humans would be limited to the bodies of infants that survived to less than one month of age [156].

To test the proposed method in a more realistic casework setting, an important aspect of this included clothing the piglets [38]. This was achieved by placing two strips of polyester/cotton blend fabric (30 x 20 cm) over the piglet (shown in Figure 5.2). It was important that this variable was kept consistent between the piglets as it has been shown that clothing serves to protect the body from sunlight, which the larvae avoid, and it assists in increasing the rate of the decay process [31,37]. Care was taken not to wrap the fabric too tightly around the piglets as experiments that involved wrapping

remains in two layers of blankets have been shown to delay oviposition by approximately two days [162].

Figure 5.2: *Piglet loosely wrapped in two strips of fabric (30 x 20 cm) to mimic clothing.*

Due to the nature of the experiment (piglets exposed to the external environment), there was a possibility that, if left unprotected, the piglets could be attacked by animal scavengers. The majority of fieldwork studies use some form of protection to prevent their subjects from being moved [35,37,146,156,160]. Therefore, cages were prepared by covering plastic milk crates with wire mesh (openings 6.5 mm in diameter). This protected the piglet from vertebrate disturbance without impeding access to arthropods (see Figure 5.3). The top surface of the milk crate was fitted with several screws to enclose the piglet. When two of these were removed, the remaining two acted as hinges which made it simple to access the piglets. These cages were installed firmly onto the ground with metal pegs, so that even large animals could not disturb the experimental set-up.

Figure 5.3: *Milk crate covered in wire mesh that was used to protect piglets from scavengers.*

A consideration when designing the fieldwork experiments was the duration of time the piglets were to be exposed to the external environment. This time is often extensive in studies that are concerned with insect succession on carrion, and examples include: 240 hours [161]; 30 days [160]; 1 month [139]; 6 weeks [35]; 50 days [21]; 11 weeks [20]; 12 weeks [146]; 3 months [36]; 207 days [145]; and 271 days [18]. However, for studies involving a PMI calculation, the exposure time is not generally as extensive, at approximately 5 days [15,22]. The fieldwork experiments described in this thesis is not concerned with calculating a PMI or in constructing a catalogue of insects attracted to carrion throughout all the stages of decomposition. The focus of this study is to collect and analyse larvae, pupae and adults for the presence of ignitable liquids. Studies involving the application of entomotoxicology, as in this thesis, tend to be of shorter duration at 3 days [19], 5 days [59,62], 8 days [49] and 12 days [43]. However, larger-scale experiments involving piglets for the purposes of toxicological analysis have never been carried out before and there is no guide as to the appropriate duration of exposure.

There were, however, some points to satisfy for the current study. The duration of the exposure of the piglets to the external environment did not need to extend to the point that the piglets' flesh was completely removed, nor did the piglets need to decompose through to the remains stage. There was, however, an interest in collecting fly larvae feeding on the carrion. It was also desirable to have arthropods remaining on the carcass that would develop through to the adult stage, so that they, together with their puparia, could be collected. It was therefore necessary to place the piglets, (following exposure), in a closed container and transfer them to a controlled laboratory environment to ensure that the fly larvae would continue to develop and so that no adventitious insects would compete with or contaminate them. This meant that it was important to capture only the first and second wave of insects from the fresh and from the bloated stages, that is, until approximately the fourth day of exposure [21].

Further to this, it was important that the extent to which the piglets were burnt remained consistent throughout the experiments. The Crow-Glassman scale was used as a guide (see Table 5.1). The piglets in the experiment described in this thesis were burnt to achieve a level 2 burn. This level of burning has been adopted by previous studies investigating the effect of burning on the PMI calculation [15,139].

Crow-Glassman Scale[163]	
Level 1	Blistering of the skin and singeing of the head and facial hair
Level 2	The body is still recognisable, but it may result in the absence of certain elements of the hands, feet and ears
Level 3	Major portions of the legs and arms are missing. The body may not be recognisable
Level 4	Extensive destruction of the body; the skull has fragmented and is absent from the body
Level 5	The body has been cremated and little if any tissue remains

Table 5.1: *Description of the Crow-Glassman scale.*

A series of trial burn experiments was conducted prior to the fieldwork experiments in order to test some of these parameters. As a result, piglets were exposed for various periods to determine the most appropriate exposure time. It was found that an exposure time of five days resulted in the piglet being so heavily infested with larvae that very little remained. It is known that when the food supply is limited, the competition between larvae intensifies and can have a significant effect on the population density and distribution [11]. Therefore, an exposure time of three days was trialled, and although insect activity was present, it was observed to be of a lesser extent than when the piglet was exposed for longer periods. Hence an exposure period of three days was selected.

It was noted during the trial burn experiments that following three days' exposure (particularly in the warmer months), although the piglets were intact, they had decayed considerably. In this state they were very difficult to remove from their cages. Therefore, an additional piece of wire mesh was added to the base of the cage in

which each piglet lay, and small wire handles were attached to this to make it easy to raise. The introduction of this wire mesh bed ensured that the piglet remained intact and that the arthropods were not disturbed when the piglet was transported from its cage to the enclosed container.

When the piglets were transferred from the cages to the controlled laboratory environment, they needed to be kept in a container that would allow easy access to the sample for the remainder of the sampling period. A 10 L plastic container (which fitted the piglet well) was purchased. The top of this container was excised and the void was sealed using insect screen (see Figure 5.4). However, during the first trial burn experiment, the insect screen was found to be an unsuitable barrier as numerous fly larvae were able to crawl through the openings. In addition, as the maggot masses formed and the temperature in the container increased, the pressure and condensation in the container caused the lid to open, and valuable fly larvae were lost. To counteract this problem, a number of changes to the design were instigated. First, the insect screen was replaced with fabric, which contained the larvae well (see Figure 5.4). Secondly, the lids of the containers were sealed with duct tape so as to prevent the lids lifting off from the internal pressure. This tape was durable and could be easily re-used. Finally, moats were installed by placing larger (30 L) containers, filled with water and hypochlorite, under the original containers. This ensured that if any larvae escaped from the containers they would be captured in this second container without being lost.

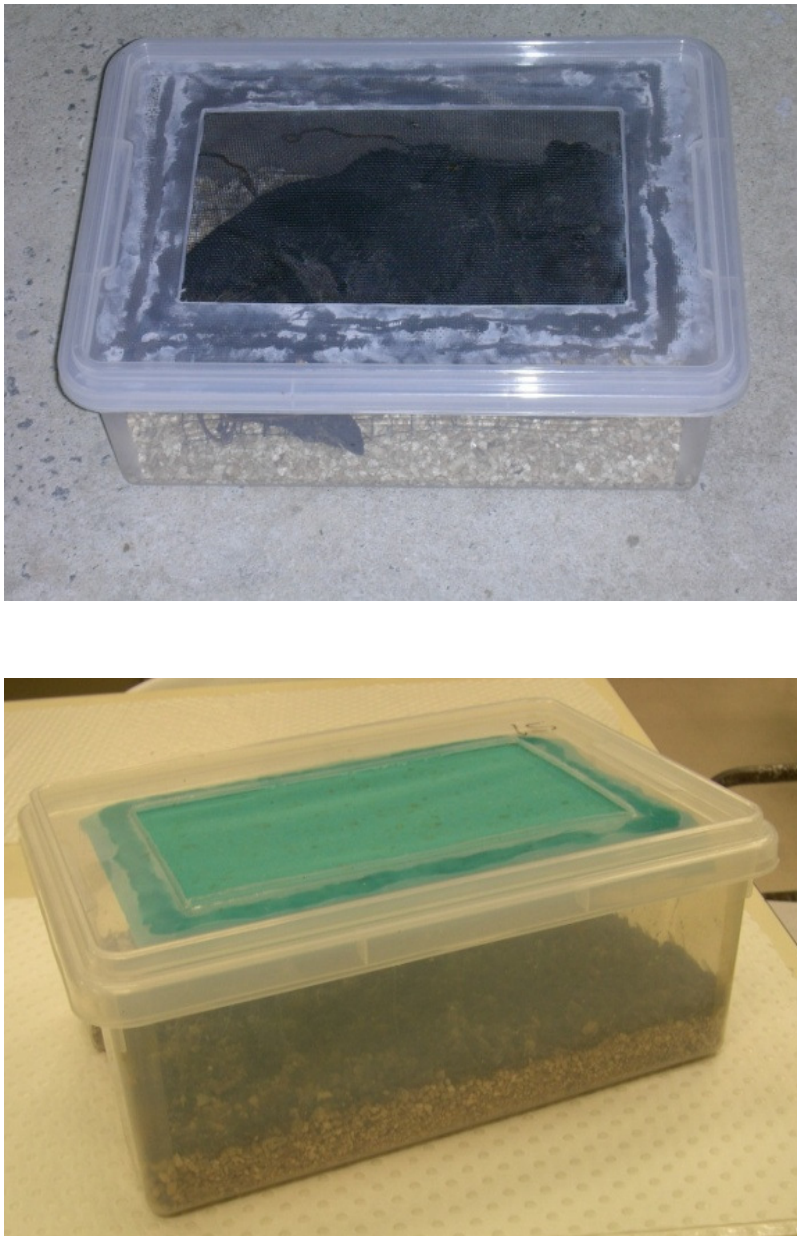


Figure 5.4: *10 L plastic containers used to house piglets fitted with insect screen (top) and fabric (bottom).*

These experiments involved four sample sets, namely petrol, kerosene, burnt and unburnt. It would have been difficult to draw a valid conclusion with an experimental design that involved a single piglet [164] and therefore more replicates were required. As mentioned previously, there was an interest in performing statistical analyses on the data from these experiments. In order to achieve this, a minimum of three piglets

was required for each of the four sample sets. The approach taken for the fieldwork experiments was to use the maximum number of replicates that could be managed with the resources available and so the number of piglets selected for each sample set was six.

To take into account the effect of variations, the fieldwork experiments were to be performed in each season over a one-year period. However, trial burn experiments in autumn yielded too few arthropods (when the piglet was exposed for three days) to warrant a full-scale experiment of this magnitude. Therefore, the fieldwork experiments were only carried out in spring and repeated in summer. Caution was exercised in terms of any seasonal conclusions made, as the experiments were not repeated in either of these seasons. As a result, rather than the experiments being referred to as spring and summer studies, the experiments were merely treated as replicate experiments, referred to as Experiment 1 and Experiment 2. As events transpired, conditions were warmer for the spring experiment than for the summer experiment, another reason not to draw seasonal conclusions.

5.3.3 Insect Samples

In the small-scale experiments, the entomological samples present were limited to one species that was added as first-instar larvae (see **Chapter 3**). Conversely, in these fieldwork experiments, the neonatal piglets were exposed to all arthropods at the fieldwork site. This meant that the experimental design mirrored a real scenario more closely and observations could be made in regards to the type of species collected from each piglet.

In the small-scale experiments, six larvae had been collected daily. During the trial burn experiments, single larvae were collected and it was noted that the chromatographic results for individual larvae varied. Therefore, it was concluded that six larvae, just as in the small-scale experiments, would be collected daily in order to obtain a sample that would be more representative of the larval population. In

addition, it has been recommended that fly larvae be collected from as many areas of the carcass as possible [7], so attempts were made to vary the locations from which the six larvae were collected.

5.3.4 Ignitable Liquids

Since the fieldwork experiments follow on from the small-scale experiment, the ignitable liquids of interest remained unchanged. Therefore the fieldwork experiments involved the testing of petrol and kerosene alone. One challenge was to determine the volume to be used for these larger-scale experiments.

An experiment reported in the literature, and described in Section 3.2 of this thesis, involved a comparison of the insect colonisation on burnt and unburnt pig carcasses [139]. This experiment was used as a guide to determine the volume of ignitable liquid to use in the fieldwork experiments. In this experiment, the authors used 3.8 L of petrol for pigs with an average weight of 25 kg, and 1.9 L for a pig with an average weight of 15.5 kg, in order to accelerate the fire. These figures were used to calculate weight to volume ratios and thus the volume required for the fieldwork experiments described in this chapter (where the average piglet weight used was calculated to be 1.39 kg). As a result, a range of volumes from 170 mL to 211 mL was obtained, and so for ease of preparation, a volume of 200 mL was selected for the fieldwork experiments.

5.4 Material and Methods

5.4.1 Study Site

The fieldwork experiments were conducted at the Holsworthy Military Area, New South Wales, Australia. The piglets were exposed from 15 - 18 October, 2007, after

which time they were transferred to a controlled laboratory environment at the University of Technology, Sydney. The experiment was then repeated from 16 - 19 February, 2008. Each site (where a piglet was placed) was equipped with a small data logger (iButtons® Maxim Integrated Products Sunnyvale, CA), that collected temperature readings every 30 minutes. In addition, the maximum and minimum daily temperatures and daily rainfall data were obtained from the local Holsworthy Control Range meteorological station (067117). When the piglets were transferred to the laboratory, a single data logger was used to record ambient temperatures at 30 minute intervals.

Another factor to consider was the placement of piglets. It has been stated that temperature has the greatest effect on the rate of decay: higher temperatures will stimulate maggot activity, which will in turn increase the rate of decay, [35,150,159,161], but larvae avoid direct sunlight [31]. Therefore, it was important that piglets were placed in areas that experienced uniform conditions in terms of temperature and direct sunlight.

5.4.2 Experimental Design

The fieldwork experiments comprised four sample sets of piglets, each with six replicates (refer to Table 5.2 for site co-ordinates). Twenty-four sites were located that were spaced a minimum 50 m apart (refer to Figure 5.5 and Table 5.3). The first set consisted of piglet burnt with petrol, the second burnt with kerosene, and the third burnt without an ignitable liquid. The final sample set was unburnt. The third and fourth sample sets acted as burnt and unburnt control groups, respectively.

Co-ordinates for All Replicates at the Fieldwork Site		
Site number	**Sample set**	**Site co-ordinates**
1	Petrol	S 33°59'56.232" E 150°55'39.324"
2	Kerosene	S 33°58'10.596" E 150°55'40.908"
3	Petrol	S 33°57'58.283" E 150°55'39.503"
4	Petrol	S 33°57'59.94" E 150°55'39.252"
5	Petrol	S 33°58'2.2874" E 150°55'38.2434"
6	Petrol	S 33°58'2.784" E 150°55'35.4"
7	Kerosene	S 33°58'5.8794" E 150°55'34.716"
8	Petrol	S 33°58'14.304" E 150°55'16.2474"
9	Kerosene	S 33°58'11.856" E 150°55'37.992"
10	Kerosene	S 33°58'11.1714" E 150°55'43.5714"
11	Kerosene	S 33°58'9.624" E 150°55'49.1154"
12	Kerosene	S 33°58'9.4074" E 150°55'54.156"
13	Burnt	S 33°58'8.5074" E 150°55'58.332"
14	Unburnt	S 33°58'10.8834" E 150°55'36.444"
15	Unburnt	S 33°58'10.5234" E 150°55'33.528"
16	Unburnt	S 33°58'13.4394" E 150°55'31.1514"
17	Unburnt	S 33°58'13.6914" E 150°55'25.5"
18	Unburnt	S 33°58'13.98" E 150°55'21.1434"
19	Unburnt	S 33°58'14.2314" E 150°55'16.1754"
20	Burnt	S 33°58'8.6154" E 150°56'39.48"
21	Burnt	S 33°58'8.472" E 150°56'8.592"
22	Burnt	S 33°58'7.428" E 150°56'13.3434"
23	Burnt	S 33°58'10.2354" E 150°56'13.9914"
24	Burnt	S 33°58'7.608" E 150°56'18.1314"

Table 5.2: *Co-ordinate and sample set information for each of the 24 fieldwork sites.*

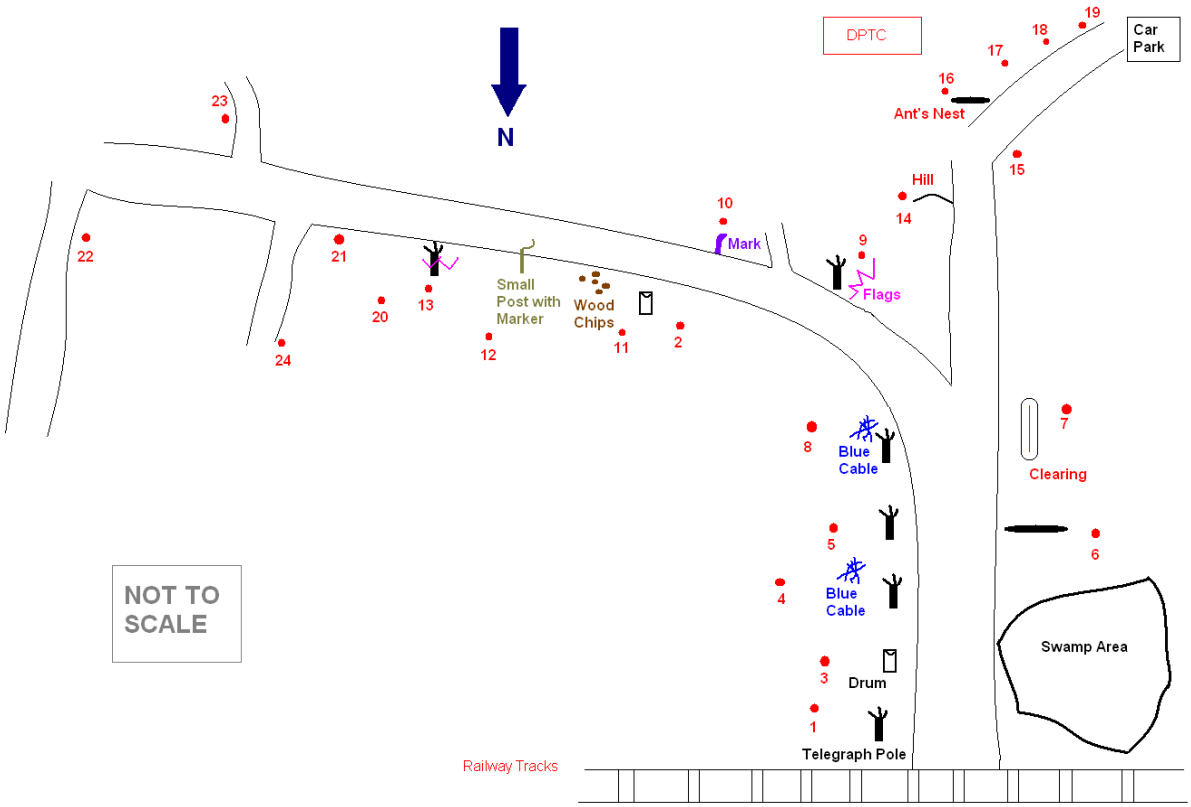


Figure 5.5: *Layout of fieldwork site indicating location of individual sites together with characteristic identifiers.*

Distance Between Individual Sites	
Site intervals	**Distance (km)**
1 to 2	3.263
1 to 3	3.643
2 to 3	0.382
3 to 4	0.051
4 to 5	0.077
5 to 6	0.075
6 to 7	0.095
7 to 8	0.540
5 to 8	0.674
8 to 9	0.562
9 to 10	0.144
10 to 11	0.150
8 to 11	0.854
11 to 12	0.130
12 to 13	0.111
9 to 14	0.051
10 to 14	0.183
13 to 14	0.566
14 to 15	0.075
15 to 16	0.108
16 to 17	0.145
17 to 18	0.112
18 to 19	0.128
13 to 20	1.0554
21 to 24	0.246
24 to 20	0.458
24 to 22	0.123

Table 5.3: *The distance between individual sites where the shortest distances have been highlighted.*

Twenty-four neo-natal piglets obtained from Boen Boe Stud pig farm in Joadja, New South Wales, Australia, weighing between 0.850 kg and 2.00 kg (mean - 1.39, standard error - 0.04) were used in these experiments (see Table 5.4 for specific weights). Piglets were refrigerated immediately after death and the carcasses were frozen within 24 hours. The carcasses, which were free of external defects, were thawed at room temperature overnight the evening prior to commencing the experiments. These piglets were transported to the fieldwork site the following day and were arranged on the metal trays with two strips of fabric (30 x 20 cm) placed around their bodies to mimic clothing.

Weight of Piglets used Throughout Fieldwork Experiments	
Weights (kg) Experiment 1	**Weights (kg) Experiment 2**
1.50	1.40
1.30	1.40
1.50	1.20
1.75	1.40
1.30	1.50
2.00	1.20
1.50	1.00
1.40	1.00
1.50	1.25
1.50	0.900
1.20	0.850
1.75	1.00
1.25	1.30
1.40	1.40
1.80	1.00
1.10	1.10
1.25	1.00
1.75	1.50
1.50	1.40
2.00	1.70
1.75	1.50
2.00	1.00
1.60	1.40
1.40	1.30

Table 5.4: *Weights of piglets used throughout fieldwork experiments.*

The ignitable liquid sample sets were prepared by adding 200 mL of the relevant ignitable liquid onto the entire surface of the clothed piglet, on the metal tray. Following this addition, the ignitable liquid was allowed to absorb for one minute prior to ignition. The piglets were ignited using a candle that was attached to the end of a long wooden broomstick (for safety purposes), and each was allowed to burn until the piglet self-extinguished. For safety reasons, the burning was conducted in a large area

that was free of trees and vegetation. In order to achieve the same approximate degree of burning for the burnt sample set as the ignitable liquid sample sets, the clothing was ignited in the former case (see Figure 5.6).

Petrol

Kerosene

Burnt

Unburnt

Figure 5.6: *Piglets indicating the degree of burning displayed for each sample set.*

Between 7:30 and 10:00 a.m., piglets were transported to their designated sites (see Table 5.2) and secured in their cages. Piglets were attended daily while exposed at the fieldwork site. Photographs were taken and observations made in regards to the piglets' appearance and the insect activity present. If fly larvae were noted, six larvae from multiple areas (if possible) were collected into labelled SPME vials and stored in cooler bags until they could be transferred to a freezer. These larvae were not washed, just as in the small-scale experiments. Attempts were made to collect larvae of consistent sizes. The vial numbers were recorded as well as the site from which the sample was taken. This was to ensure that when the samples were analysed, the sample set from which they were derived could not easily be identified without referring to the notes.

Once the piglets had been exposed for three full days, they were transferred to 10 L plastic containers from which a portion of the lid had been removed and replaced with fabric, while the base of each container was covered in vermiculite. These containers

were sealed with duct tape and clearly marked with the site from which they had been retrieved. These containers were then transported to a controlled laboratory where each was placed into a moat. Attempts were made to use the same containers for all of the sites in both experiments in order to reduce the possibility of cross contamination.

At the laboratory, the piglets were also photographed and samples collected daily until the larvae reared on the piglets pupated. Once the larvae had reached the pupation stage, the carcasses were removed and the container was sealed until the adults emerged, and could be collected along with their puparia. The species of all the adults that emerged were also identified.

5.4.3 Sample Preparation

The extraction conditions for the SPME technique described in **Chapter 3** remained unchanged; that is, the headspace was heated to 90 ^{o}C and the extraction time was three minutes. The only change that was made was that an SPME vial was used instead of a metal can as the sampling container. As the SPME vials were removed from a freezer, care was taken to ensure that the headspace had been adequately heated prior to introducing the fibre (on average the vial needed to be in the oven for one hour prior to extraction). In addition, due to the small size of the vial, it was important to use a retort stand to fasten the fibre and vial in place. If this is not performed, the chances of fibre breakage were increased as the build-up of pressure caused the septum to expand, which in turn caused the fibre holder to fall. Following adsorption, the fibre was inserted directly into the GC-MS injection port for three minutes (the duration of the solvent delay) to thermally desorb.

5.4.4 Sample Analysis

The analysis was carried out on an Agilent 6890 series GC system, coupled to an Agilent 5973 network mass selective (MS) detector using a Zebron ZB – 5 ms 30 m × 0.25 mm ID × 0.25 µm 5% polysiloxene 95% polydimethylsiloxane column (see Table 5.5 for a list of the parameters used).

GC-MS System Parameters	
Carrier gas	helium
Injector temperature	250 °C
Nominal initial flow rate	1.8 mL/min
Pressure	85.2 kPa
Acquisition mode	SIM
Split injection system	Pulsed splitless
Pulse pressure	200 kPa
Pulse time	0.5 minutes
Purge time	1 minute
Oven program initial ramp 1 ramp 2	 35 °C, held 2 minutes 10 °C/min to 190 °C 70 °C/min to 290 °C, held 4 minutes
Solvent delay	3 minutes
Multiplier voltage	Autotune + 400 volts

Table 5.5: *GC-MS instrument parameters used throughout the fieldwork experiments.*

5.4.5 Data Analysis

Total ion chromatograms (TIC) were produced for all the fly larvae, puparia and adults tested. Each chromatogram was only labelled with the sample number so that the

analyst had no prior knowledge of which group the sample was derived from. These chromatograms were evaluated as being positive for either petrol (P), kerosene (K) or negative (N), for an ignitable liquid, using visual pattern matching against known reference chromatograms, based in turn on the presence of target compounds and guidelines set out by the current ASTM method [82]. The target compounds in this experiment did not include all of those specified in **Chapter 3**, as these were not all present in the standards run using the new SIM method. In addition, retention times also changed slightly (see Table 5.6).

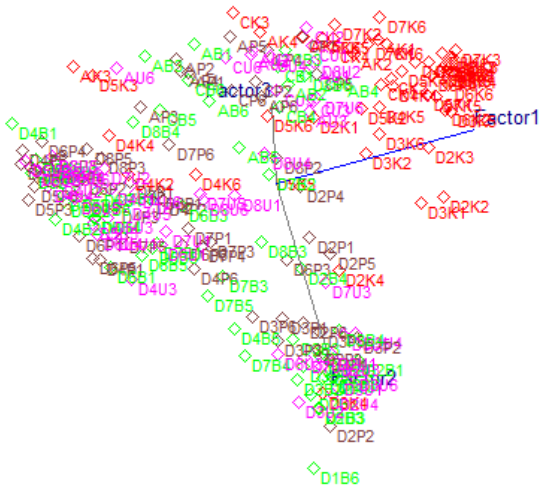
Target Compounds for the Ignitable Liquids of Interest using SIM Method			
Petrol		**Kerosene**	
1,3,5-trimethylbenzene	7.591	decane	8.186
1,2,4-trimethylbenzene	8.072	undecane	10.004
1,2,3-trimethylbenzene	8.577	1,2,3,5-tetramethylbenzene	10.370
1,2,4,5-tetramethylbenzene	10.305	dodecane	11.670
1,2,3,5-tetramethylbenzene	10.370	2-methylnaphthalene	13.288
dodecane	11.670	1-methylnaphthalene	13.524
4,7-dimethylindane	12.505	tridecane	13.255
2-methylnaphthalene	13.288	tetradecane	14.717
1-methylnaphthalene	13.524	pentadecane	16.083
ethylnaphthalenes (mixed)	14.738	hexadecane	17.362
1,3-dimethylnaphthalene	14.893	heptadecane	18.299
2,3-dimethylnaphthalene	15.088	pristane	18.324
		octadecane	18.951
		phytane	18.984
		nonadecane	19.465
		eicosane	19.896
		heneicosane	20.288

Table 5.6: *The target compounds and corresponding retention times (min) for petrol and kerosene that were used throughout the fieldwork experiments for identification purposes.*

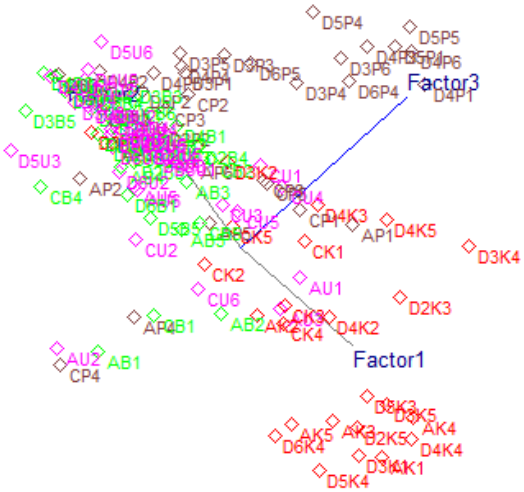
An in-house program (a Visual Basic macro in Microsoft Excel) was used to perform batch integration on the chromatographic data, in order to avoid manual integration of every chromatogram. This program performed a simple fixed-width integration of the

peaks corresponding to 25 target compounds in Table 5.6 (see Appendix 3 for details of the macro used); although a 5-point integration width was selected for the small-scale data, a number of different integration widths (see Figure 5.7) were trialled to determine which gave the best result.

Experiment 1: 3-point integration



Experiment 2: 3-point integration



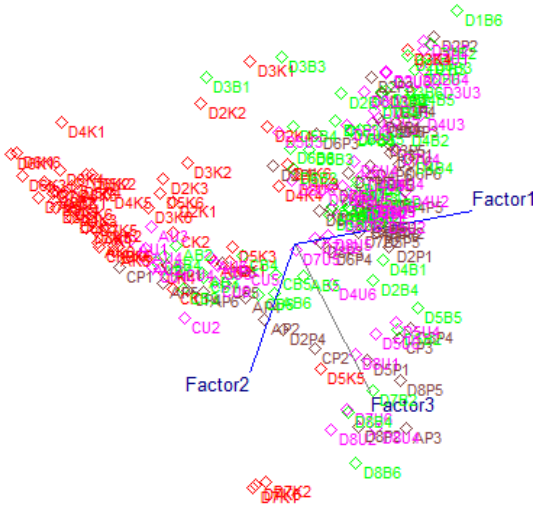
◇ **Petrol sample**

◇ **Kerosene sample**

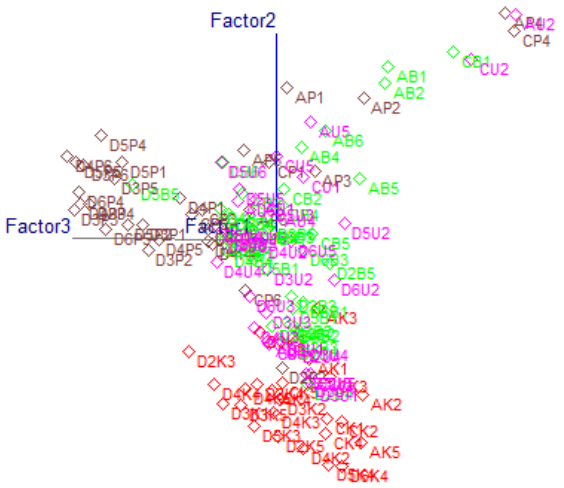
◇ **Burnt sample**

◇ **Unburnt sample**

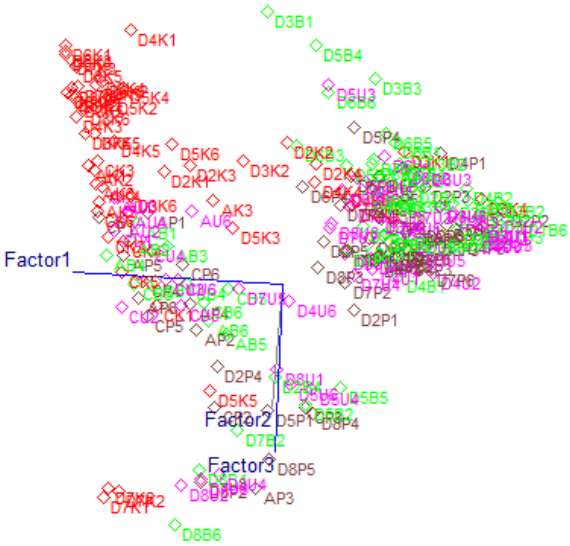
Experiment 1: 11-point integration



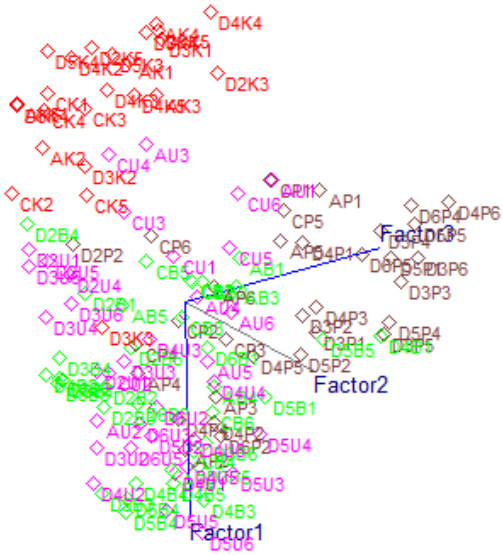
Experiment 2: 11-point integration



Experiment 1: 17-point integration



Experiment 2: 17-point integration



Experiment 1: 25-point integration **Experiment 2: 25-point integration**

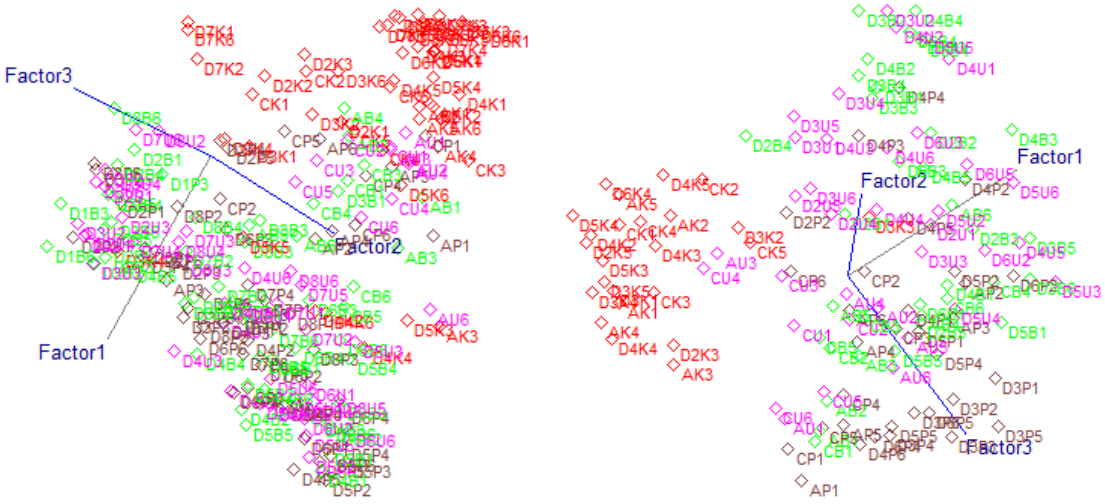


Figure 5.7: *Scores plots constructed for the fieldwork data using 3-point, 11-point, 17-point and 25-point integration widths.*

It was concluded that the 3-point and 25-point integration widths resulted in the least separation between sample sets. Conversely, the 11-point, and to a lesser extent, the 17-point integration width, offered the best separation of kerosene, petrol and control groups and the tightest clustering within these groups, the 11-point integration width was therefore adopted. The areas obtained formed the variables for principal component analysis (PCA). Pre-processing of this data involved vector normalisation and mean centring (just as in **Chapter 3**). PCA was then performed on this dataset using Pirouette® 4.0 software.

The scree plot (Figure 5.8) gives the eigenvalue (a measure of the variability) of each of the principal component transformations. From this plot it is clear that most of variability in data can be explained by the first seven principal components. This successfully reduced the data matrix from 25 variables to seven, while maintaining the important information [79].

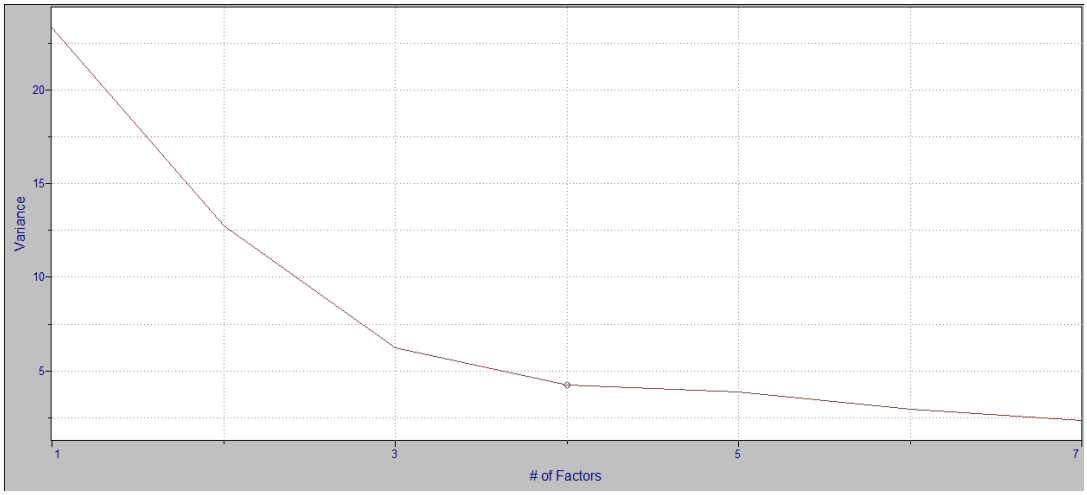


Figure 5.8: *Scree plot that provides a measure of the variability of each of the principal component transformations.*

Further investigation of the statistical differences between the four sample sets was performed through a one-way multivariate analysis of variance (MANOVA) using SPSS® on selected principal components that Pirouette® had calculated. Post-hoc analysis was subsequently carried out to further investigate the differences found and to determine which principal component was responsible for these differences.

5.5 Results and Discussion

5.5.1 Study Site

The temperature results discussed in this section were derived specifically from the period when the piglets were exposed at the fieldwork site. The temperatures recorded at the laboratory where the piglets were taken following exposure are not discussed. This is because all the piglets were stored in close proximity and thus experienced the same conditions, which ranged from 19 ^{o}C to 23 ^{o}C during the fieldwork experiments.

The minimum daily temperatures recorded for each of the sites throughout the three days of exposure were plotted for both Experiments 1 and 2 (see Figure 5.9 and 5.10). The graphs produced from the minimum daily temperature data indicate that generally each of the sites experienced the same trends in terms of their minimum temperatures (similar rises and falls) and at most differed by only approximately 3 ^{o}C. These observations (for each experiment) were investigated further through a one-way analysis of variance (ANOVA) calculation using the statistical software SPSS® for each day of exposure. The results obtained indicate that the mean minimum daily temperatures of the four sample sets in Experiment 1 are significantly different on the first and second days of exposure (15 and 16 October: $F_{3,20} = 3.76$, $p<0.05$ and $F_{3,20} = 6.1$, $p<0.05$, respectively). Conversely, the four sample sets were not significantly different based on their mean minimum daily temperatures on the third day and fourth day of exposure (17 October: $F_{3,20} = 1.322$, $p>0.05$ and 18 October $F_{3,20} = 1.712$, $p>0.05$, respectively). The results obtained for Experiment 2 indicate that the four sample sets were not significantly different based on their mean minimum daily temperatures on the first day of exposure (16 February: $F_{3,20} = 1.236$, $p>0.05$), whereas on all the remaining days of exposure the four sample sets were significantly different (17 February: $F_{3,20} = 5.053$, $p<0.05$, 18 February: $F_{3,20} = 5.879$, $p<0.05$ and 19 February: $F_{3,20} = 6.422$, $p<0.05$).

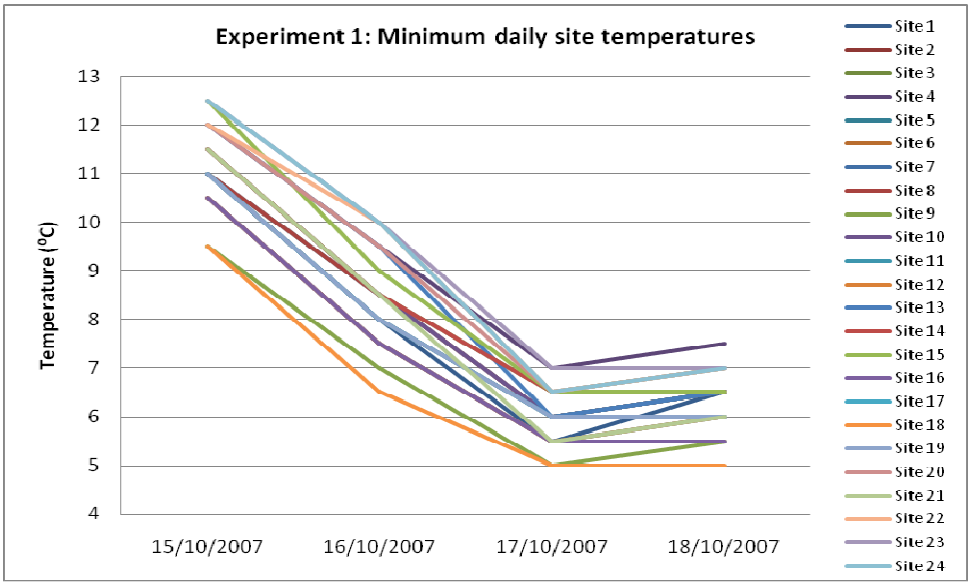


Figure 5.9: *Minimum daily temperatures recorded for the 24 sites during Experiment 1.*

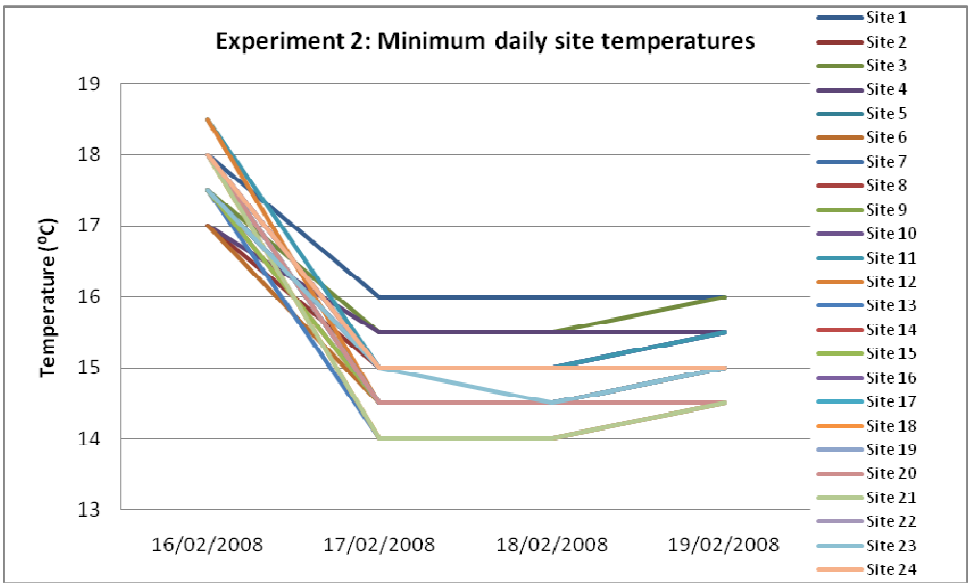


Figure 5.10: *Minimum daily temperatures recorded for the 24 sites during Experiment 2.*

The maximum daily temperature data were plotted for both Experiments 1 and 2 (see Figure 5.11 and 5.12). In these experiments, the spread of maximum daily temperatures among the sites was significantly greater than the daily minima. Certain

sites experienced much warmer temperatures than others. The sites did, however, experience similar trends, except for the final day of exposure. These graphs indicate that on the final day of each experiment, most of the sites' maximum temperatures were clustered together in one group (Experiment 2) or two groups (Experiment 1). This data was examined further through the use of an ANOVA calculation. The results obtained were the same for both experiments. This indicates that the four sample sets were not significantly different based on their mean maximum daily temperatures on the first three days of exposure (Experiment 1: 16 February: $F_{3,20} = 0.842$, $p>0.05$, 17 February: $F_{3,20} = 2.318$, $p>0.05$, 18 February: $F_{3,20} = 1.878$, $p>0.05$ and Experiment 2: 16 February: $F_{3,20} = 1.44$, $p>0.05$, 17 February: $F_{3,20} = 1.065$, $p>0.05$, 18 February: $F_{3,20} = 0.703$, $p>0.05$). The only day on which there was a significant difference between the sample sets in mean maximum temperatures was on the final day the piglets were exposed at the fieldwork site (Experiment 1: 19 February: $F_{3,20} = 6.418$, $p<0.05$ and Experiment 2: 19 February: $F_{3,20} = 3.477$, $p<0.05$). These observations can be explained in terms of the activities conducted on the final day. Firstly, the piglets were removed from the fieldwork site on this day, and therefore the temperature was not recorded for a full day. In addition, due to the area over which the piglets ranged, it took a number of hours to retrieve all the piglets and place them in a vehicle. This meant that some piglets were stored in a hot vehicle for longer periods than others, and this would explain the differences in maximum temperatures observed on the final day of exposure at the fieldwork site.

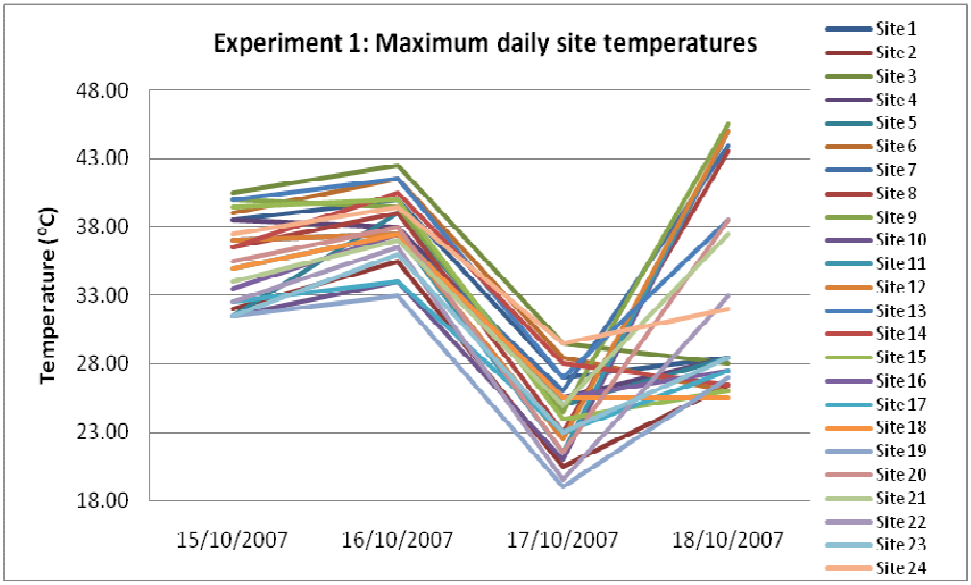


Figure 5.11: *Maximum daily temperatures recorded for the 24 sites during Experiment 1.*

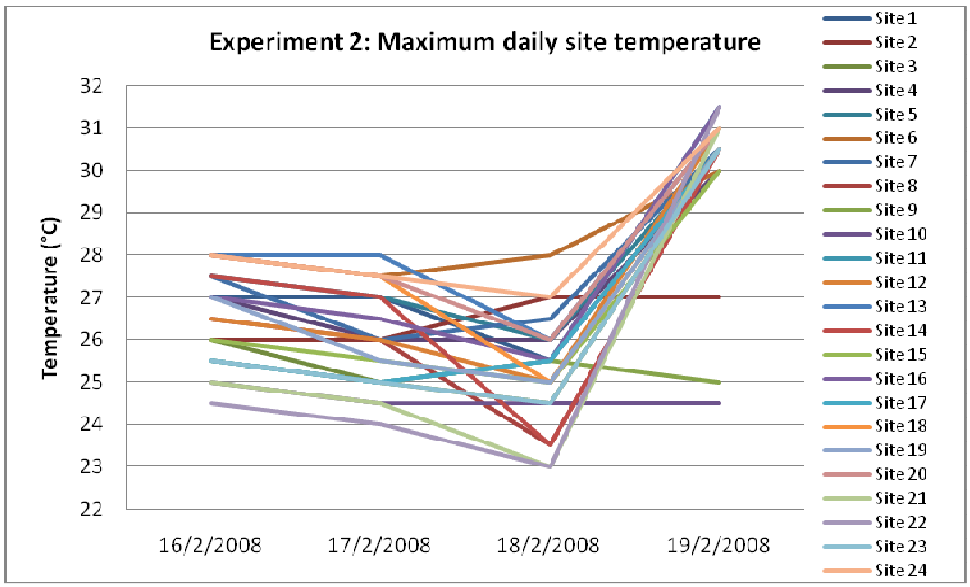


Figure 5.12: *Maximum daily temperatures recorded for the 24 sites during Experiment 2.*

The mean daily temperatures were plotted for each day of exposure for Experiments 1 and 2 (see Figures 5.13 and 5.14). The tightly clustered data collected for Experiment 1, and (to a lesser extent) Experiment 2 indicates that the sites experienced comparable mean daily temperatures in each experiment. This observation was

investigated further through an ANOVA calculation. In this case, this calculation was done to determine if the four sample sets were significantly different in their daily mean temperatures on each day they were exposed at the fieldwork site. The results indicate that in Experiment 1 on 15, 17 and 18 October, the four sample sets did not differ in their mean daily temperatures (15 October: $F_{3,20} = 1.766$, $p>0.05$, 17 October: $F_{3,20} = 1.730$, $p>0.05$ and 18 October: $F_{3,20} = 1.278$, $p>0.05$). The only day in which the sample sets differed in their mean daily temperatures in Experiment 1 was on 16 October ($F_{3,20} = 3.605$, $p<0.05$). Conversely, the sample sets differed in their mean daily temperatures in Experiment 2 on all days except 16 February (16 February: $F_{3,20} = 1.324$, $p>0.05$, 17 February: $F_{3,20} = 4.276$, $p<0.05$, 18 February: $F_{3,20} = 6.224$, $p<0.05$, 19 February: $F_{3,20} = 12.984$, $p<0.05$).

There are a number of possible explanations for these observations in Experiment 2. Firstly, there was rainfall during this experiment (between 0.2 mm to 0.4 mm) and there had been several millimetres of rain in the lead-up to the experiment. It was observed that some of the sites (particularly the petrol and kerosene piglets) were more susceptible to flooding than others. This may have contributed to the differences in temperature. In addition, the level of insect activity varied from piglet to piglet, irrespective of the sample set (discussed further in Section 5.52). This could have led to differences in the recorded temperatures, due to the formation of maggot masses of different sizes.

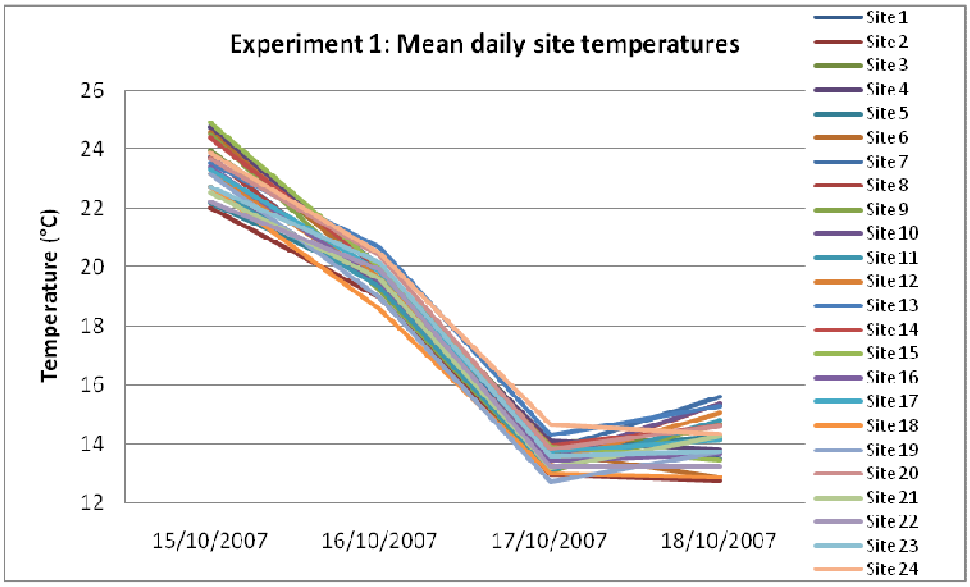


Figure 5.13: *Mean daily temperatures recorded for the 24 sites during Experiment 1.*

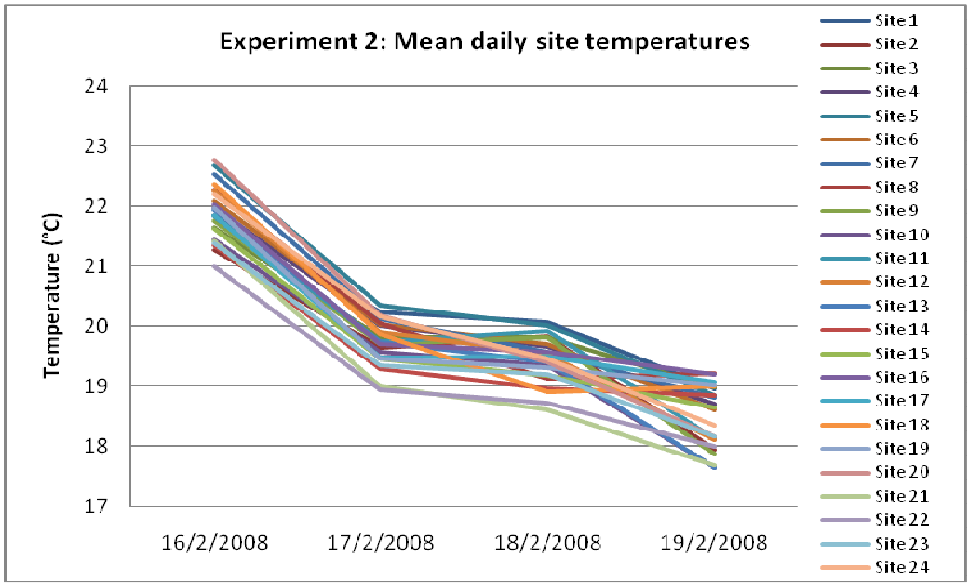


Figure 5.14: *Mean daily temperatures recorded for the 24 sites during Experiment 2.*

The 24 sites selected for the fieldwork experiments were chosen as they appeared to share the same levels of direct sunlight and shade. This was an important point to consider as it is known that decomposition occurs at a faster rate in warmer

temperatures, but larvae are deterred by direct sunlight [161]. However, it was observed that the sites differed based on the minimum and mean daily temperatures and, in some cases, maximum daily temperatures. This highlights that although areas appeared to experience the same conditions, other factors may also have contributed to temperature variability. For future experiments it is recommended that the temperature at sites be monitored prior to any experiment to ensure that sites do not experience significant temperature differences.

The temperature recorded from the closest weather station (Holsworthy Control Range) was also examined in order to interpret the results. Interestingly, the minimum temperatures at the fieldwork site were 2 ^{o}C to 3 ^{o}C lower than those recorded at the weather station, but the maximum daily temperatures obtained were comparable. These observations reveal that efforts should be taken to take temperature readings at the site at which the experiments were conducted. If this cannot be performed and data from a nearby weather station is required, appropriate uncertainty should be factored into those data. This conclusion was also reached by a separate study [4]. Weather stations, however, are still important for obtaining rainfall information, as was the case in these experiments.

5.5.2 Observations

Insects colonised the piglets earlier in Experiment 1 than in Experiment 2. In Experiment 1, five piglets contained insect activity after one day of exposure and 19 piglets contained insect activity after two days of exposure (Days 1 and 2). In Experiment 2, no piglets contained insect activity after only one day of exposure and 15 piglets contained insect activity following two days of exposure. The differences can be explained by the finding that Experiment 1 experienced ambient temperatures that were on average 3 ^{o}C higher than those experienced during Experiment 2. Further to this, there was considerable cloud cover during Experiment 2, whereas the piglets in Experiment 1 would have been exposed to more direct sunlight, which may have created more heat, and this increased maggot growth and activity [159].

Minor differences in the sample sets were noted in terms of the onset of insect activity. In Experiment 1, one piglet from each of the sample sets contained insect activity after one day, except for the burnt group, where two piglets contained insect activity. However, following two days of exposure, all six of the piglets derived from the petrol and burnt groups, four from the kerosene group and three from the unburnt group contained insect activity. In Experiment 2, a similar pattern was observed where, after two days of exposure, four and five piglets from the petrol and burnt groups, respectively, contained insect activity, whereas only two and four piglets contained insect activity from the kerosene and unburnt groups, respectively.

Although the sample sizes are small, these findings suggest that the petrol and burnt groups were colonised faster than the kerosene and unburnt sample sets in both Experiment 1 and 2. This finding supports the conclusion drawn by other authors that insects colonised pigs burnt with petrol earlier than unburnt pigs [139]. It is possible that the chemicals created by burning a piglet (with or without the use of petrol) trigger an olfactory response in insects, but it is outside of the scope of this study to investigate this further or the effect these chemicals might have on insect growth rates. However, this is an important point to consider particularly if a PMI calculation is to be performed on burnt remains.

One piglet from the kerosene sample set in Experiment 2 contained no larvae at all, and the corresponding piglet in equivalent location from Experiment 1 showed only limited numbers of larvae. These observations can be explained by the presence of ants at this site. However, this piglet was not the only subject that was affected by these predators. Throughout Experiment 1, more than half of the piglets were infested by ants, while in Experiment 2, the proportion was approximately a quarter. This difference is most likely explained by the cooler, wetter conditions experienced during the second experiment. It is known that ants are serious predators as they remove dipteran eggs, and as a result retard the rate of decomposition [4,18,20]. Studies have shown that a major factor affecting the rate a carcass is removed is the presence of ants [20]. Additionally, in these experiments the ants did not limit their

interference to the collection of eggs and larvae alone. They also fed on the piglets directly (see Figure 5.15). Therefore, it is highly recommended that in future experiments if possible care is taken to ensure that no large ant colonies are present in the area, as they will introduce an extra variable into the experiments.

Figure 5.15: *Piglet from Experiment 2 that was severely affected by ants removing dipteran eggs, larvae and feeding directly on the surface of the piglet.*

Differences were noted between Experiments 1 and 2 in terms of the percentage of the tissue that had been removed by insect activity by the pupation stage (see Table 5.7). This was based purely on visual observations, as piglets were not weighed due to the potential threat of cross-contamination. In addition, according to a recent study, weighing carrion disturbs the feeding insects, and could result in inaccuracies due to biomass loss [33]. It was observed that a higher percentage of the piglets' tissue had been removed in Experiment 1, compared with Experiment 2. However, this pattern was not consistent as the approximate percentage of tissue lost in seven piglets was the same in both experiments, and some of the piglets from the second experiment showed greater tissue removal than in the first. This was despite the fact that the

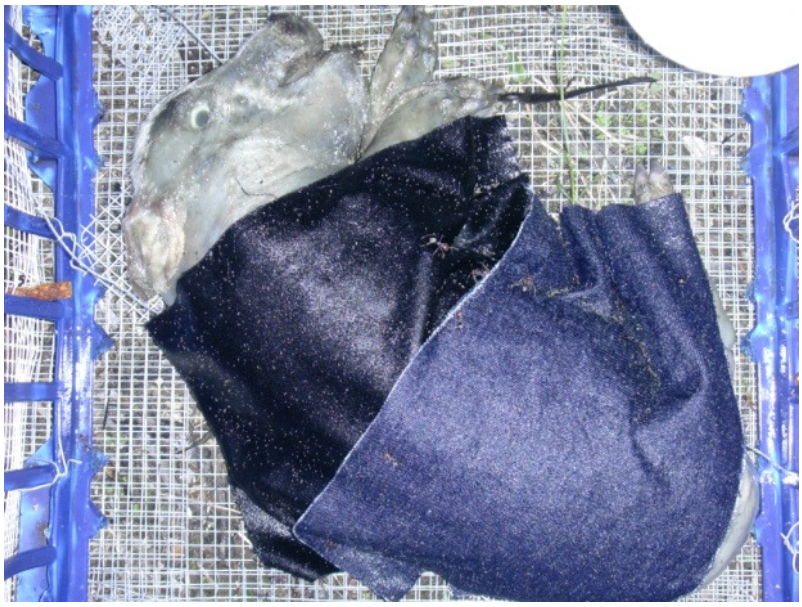
highest temperatures were recorded throughout Experiment 1. It is interesting to note that the piglet that was reduced to bones at the fastest rate (five days) throughout the entire study was from Experiment 2 (see Figure 5.16). The possible explanation for this observation was that during Experiment 2, there was an average of 0.2 mm of rain on each day. The piglet in question was from the unburnt sample set. Therefore, the 'clothing' present on the piglet remained intact and provided protection, which was not true of any of the other sample sets, where the fabric was either destroyed by burning or became embedded on the skin. This observation is also consistent with the finding that, as a group, the piglets derived from the unburnt sample set were found to have decomposed to a greater extent in this experiment compared with other sample sets. Another study concluded that rainfall increases the rate of decomposition by increasing mass loss and reducing the length of decay stages in neo-natal piglets [156]. This author also hypothesised that rain keeps the soil surrounding the carcass moist, which in turn encourages maggot and bacterial action. Although these specific observations are not relevant to the detection of ignitable liquids, they are important for future decomposition studies in this area.

Approximate Percentage of Tissue Removed Through Insect Activity at the Pupation Stage		
Site	**Piglet lost (%) experiment 1**	**Piglet lost (%) experiment 2**
1 (Petrol)	50	0
3 (Petrol)	25	100
4 (Petrol)	50	25
5 (Petrol)	100	25
6 (Petrol)	25	25
8 (Petrol)	25	50
2 (Kerosene)	0	25
7 (Kerosene)	25	0
9 (Kerosene)	100	0
10 (Kerosene)	25	50
11 (Kerosene)	0	25
12 (Kerosene)	0	0
13 (Burnt)	100	50
20 (Burnt)	100	0
21 (Burnt)	100	0
22 (Burnt)	25	0
23 (Burnt)	100	100
24 (Burnt)	25	25
14 (Unburnt)	25	25
15 (Unburnt)	25	100
16 (Unburnt)	0	25
17 (Unburnt)	100	100
18 (Unburnt)	25	100
19 (Unburnt)	25	25

Table 5.7: *The approximate percentage of tissue removed by insect activity when the majority of insects present entered the pupation stage throughout the fieldwork experiments.*

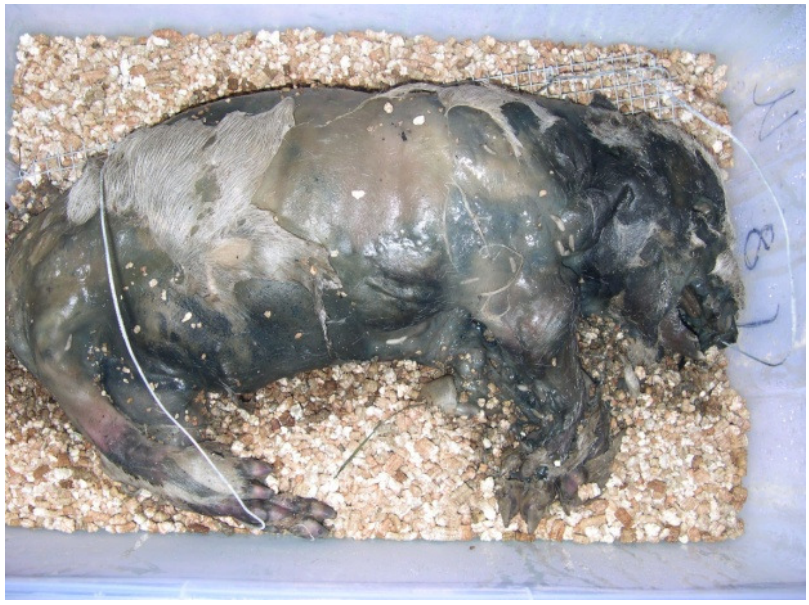
Day 1

Day 2



Day 3

Day 4



Day 5

Figure 5.16: *Piglet derived from the unburnt sample set in Experiment 2 that was reduced to bones at the fastest rate (five days) of all the piglets in the fieldwork experiments.*

It was also noted that twice the number of piglets in Experiment 2 (compared with Experiment 1) contained visible fungal growth (see Figure 5.17). This was more than likely due to the wet conditions in Experiment 2. The piglets that exhibited the greatest fungal growth were those derived from the petrol and kerosene sample sets. Further experiments should be conducted to ascertain if the presence of an ignitable liquid effects the fungal growth on carrion. In addition, the effect of carrion fungal growth on insect activity and decomposition has not been explored here or in any other study. However, it has been suggested that frozen-thawed subjects are more susceptible to aerobic decay of skin and invasion of insects and micro-organisms compared with freshly-sourced subjects [37]. Elsewhere, it has been suggested that freezing (as was the case in this project) can impact on the bacterial decomposition, but not the insect succession on carrion [18]. However, due to the number of piglets required for the fieldwork experiments, freezing was the only viable option to preserve the piglets until a sufficient number of subjects were obtained.

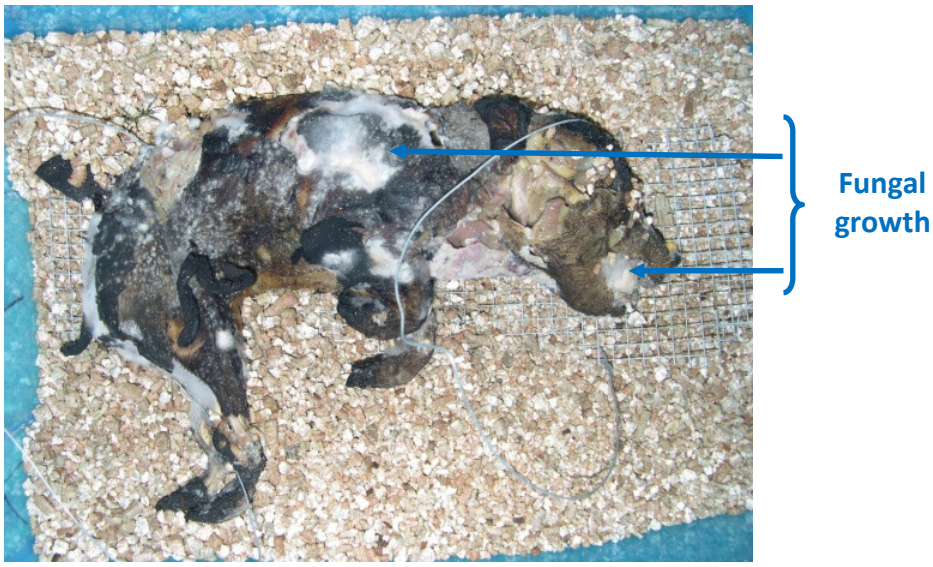


Figure 5.17: *Piglet with fungal growth on the surface of its body.*

5.5.3 Insect Samples

The insects that emerged from each piglet (following storage in a controlled laboratory) were identified (see Table 5.8). Of the ten species that emerged, the blow flies were the most common group encountered, although the flesh flies were also well represented, particularly in Experiment 2. A review conducted of cases involving entomological evidence over a 17 year period in New South Wales also concluded that the blow flies were the most commonly encountered insects, as they were present in 86% of cases [142]. These insects correspond to the first and second wave of insects visiting carrion after death, and given the odorous stage that the piglets reached when they were transferred to the laboratory, these findings do not deviate from the literature on what insects should be present [11].

Insects that Emerged from Each Piglet Throughout the Fieldwork Experiments		
Site	**Experiment 1**	**Experiment 2**
3 (Petrol)	*Calliphora augur*	*Calliphora augur* *Sarcophaga impatiens*
1 (Petrol)	*Calliphora augur* *Calliphora stygia*	*Calliphora augur*
4 (Petrol)	*Calliphora augur*	*Sarcophaga impatiens*
5 (Petrol)	*Calliphora augur* *Calliphora stygia*	*Calliphora augur*
6 (Petrol)	*Calliphora augur*	*Calliphora augur* *Sarcophaga impatiens* *Lucilia cuprina*
8 (Petrol)	*Calliphora augur*	*Calliphora augur* *Sarcophaga impatiens*
2 (Kerosene)	*Calliphora augur*	*Calliphora augur* *Sarcophaga impatiens*
7 (Kerosene)	*Calliphora augur*	*Calliphora augur*
9 (Kerosene)	*Calliphora augur*	*Calliphora augur* *Sarcophaga impatiens*
10 (Kerosene)	*Calliphora augur*	*Calliphora augur*
11 (Kerosene)	*Calliphora augur*	*Calliphora augur*
12 (Kerosene)	*Calliphora augur*	No insects present
13 (Burnt)	*Calliphora augur* *Calliphora dubia*	*Calliphora augur* *Sarcophaga impatiens* *Calliphora dubia*
20 (Burnt)	*Calliphora augur* *Calliphora hilli* *Calliphora dubia*	*Calliphora augur* *Sarcophaga impatiens*
21 (Burnt)	*Calliphora augur* *Hydrotaea rostrata* *Sarcophaga impatiens*	*Calliphora augur* *Sarcophaga impatiens*
22 (Burnt)	*Calliphora augur* *Calliphora hilli*	*Calliphora augur* *Sarcophaga impatiens*
23 (Burnt)	*Sarcophaga impatiens* *Calliphora hilli* *Hydrotaea rostrata* *Calliphora augur* *Calliphora dubia*	*Sarcophaga impatiens* *Chrysomya incisuralis* *Hydrotaea rostrate* *Chrysomya rufifacies*
24 (Burnt)	*Calliphora augur*	*Chrysomya incisuralis* *Calliphora augur*
14 (Unburnt)	*Calliphora augur* *Sarcophaga impatiens*	*Calliphora augur* *Sarcophaga impatiens*
15 (Unburnt)	*Calliphora augur*	*Sarcophaga impatiens*
16 (Unburnt)	*Calliphora augur* *Sarcophaga impatiens* *Calliphora hilli*	*Calliphora augur* *Sarcophaga impatiens*
17 (Unburnt)	*Calliphora augur* *Calliphora stygia*	*Calliphora augur* *Sarcophaga impatiens*
18 (Unburnt)	*Calliphora augur*	*Sarcophaga impatiens* *Hydrotaea rostrata*
19 (Unburnt)	*Calliphora augur* *Calliphora hilli*	*Calliphora augur* *Sarcophaga impatiens*

Table 5.8: *A list of the insects that were reared on, and subsequently emerged from the piglets that were exposed to the elements at the fieldwork site.*

Calliphora augur (Fabricius) (Diptera: Calliphoridae) (which is active in the months during which the experiments were conducted [146]) was the most common species to emerge from the piglets throughout the fieldwork experiments, irrespective of the sample set. Although *Sarcophaga impatiens* (Walker) (Diptera: Sarcophagidae) are active during the same months of the year as *Calliphora augur* and are attracted to highly decomposed carrion, this species emerged from only four piglets in Experiment 1, but from 17 piglets in Experiment 2. This may be due to the differences in temperatures between the experiments. This may also explain why *Calliphora stygia* (Fabricius) (Diptera: Calliphoridae) and *Calliphora hilli* (Patton) (Diptera: Calliphoridae) were only observed in Experiment 1 and why *Chrysomya rufifacies* (Macquart) (Diptera: Calliphoridae) *and Lucilia cuprina* were only observed in Experiment 2. Despite this, no obvious differences were observed in terms of certain species preferentially colonising a sample set over another. However, *Chrysomya rufifacies*, *Lucilia cuprina*, *Calliphora dubia* (Macquart) (Diptera: Calliphoridae) and *Chrysomya incisuralis* (Macquart) (Diptera: Calliphoridae) only emerged from one or two piglets, and so a conclusion cannot be drawn as to their preference for a particular sample set. Further investigation is required to determine if particular species preferentially colonise remains that have been burnt with petrol or kerosene, or if unburnt remains are preferred.

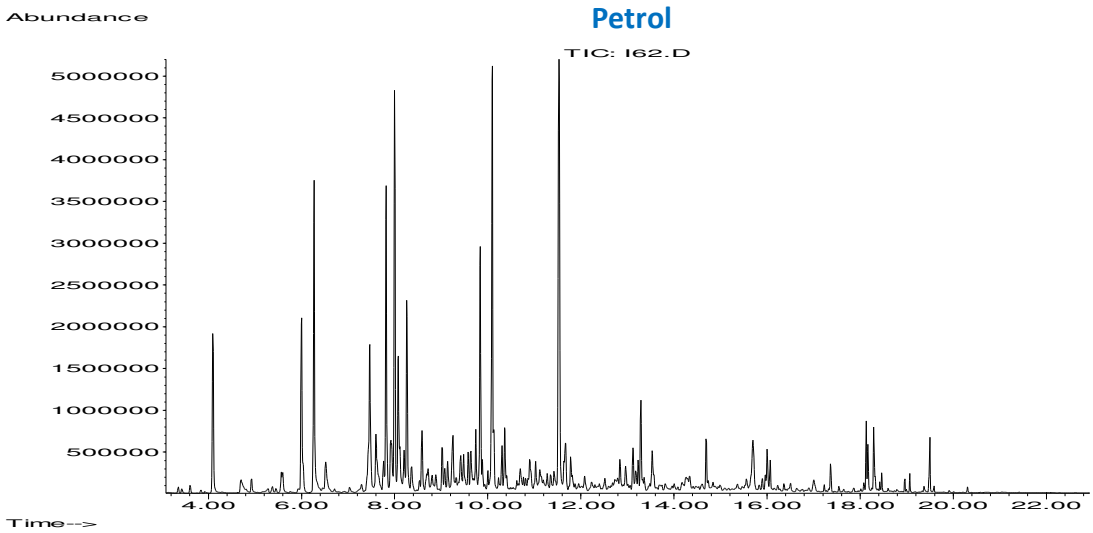
A review of samples taken from human remains between 1984 and 2001 in New South Wales revealed that the most common calliphorids encountered were *Chrysomya rufifacies*, *Calliphora augur*, *Calliphora stygia*, *Chrysomya varipes* (Macquart) (Diptera: Calliphoridae), *Chrysomya nigripes* (Aubertin) (Diptera: Calliphoridae), *Lucilia cuprina*, *Lucilia sericata* (Meigen) (Diptera: Calliphoridae), *Chrysomya megacephala* (Fabricius) (Diptera: Calliphoridae), *Calliphora ochracea* (Schiner) (Diptera: Calliphoridae) and *Calliphora vicina* (Robineau-Desvoidy) (Diptera: Calliphoridae) [142]. Six of the ten species mentioned in the review were not encountered during the fieldwork experiments and many of those that were are not mentioned in the review. This review observed that *Calliphora augur* and *Calliphora stygia* developed on remains and were often the sole species present. In the fieldwork experiments reported in this

thesis, *Calliphora augur* was observed to be the sole species that emerged from three piglets in Experiment 1 and five piglets in Experiment 2. Conversely, *Calliphora stygia* was never the sole species to emerge in the fieldwork experiments.

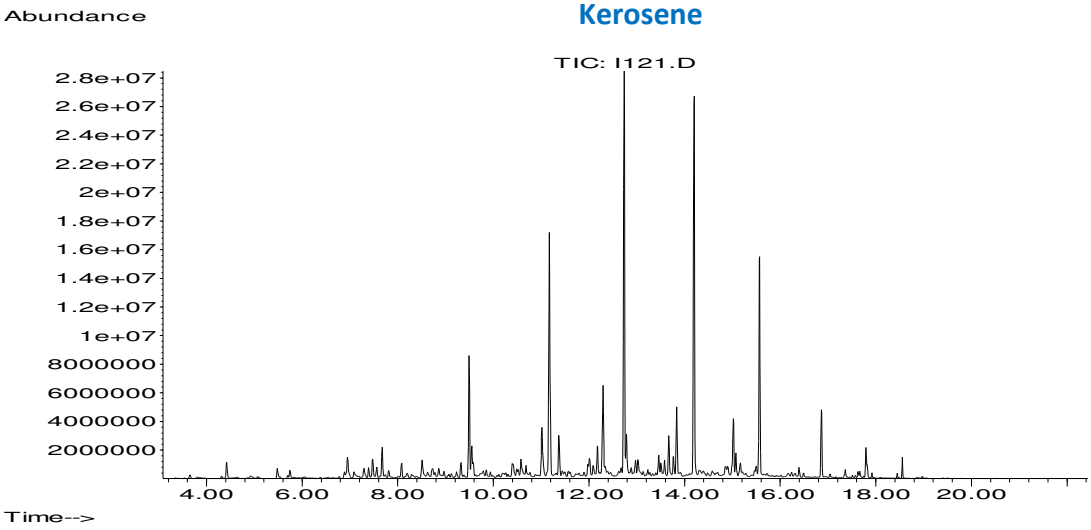
The most significant challenge encountered throughout this study in terms of the insects collected was the presence of the parasitic wasps. These are tiny wasps that lay their eggs in the pupae of other insects, which causes the host to die. A number of the puparia in the fieldwork experiments demonstrated evidence of the puparium having been attacked by this predator. The predatory action of these wasps reduced the numbers of flies that emerged from each piglet (10 piglets were affected in Experiment 1 and nine piglets in Experiment 2). Therefore, it is unclear if other fly species may have been present at particular sites.

5.5.4 Chemical Analysis

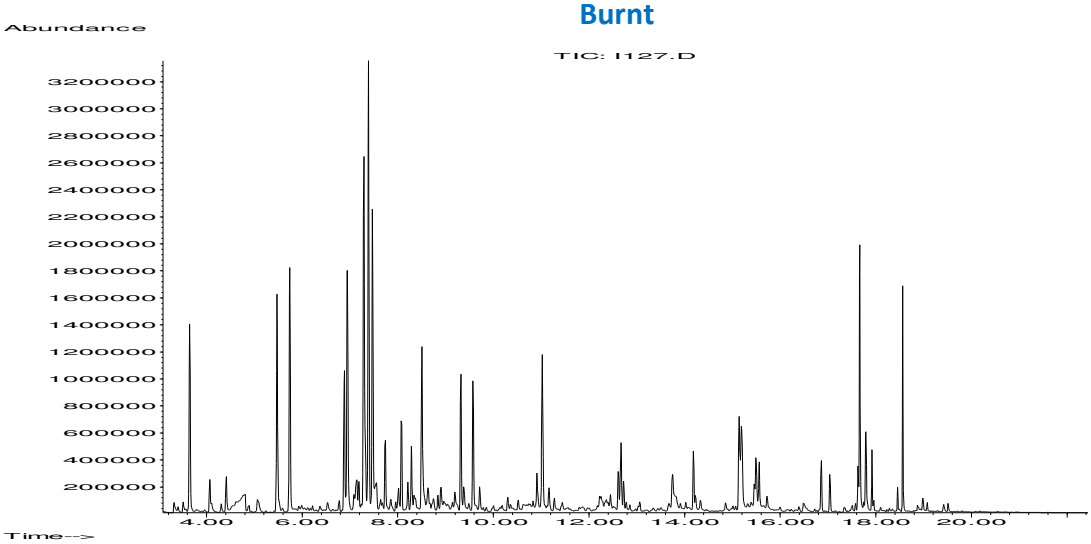
Chromatograms were obtained from all sample sets on each day that entomological evidence was present (see Figure 5.18 for an example of the chromatograms obtained).



Kerosene



Burnt



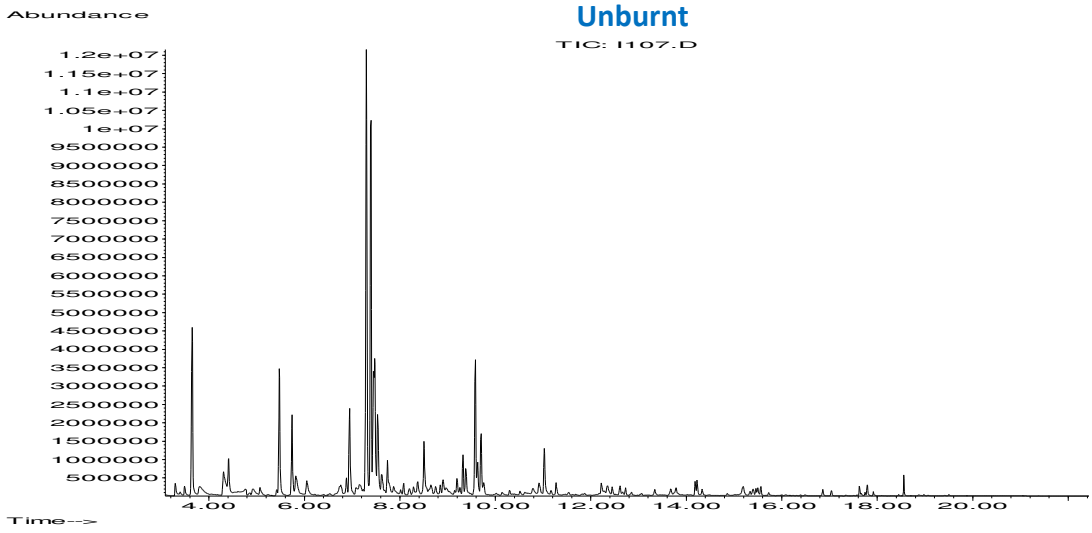


Figure 5.18: *Chromatograms obtained on Day 5 of Experiment 1 from larval samples reared on the petrol, kerosene, burnt and unburnt sample sets, respectively.*

The results obtained from the petrol sample set in Experiment 1 (see Table 5.9) indicate that 90% of larvae, 50% of adults and 66.7% of pupae contained traces of petrol. Similarly, the results obtained from the kerosene sample set indicate that 97.4% of larvae and 100% of both adults and pupae contained traces of kerosene. In Experiment 2 (see Table 5.10), the results obtained for the petrol sample set indicate that 76.1% of larvae, 83.3% of adults and 66.7% of pupae contained traces of petrol and all the samples collected from the piglets burnt with kerosene contained traces of kerosene. These findings are particularly noteworthy as the analyst had no prior knowledge of the sample set from which the samples were derived (as they were merely labelled as numbers) without referring to the fieldwork notes. Therefore, the results are based purely on the chromatographic pattern produced by these samples. This indicates that this method can successfully differentiate between samples that do not contain ignitable liquids and those that do.

Chromatographic Results for Experiment 1										
Site	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**	**Day 8**	**Adults**	**Cases**
1 (Petrol)	N	P	P	P	P	P	P	P	P	P
3 (Petrol)	-	P	P	P	P	P	P	P	P	N
4 (Petrol)	-	N	N	P	P	P	N	P	N	N
5 (Petrol)	-	P	P	P	P	P	P	-	N	P
6 (Petrol)	-	N	P	P	P	P	P	P	P	P
8 (Petrol)	-	P	P	P	P	P	P	P	P	N
2 (Kerosene)	-	K	-	K	K	K	K	K	K	K
7 (Kerosene)	-	K	K	K	K	K	K	-	K	K
9 (Kerosene)	-	K	K	K	K	K	K	K	K	K
10 (Kerosene)	-	K	N	K	K	K	K	K	K	K
11 (Kerosene)	-	-	K	K	K	K	K	K	K	K
12 (Kerosene)	-	-	K	K	K	K	K	K	K	K
13 (Burnt)	-	N	N	N	N	N	N	-	N	N
20 (Burnt)	-	N	N	N	P	N	N	-	N	N
21 (Burnt)	N	N	N	N	N	N	N	N	N	N
22 (Burnt)	N	N	N	N	P	N	N	N	N	N
23 (Burnt)	-	N	N	P	P	N	N	N	N	N
24 (Burnt)	-	N	N	N	N	N	N	P	N	N
14 (Unburnt)	-	N	N	N	N	N	N	N	N	N
15 (Unburnt)	-	-	N	N	N	P	N	N	N	N
16 (Unburnt)	-	-	N	N	N	N	N	N	N	P
17 (Unburnt)	-	N	P	N	N	N	N		N	N
18 (Unburnt)	-	N	N	N	P	N	N	N	N	N
19 (Unburnt)	-	-	P	N	N	P	N	-	N	N

Table 5.9: *Summary of the chromatographic results obtained for all the entomological samples collected throughout Experiment 1. Samples are denoted as positive for petrol (P), kerosene (K), negative for an ignitable liquid (N), or (-) where a sample was not collected.*

Chromatographic Results for Experiment 2								
Site	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Adults**	**Cases**
1 (Petrol)	-	-	P	P	-	-	P	P
3 (Petrol)	-	-	P	P	P	P	P	N
4 (Petrol)	-	-	P	P	P	N	N	N
5 (Petrol)	-	-	P	P	-	-	P	P
6 (Petrol)	-	N	N	N	P	N	P	P
8 (Petrol)	-	P	N	P	P	-	P	P
2 (Kerosene)	-	K	K	K	K	-	K	K
7 (Kerosene)	-	-	K	-	-	-	K	K
9 (Kerosene)	-	-	K	K	-	-	K	K
10 (Kerosene)	-	-	K	K	K	K	K	K
11 (Kerosene)	-	K	K	K	-	-	K	K
12 (Kerosene)	-	-	-	-	-	-	-	-
13 (Burnt)	-	N	N	N	N	N	N	N
20 (Burnt)	-	N	N	N	-	-	N	N
21 (Burnt)	-	N	N	N	N	N	N	N
22 (Burnt)	-	-	N	-	-	-	P	N
23 (Burnt)	-	N	N	N	N	-	N	N
24 (Burnt)	-	N	N	N	N	N	N	N
14 (Unburnt)	-	N	N	N	-	-	N	N
15 (Unburnt)	-	-	N	N	N	-	N	N
16 (Unburnt)	-	-	N	N	N	N	N	N
17 (Unburnt)	-	-	N	N	N	N	N	K
18 (Unburnt)	-	N	N	N	N	-	P	P
19 (Unburnt)	-	N	N	N	N	N	N	N

Table 5.10: *Summary of the chromatographic results obtained for all the entomological samples collected throughout Experiment 2. Samples are denoted as positive for petrol (P), kerosene (K), negative for an ignitable liquid (N), or (-) where a sample was not collected.*

The results obtained for the piglets burnt with petrol indicate that in Experiments 1 and 2, 90% and 76.1% of larvae contained traces of petrol, respectively. These findings

are important given the volatile nature of petrol, and the experimental set-up in these fieldwork experiments. Additionally (as was also the case in the small-scale experiments), virtually all the larval samples that were collected from the piglets burnt with kerosene contained traces of this ignitable liquid. These results indicate that both petrol and kerosene were still present at reportable levels in the insects collected, even after having been exposed to the external environment for up to eight days.

Ignitable liquids were also detected in both the adult and puparial samples that were analysed. In Experiments 1 and 2, for the petrol sample sets, 50% and 83.3% of adults, respectively, and 66.7% of pupae (in both experiments) contained traces of petrol. However, all of the adult and puparial samples collected from the piglets burnt with kerosene contained traces of this ignitable liquid. These results are not dissimilar to those obtained in the small-scale experiments, in that kerosene was detected in more of the samples than petrol. Despite this there is little difference between the entomological samples (in each ignitable liquid) in terms of the results obtained. This finding is significant given the behaviour of a post-feeding larva. That is, any remaining food is expelled from its crop. Irrespective of this, a high percentage of adult and puparial samples still tested positive for an ignitable liquid. This suggests that the larvae may possess the ability to bioaccumulate the ignitable liquid residues. This is potentially a significant finding in terms of a fire investigation, hence further testing is required to confirm this. In addition, further investigation into the mechanism of metamorphosis and how it affects different ignitable liquids should be undertaken.

The results obtained from the larval, adult and puparia samples collected from the piglets burnt with an ignitable liquid highlight the potential use of these sample types in casework. It has been discussed previously that both petrol and kerosene comprise volatile components that evaporate rapidly rather than remain in fire debris. The results obtained, however, indicate that these ignitable liquids can be detected for as long as eight days in larval samples, as well as in flies and pupal samples for at least one month (representing the approximate time when these samples were collected). The puparia have the potential to extend the sampling period further as they do not

breakdown and, unless otherwise moved, will remain *in situ* for sometime [12]. This has the potential to be a significant advantage of utilising this new sample type. However, it is important that this claim be further confirmed through testing of this sample type over an extended period to determine the maximum period ignitable liquids can be detected. Moreover, if this type of sample is to be collected for casework purposes, it is important that the area where the remains are located be searched for presence of puparia, as it is known that post feeding larvae can travel considerable distances in order to pupate [38].

It has been well documented that petrol and kerosene are highly volatile ignitable liquids (particularly petrol) and evaporate over time. Therefore, it is unclear why the profiles obtained from the entomological samples did not demonstrate consistent evaporation from the first day they were exposed to ambient conditions, as is observed with traditional fire debris. This is another significant advantage of using entomological samples. It is postulated that this could be due to the fact that larvae feed in protected areas such as the mouth, which would not be as susceptible to the effects of evaporation. However, by the third day of exposure, many of these larvae were no longer feeding in protected areas, as many of the organs were exposed. Again this suggests that the larvae may possess a mechanism for bioaccumulation (as has been previously noted for mercury), that allows them to retain these ignitable liquids [40,41]. Further investigation is required to confirm this hypothesis.

Further to this, the results obtained are important given Experiment 1 (conducted in spring) experienced temperatures as high as approximately 40 ^{o}C, and approximately 30 ^{o}C during Experiment 2 (conducted in summer). These extreme temperatures would have accelerated the evaporation of the ignitable liquids present, despite this a high percentage of entomological samples tested positive for an ignitable liquid.

It is important to note that all of the piglets from individual sample sets were prepared in the same way. The only difference was that they were stored in different locations. Analysis of the temperature data has shown that the sites experienced different

minimum, mean and (to a lesser extent) maximum temperatures. Since it is known that temperature has the greatest effect on decomposition, this may explain why certain piglets were colonised earlier than others. Although the ambient conditions experienced in the first experiment (compared with the second) were much warmer, and there was no rainfall, there does not appear to be a major difference between the results obtained from each experiment in terms of the ignitable liquids detected. In the first experiment, when the conditions were warmer, oviposition was noted earlier than in the second experiment, and as a result more samples were collected in this experiment. This follows the finding that rainfall may reduce fly activity [31].

In the small-scale experiments, considerable sample-to-sample variation was observed. For example, a larval sample was observed to be negative for an ignitable liquid, but in the days immediately preceding it and following it, the samples were positive. This phenomenon was only observed once in the fieldwork experiments. Therefore, it was concluded that such sample-to-sample variations decreased because of the larger-scale of the experiments.

The samples derived from the control groups were generally negative for any traces of ignitable liquids; however, 7.7% of these samples contained traces of an ignitable liquid. It is important to note that these samples were derived from different piglets, demonstrating that the entire population was not affected. In addition, many control measures were set in place to ensure no physical contamination could occur. All but one of these apparently contaminated samples contained traces of petrol, the other was contaminated with traces of kerosene. However, the compounds observed in the control sample sets included 1,2,3-trimethylbenzene and 1,3,5-trimethylbenzene. These compounds can be classified as matrix interferences in the form of volatile organic compounds that are known to evolve from decaying remains [150,151].

In the majority of samples, despite the presence of these compounds, the control groups could be differentiated from groups containing ignitable liquids. However, this became much more challenging as the larval growth stages progressed: as the

abundance of petrol and kerosene dropped, it was increasingly difficult to differentiate between samples that contained possible matrix interferences and samples that did not contain any detectable levels of ignitable liquid. This could therefore explain the number of samples classified as positive for petrol in the control samples.

One of the control samples contained high levels of kerosene, and was the only sample in which it is likely that a contamination event had occurred. This is most likely due to carryover (in the SPME-GC-MS analysis) from a high-kerosene sample that had been analysed immediately preceding this sample. Obviously, if this method was to be introduced for casework purposes, a limited number of samples would be analysed at a time. Therefore, it would be possible to analyse a blank or even several blanks in between samples to demonstrate that no such carryover had occurred. These findings highlight several issues. First, extreme caution should be taken when interpreting results that contain only low level peaks. It may be necessary to introduce a cut-off threshold, below which peaks cannot be relied upon. Further to this, it is recommended that blanks be run in between samples and are inspected for any target compounds. If they are indeed present then a second blank may be required to eliminate further carryover.

Additionally, it has been reported that chemical results obtained from fly larvae are less likely to contain interferences from decomposition by-products compared with other traditional specimens [47,48,50]. This was observed to be the case in the current study, as only two volatile organic compounds associated with decomposition (from a vast number that have been observed in other studies) were found here. Therefore, it seems reasonable to conclude that this method has decreased the interference from decomposition products.

The results obtained from this experiment indicate that the SPME-GC-MS method presented can be used to successfully detect petrol and kerosene in entomological samples. These findings have highlighted the potential use of entomological samples

in cases involving burnt remains and thus demonstrate their use in the field of fire investigation.

5.5.5 Data Analysis

In the small-scale experiments, the main reason for performing PCA was to remove the bias that may have been introduced through the analyst having knowledge of the sample set that each chromatogram was derived from. This was not an issue in the fieldwork experiments, as the analyst had no prior knowledge of the sample. However, PCA was still employed for exploratory analysis of the data.

As discussed in Section 5.45, an in-house program was used on the chromatographic data to integrate the peaks corresponding to the 25 target compounds in Table 5.6, using an 11-point integration.

A number of volatile organic compounds associated with decomposition that have been reported in the literature were detected in the sample sets burnt with and without an ignitable liquid. Therefore, PCA was performed with the variables representing 1,2,3-trimethylbenzene and 1,3,5-trimethylbenzene removed (see Figure 5.19). The graphical representation (scores plot) of the first three principal components (representing the greatest variability) obtained have not separated the sample sets any further or resulted in the groups clustering any tighter than the scores plots constructed with these volatile organic compounds present. These results indicate that the volatile organic compounds that were detected are not responsible for the differences observed between the groups. Based on this finding, the scores plot containing all of the target compounds was used for further interpretation.

Experiment 1: 11-point integration **Experiment 2: 11-point integration**

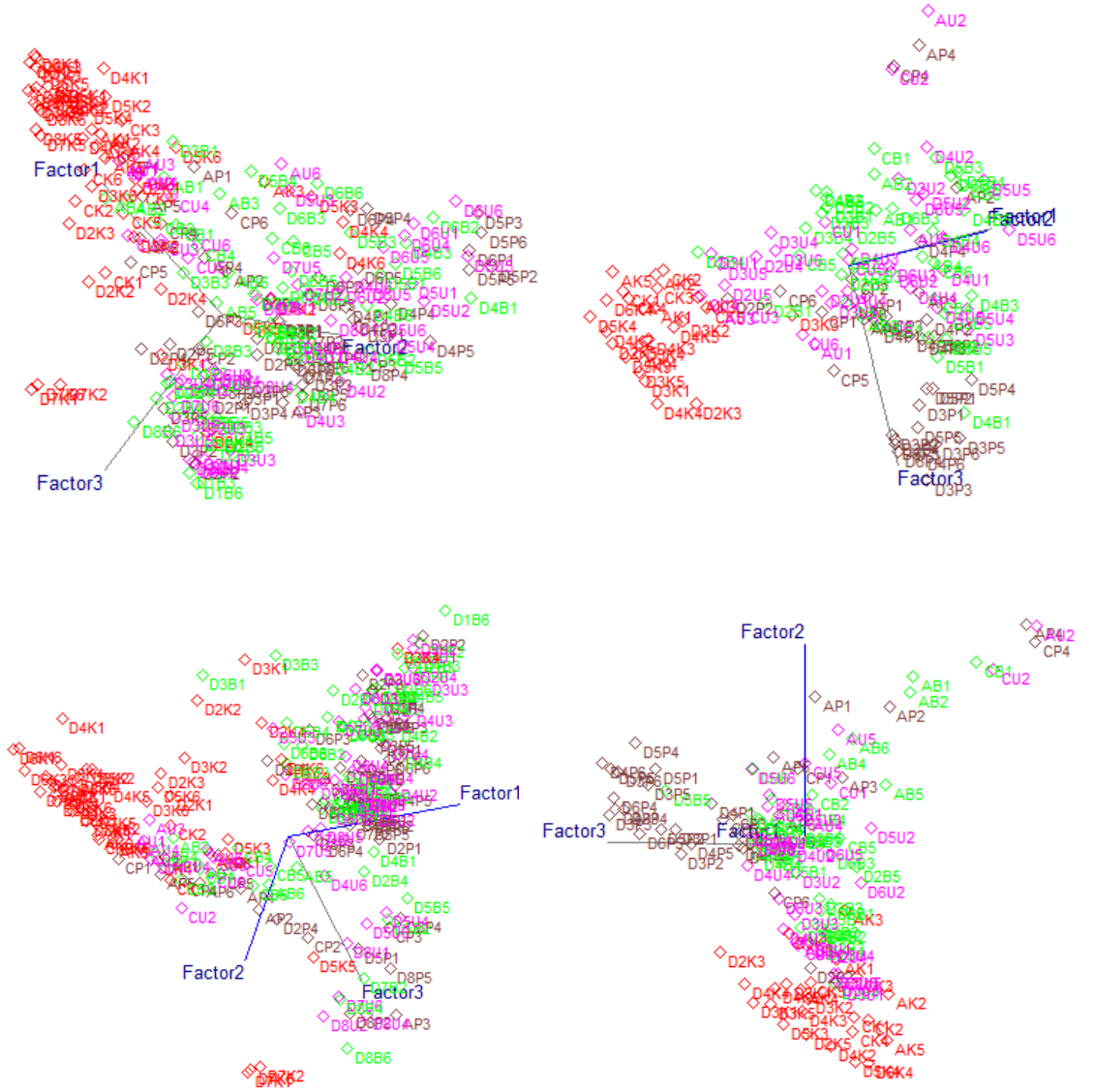


Figure 5.19: *Scores plots constructed for Experiments 1 and 2 with the volatile organic compounds removed (top) and with the volatile organic compounds present (bottom), constructed using an 11-point integration width.*

◇ **Petrol sample**

◇ **Kerosene sample**

◇ **Burnt sample**

◇ **Unburnt sample**

The PCA scores plots obtained for Experiment 1 demonstrate that the petrol, kerosene and control groups can be distinguished from each other (see Figure 5.19) although some overlapping of groups is evident. The close association (clustering together) of the control groups, even though they are two separate sample sets, was not unexpected given that these groups contained few, if any, target compounds that corresponded to the ignitable liquids of interest.

The scores plot obtained for Experiment 2 demonstrates better separation between sample sets. However, there appears to be more sample-to-sample variation within the groups, as the individual sample sets are not as tightly clustered as was observed in Experiment 1. This observation can be rationalised using the differences between the experiments. Experiment 1 experienced higher temperatures than Experiment 2. It is known that higher temperatures will increase the rate of evaporation of volatile compounds, and since petrol comprises more volatile compounds than kerosene, it would be more greatly affected by these conditions. In addition, the differences noted between the ignitable liquids may also be attributed to variability in the extraction process.

In both experiments, the control groups are fairly diffuse, and there is some overlap with the ignitable liquid groups. This overlap can be attributed to results where an insufficient number of target compounds were detected from the petrol and kerosene samples (possibly due to evaporation), and thus the samples were categorised as negative. The sample derived from the control group that was positive for kerosene has clustered with the other kerosene samples. Interestingly, the samples from the control groups that were positive for petrol have clustered with the diffuse group of negative samples. This finding supports the conclusion drawn from the chemical analysis data that, although these samples were considered positive for petrol residues, only low levels of the target compounds were present. This further supports the recommendation that a threshold should be applied, below which peaks cannot be relied upon.

A one-way multivariate analysis of variance (MANOVA) was performed for Experiments 1 and 2 to investigate the differences between the sample sets. The scores for the first seven principal components (PC1-PC7) were used as the dependent variables, as these represented the greatest variability in the data. In fact, these components together explained 90% of the variability in the data. The independent variable, *sample set,* comprised four groups: petrol, kerosene, burnt and unburnt. (Preliminary assumption testing was conducted to check normality, linearity, univariate and multivariate outliers, homogeneity of variance-covariance matrices, and multicollinearity, with no serious violations noted.)

In Experiment 1, a statistically significant difference was found between the four sample sets on the combined dependent variables: $F_{21,594}$ =8.82, $p<0.05$, Pillai's Trace=0.713, partial eta squared = 0.238. When the results for the dependent variables (PC1-PC7) were considered separately, the only difference to reach statistical significance, using a Bonferroni adjusted alpha value of 0.007, was PC1 and PC4: (PC1: $F_{3,202}$ = 72.19, $p<0.001$, partial eta squared = 0.517; PC4: $F_{3, 202}$ = 8.211, $p<0.001$, partial eta squared = 0.109). This indicates that although seven principal components were chosen, only PC1 and PC4 were responsible for the differences between the sample sets.

Post hoc analysis for PC1 and PC4 consisted of conducting pair-wise comparisons. This revealed that, based on PC1 and PC4, the petrol, burnt and unburnt sample sets did not differ based on their means, whereas the kerosene sample set resulted in statistical differences in the means when paired with the petrol, burnt and unburnt sample sets. This indicates that kerosene is the only group that is statistically different from the other sample sets in Experiment 1. This result is more than likely due to the number of evaporated samples present in the petrol sample set that did not contain an ignitable liquid. Obviously, these samples would not be significantly different from the samples derived from the control groups. If we consider the chromatographic results, the majority of burnt and unburnt samples could be differentiated from the petrol

samples. Furthermore, these results emphasise that, based on PC1 and PC4, the control groups do not differ.

A statistically significant difference was also found between the four sample sets on the combined dependent variables in Experiment 2: $F_{21,354}$ =9.91, p<0.05, Pillai's Trace=1.11, partial eta squared = 0.370. When the results for the dependent variables (PC1-PC7) were considered separately, the only difference to reach statistical significance, using a Bonferroni adjusted alpha value of 0.007, was PC1, PC2 and PC3: (PC1: $F_{3,122}$ = 16.27, p<0.001, partial eta squared = 0.286; PC2: $F_{3,122}$ = 39.79, p<0.001, partial eta squared = 0.495); PC3: $F_{3,122}$ = 13.05, p<0.001, partial eta squared = 0.243). This indicates that although seven principal components were chosen, only PC1, PC2 and PC3 were responsible for the differences between the sample sets.

Post hoc analysis for PC1, PC2 and PC3 consisted of conducting pair-wise comparisons. This revealed that, based on PC1 and PC2, petrol differed from all other groups in terms of the sample means, as did kerosene. Conversely, the control groups did not differ from each other based on their means. Further to this, based on PC3, the petrol sample set was considered statistically different from the other sample sets based on the sample means. However, a comparison of the kerosene and control groups based on this factor did not result in differences.

Although the MANOVA results cannot be compared between the experiments as the principal components in each experiment represent different factors, the results obtained for Experiment 2 correspond with the differences observed in the scores plot and the fact that evaporation of the ignitable liquids would have been more pronounced in Experiment 1 due to the ambient conditions experienced. These results indicate that, based on PC1 and PC2 (the factors that contain the greatest variability in the data set), the ignitable liquid groups differ both from the control groups and each other. Hence, this method is able to successfully differentiate between the three groups. Based on PC3, petrol is found to be statistically different from the remaining groups, although kerosene was not found to be statistically different based on this

factor. This finding differs from Experiment 1, where petrol was found not to differ from the control groups. It is postulated that the ambient conditions are responsible for this difference.

In summary, statistical differences were noted between the petrol and kerosene sample sets using principal component analysis (PCA) and multivariate analysis of variance (MANOVA) in both Experiment 1 and 2. This is an important finding as it indicates that even though these ignitable liquids share a number of common target compounds within their distinctive profiles, these ignitable liquids can still be identified as separate sample sets. Conversely, the PCA results from the burnt and unburnt control groups are fairly diffuse, particularly throughout Experiment 1 and there is some overlap with the ignitable liquid groups. However, according to the MANOVA results in both experiments, these control groups were not considered statistically different from each other based on any of the principal components. This indicates that although volatile organic compounds were detected in many of these control samples and some of these were even categorised as petrol, this did not result in a statistical difference between the groups.

5.6 Conclusion

The method designed and tested in these fieldwork experiments was successful in detecting petrol and kerosene in entomological samples, as well as identifying the control groups as negative for these ignitable liquid residues. This project was the first large-scale study carried out using entomological samples for the purposes of detecting ignitable liquids that have been used to burn remains. These findings are significant as they have potential implications for casework. They demonstrate that entomological evidence may be a good alternative to traditional fire debris analysis when burnt human remains are involved, and therefore they can play an important role in the field of fire investigation. Further to this, the results obtained indicate that

entomological samples have the ability to potentially increase the sampling period. In addition, a novel use for the SPME-GC-MS techniques has been explored.

Chapter 6:

Conclusions and Further Work

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6.1 Summary of Findings

Entomological evidence has been shown to be valuable in the field of forensic science to assist with the determination of toxic substances such as drugs, environmental pollutants and pesticides in carrion feeding insects. To date, studies in this area have focused on the detection of substances that may have contributed to the cause of death. In this thesis, the idea was extended to consider the detection of ignitable liquids, such as petrol and kerosene, used to accelerate the spread of a fire and possibly contribute to death in this way. While there are a number of techniques designed to detect such substances in fire debris, such analysis still presents some challenges. Firstly, ignitable liquids are extremely volatile and will therefore begin to evaporate as soon as they are exposed to the elements. Typical fire scene conditions (high temperatures) will cause ignitable liquids to evaporate more rapidly than usual. Subsequent exposure to the elements will only cause further evaporation. This evaporation modifies the GC profile of ignitable liquids such that the lighter, more volatile components (which diffuse into the air first) will be absent and the heavier, less volatile components will dominate the profile. Consequently, too few compounds will be present to report the presence of an ignitable liquid. Therefore, time is the critical factor when attempting to recover ignitable liquids.

Due to these significant downfalls, an alternative type of sample was sought where the drawbacks of the chemical analysis of ignitable liquids were not so pronounced. Therefore, the purpose of this project was to investigate the potential use of entomological samples (as an alternative to fire debris evidence) at fire scenes involving burnt human remains. This was successfully achieved through two major studies: small-scale laboratory controlled experiments and larger-scale fieldwork experiments.

6.1.1 Small-Scale Experiment

The results obtained from the small-scale experiments, using the SPME-GC-MS technique developed that was optimised for this project, indicated that both petrol and kerosene could be successfully detected in the larvae of the blow fly *Lucilia cuprina* (Wiedemann) (Diptera: Calliphoridae) for up to five days after the initial burn. In addition, petrol and kerosene could also be detected in a limited number of adult and puparial samples for up to four. These results were important as they demonstrated that the method could successfully detect ignitable liquids in entomological samples even after these volatiles had been ignited and exposed in a controlled environment. Moreover, these findings were significant to the field of fire investigation and entomotoxicology as they demonstrated a novel use for SPME and highlighted another foreign substance that could be detected in entomological samples.

6.1.2 Fieldwork Experiments

The preliminary findings obtained from the small-scale experiments indicated that there was scope for further research. Therefore, a second study was performed larger-scale fieldwork experiments that mirrored a real casework scenario more closely. The purpose of extending this study to encompass a larger fieldwork design was to determine whether the method had the ability to withstand the greatly increased variability presented by the external environment. This demonstration is important if the method is to be adopted for casework purposes.

During the course of this study, a systematic methodology was optimised and validated that involved a SPME-GC-MS technique using a selective ion monitoring system. It was found during these fieldwork experiments that petrol and kerosene could be successfully detected in larvae after as many as eight days, in spring and summer conditions, as well as in adult and puparial samples, which were collected in

excess of one month after the initial exposure of the piglet. These findings demonstrate the significant advantage of using insects as an alternative to fire debris in that they extend the sampling period. That is, due to the volatile nature of petrol and kerosene, it would be extremely unlikely that these ignitable liquids would otherwise persist on traditional fire debris for such an extended period. Further to this, puparia can withstand changing climatic conditions, and unlike the larvae and adults, are immobile and unless otherwise disturbed also have the potential to remain in the vicinity of human remains for extended periods. This aspect of using entomological samples will be of importance when the remains are located in a remote area, as it is likely considerable time will elapse before they are discovered. Therefore, these findings are significant for future fire investigations.

Due to the number of replicates involved in the experiments, the chromatographic data could be evaluated both visually (as is common practice) and through a chemometric technique (PCA). This technique successfully removed any bias from the analyst, issues associated with the weathering of the ignitable liquids and any matrix interferences. Therefore, it is highly recommended that PCA be used for future experiments involving large data sets.

In addition, the experiments conducted in this project are important as they not only highlight a novel type of sample that can be used for the chemical analysis of fire debris and demonstrate a new application for SPME, but they also represent the first large-scale study to be conducted in New South Wales, and one of only a few conducted in Australia.

6.2 Collection and Storage Protocol

The research presented in this thesis has direct implications for casework. It is important when proposing a new method, particularly for casework purposes, to devise a protocol for the appropriate collection and preservation of entomological samples. If the entomological samples are located in a number of areas surrounding

the remains, it is recommended that a number of disposable forceps be used to avoid cross contamination. It is also important that if entomological samples are present in a number of areas on the remains, then a representative sample, comprising approximately six larvae, be collected into SPME vials. These vials should be stored in a freezer until they are ready for analysis. When conducting the chemical analysis (GC-MS), it is important that two fibre blanks be run initially and that at least one blank be run in between successive samples. Furthermore, the SPME vials should be placed in an oven set at 90 ^{o}C for approximately one hour to ensure that the headspace has achieved this temperature. It is recommended that if the SPME-GC-MS method presented in Sections 5.43 and 5.44 is to be adopted for casework purposes, additional experiments should be carried out to ensure the method specifications are optimised based on the available instrumentation. The results obtained should be interpreted through visual pattern matching against known reference chromatograms, based on the presence of target compounds and guidelines set out by the current ASTM method. However, extreme caution should be taken when interpreting results that contain only low level peaks. It is highly recommended that a cut-off threshold be introduced, further research is required to determine an appropriate threshold.

6.3 Further Work

The purpose of this project was to investigate the detection of ignitable liquids in entomological samples. This was achieved by designing and optimising a technique using SPME-GC-MS and testing this through two major studies. In order for this method to be integrated into the field of fire investigation further research is required. Areas of further research include, a study involving washed larvae in order to construct evaporation over time profiles. Further to this, a study should be conducted involving burnt piglets where entomological samples as well as traditional fire debris sample are collected over a number of months and their results compared. This will provide a direct comparison on how this potentially new type of evidence performs against traditional fire debris. If tissue samples are included in any further studies, the claim that entomological samples contain fewer decomposition by-products can be

investigated. In addition, it would be interesting to collect data from all seasons of the year, not merely spring and summer to determine if higher abundances of the ignitable liquids are obtained in the cooler months of the year.

The results obtained in this thesis, particularly with regard to the adults and puparia, are significant due to the actions that precede the pupation stage. Post-feeding larvae will expel any remaining food from their crops. Since the ignitable liquids of interest could be identified in a number of adults and puparia, it is possible that the larvae bioaccumulate these chemicals. Therefore the possible bioaccumulation of these residues and the effect that metamorphosis may have on entomological samples should be researched further, as these factors may also be responsible for extending the sampling period.

Further to this, it will be important to determine (under different conditions) how long an ignitable liquid can be detected in the puparia. Moreover, it will be important to extend this study to include a larger number of common ignitable liquids. Such a study will be significant in providing a true indication of the sampling period of the entomological samples.

In this project insects were found to colonise the petrol and burnt sample sets faster than the kerosene and unburnt sample sets. It is possible that the chemicals created by burning a piglet (with or without the use of petrol) trigger an olfactory response in insects. It is recommended that further research be conducted into the chemicals that trigger an olfactory response in flies, and those that result in a delay in oviposition, as observed in these experiments.

It was beyond the scope of this study to determine if insect growth is affected by the presence of ignitable liquids present on the tissue in which they are feeding. If these samples are to be adopted for casework purposes, this is an important factor to consider further, as it has the potential to affect a PMI calculation.

Finally, the insects that were identified from the fieldwork experiments were compared with insect activity data compiled from Victoria, Australia, and were consistent with insects normally present at those times of the year. Ideally, data from New South Wales should have been used, but no such study has been conducted in this state. It is therefore recommended that yearly activity patterns of carrion-breeding flies in New South Wales be compiled.

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Appendix A

Appendix A: Macro used for GC-MS Analysis

```
!File   : deuser.mac
!Macros : CustomAnalysis

NAME CustomAnalysis
        remove x
        ! file
        TIC 1.75:20
        chromat 1.75:20,57
        chromat 1.75:20,71
        add
        chromat 1.75:20,85
        add
        chromat 1.75:20,99
        add
        merge
        chromat 1.75:20,55
        chromat 1.75:20,69
        add
        chromat 1.75:20,83
        add
        chromat 1.75:20,97
        add
        merge
        chromat 1.75:20,91
        chromat 1.75:20,105
        add
        chromat 1.75:20,133
        add
        merge
        chromat 1.75:20,104
        chromat 1.75:20,118
        add
        chromat 1.75:20,132
        add
        chromat 1.75:20,146
        add
        merge
        chromat 1.75:20,128
        chromat 1.75:20,142
        add
        chromat 1.75:20,156
        add
        chromat 1.75:20,170
        add
        merge
        ! chromat 1.75:20,154
        ! chromat 1.75:20,168
        ! add
        ! chromat 1.75:20,182
        ! add
        ! merge
        format separated,labelpeaks,1,1
        draw 3,x

        pagesize char,line
        startprint
        winprint 3,0,0,char,line
        endprint
        !REMOVE CustomAnalysis
        ! return
```

Appendix B

Appendix B: Macro used for Small-Scale Experiments

```
Sub LisaPetrolKeroPeakIntegrate()
'
' LisaPetrolKeroPeakIntegrate Macro
' Macro to add intensities around chosen retention times for petrol and kerosene
'
' Keyboard Shortcut: Ctrl+Shift+P
'
Dim Path As String
Dim CurrentFile As String
Dim RetentionTimes(33) As Integer

RetentionTimes(0) = 1077
RetentionTimes(1) = 1188
RetentionTimes(2) = 1216
RetentionTimes(3) = 1306
RetentionTimes(4) = 1363
RetentionTimes(5) = 1366
RetentionTimes(6) = 1480
RetentionTimes(7) = 1614
RetentionTimes(8) = 1709
RetentionTimes(9) = 1725
RetentionTimes(10) = 1792
RetentionTimes(11) = 1807
RetentionTimes(12) = 1842
RetentionTimes(13) = 2037
RetentionTimes(14) = 2195
RetentionTimes(15) = 2231
RetentionTimes(16) = 2408
RetentionTimes(17) = 2413
RetentionTimes(18) = 2470
RetentionTimes(19) = 2572
RetentionTimes(20) = 2753
RetentionTimes(21) = 2757
RetentionTimes(22) = 2797
RetentionTimes(23) = 2840
RetentionTimes(24) = 2923
RetentionTimes(25) = 3077
RetentionTimes(26) = 3384
RetentionTimes(27) = 3606
RetentionTimes(28) = 3610
RetentionTimes(29) = 3760
RetentionTimes(30) = 3770
RetentionTimes(31) = 3885
RetentionTimes(32) = 3988
RetentionTimes(33) = 4081

Dim Count As Integer
Dim FileID As String
Dim LineCount As Integer
Dim IntRange As Integer

IntRange = InputBox("Enter the number of points (on either side of peaks) to be integrated:")
Path = InputBox("Enter the pathname:")
If Right(Path, 1) <> "\" Then Path = Path & "\"
LineCount = 0
Workbooks.Add

CurrentFile = Dir(Path & "*.*")
ChDir Path

Do While CurrentFile <> ""
```

```
    Workbooks.Open FileName:=CurrentFile

'Remove the .D from data file name and store the rest for pasting later
    FileID = ActiveSheet.Range("B2").Value
    FileID = Left(FileID, Len(FileID) - 2)

'Delete first 3 rows
    ActiveSheet.Rows("1:3").Select
    Selection.Delete Shift:=xlUp
    Range("C1").Select

'Add (bin) intensities for specified number of points (IntRange) on either side of retention time
    For Count = 0 To 33
    Cells(Count + 1, 3).Value = Application.Sum(Range("B" & (RetentionTimes(Count) - IntRange), "B" & (RetentionTimes(Count) +
IntRange)))
    Next

'Keep track of number of files so far (and rows in the master workbook)
    LineCount = LineCount + 1

'Copy binned intensities
    Range("C1:C34").Select
    Selection.Copy

'Paste binned intensities into master workbook, along with data file name
    Workbooks("Book1").Worksheets("Sheet1").Range("B" & LineCount).PasteSpecial Transpose:=True
    Workbooks("Book1").Worksheets("Sheet1").Range("A" & LineCount).Value = FileID
    Application.CutCopyMode = False

'Save and close original file
    Workbooks(CurrentFile).Close SaveChanges:=True

    CurrentFile = Dir
Loop

End Sub
```

Appendix C

Appendix C: Macro used for Fieldwork Experiments

```
Sub LisaPetrolKeroPeakIntegrate2()
'
' LisaPetrolKeroPeakIntegrate2 Macro
' Macro to add intensities around chosen retention times for petrol and kerosene (field work data)
'
' Keyboard Shortcut: Ctrl+Shift+Q
'
Dim Path As String
Dim CurrentFile As String
Dim RetentionTimes(24) As Integer

RetentionTimes(0) = 554
RetentionTimes(1) = 613
RetentionTimes(2) = 627
RetentionTimes(3) = 675
RetentionTimes(4) = 850
RetentionTimes(5) = 887
RetentionTimes(6) = 895
RetentionTimes(7) = 1055
RetentionTimes(8) = 1157
RetentionTimes(9) = 1249
RetentionTimes(10) = 1253
RetentionTimes(11) = 1282
RetentionTimes(12) = 1428
RetentionTimes(13) = 1431
RetentionTimes(14) = 1450
RetentionTimes(15) = 1474
RetentionTimes(16) = 1596
RetentionTimes(17) = 1753
RetentionTimes(18) = 1868
RetentionTimes(19) = 1871
RetentionTimes(20) = 1948
RetentionTimes(21) = 1952
RetentionTimes(22) = 2011
RetentionTimes(23) = 2064
RetentionTimes(24) = 2112

Dim Count As Integer
Dim FileID As String
Dim LineCount As Integer
Dim IntRange As Integer

IntRange = InputBox("Enter the number of points (on either side of peaks) to be integrated:")
Path = InputBox("Enter the pathname:")
If Right(Path, 1) <> "\" Then Path = Path & "\"
LineCount = 0
Workbooks.Add

CurrentFile = Dir(Path & "*.*")
ChDir Path

Do While CurrentFile <> ""

   Workbooks.Open FileName:=CurrentFile

'Store the filename without extension for pasting later
   FileID = Left(CurrentFile, Len(CurrentFile) - 4)

'Delete first 3 rows
   ActiveSheet.Rows("1:3").Select
```

```
    Selection.Delete Shift:=xlUp
    Range("C1").Select

'Add (bin) intensities for specified number of points (IntRange) on either side of retention time
    For Count = 0 To 24
    Cells(Count + 1, 3).Value = Application.Sum(Range("B" & (RetentionTimes(Count) - IntRange), "B" & (RetentionTimes(Count) +
IntRange)))
    Next

'Keep track of number of files so far (and rows in the master workbook)
    LineCount = LineCount + 1

'Copy binned intensities
    Range("C1:C25").Select
    Selection.Copy

'Paste binned intensities into master workbook, along with data file name
    Workbooks("Book1").Worksheets("Sheet1").Range("B" & LineCount).PasteSpecial Transpose:=True
    Workbooks("Book1").Worksheets("Sheet1").Range("A" & LineCount).Value = FileID
    Application.CutCopyMode = False

'Save and close original file
    Workbooks(CurrentFile).Close SaveChanges:=True

    CurrentFile = Dir
Loop

End Sub
```

Appendix D

Appendix D: Publications

The following manuscripts relate directly to the research presented in this thesis

1) L. Mingari, Is forensic entomology a viable part of fire investigation? Fire and Arson Investigator April (2008) 48-49.
2) L. Mingari, B. Reedy, J. Wallman, The detection of ignitable liquids in blow fly larvae. *Submitted to Science & Justice.*

IS FORENSIC ENTOMOLOGY A VIABLE PART OF FIRE INVESTIGATION?

BY LISA MINGARI, UNIVERSITY OF TECHNOLOGY, SYDNEY, NEW SOUTH WALES, AUSTRALIA

Forensic entomology is a branch of forensic science that applies entomology, the study of insects, to legal problems[1]. With this definition in mind, it may seem unusual that forensic entomology is proposed as a tool to compliment fire investigation. The potential use of forensic entomology in fire investigation is what I will be highlighting throughout my post graduate research project, at the University of Technology, Sydney.

Fire and forensic entomology are by no means synonymous research topics. In fact, few studies have incorporated the two areas. There are but two published studies that link the areas. The first examined the succession of insects on burnt and unburnt pig carcasses[2]. This study indicated that insects were just as attracted to pig carcasses burnt with petrol, compared with unburnt carcasses. The second study explored the survival of entomological evidence after a fire, and the type of evidence entomologists should search for[3]. Despite the definite lack of research in this area, there have not been any studies conducted to rectify the issue. This gap in the research was what inspired me to embark on this project.

Entomotoxicology, the toxicological analysis of larvae, has been the subject of a vast amount of scientific research. Entomotoxicology has seen larvae used as a means to detect countless drugs and toxins including: copper, iron, zinc and calcium[4]; mercury[5]; barbiturates[6]; opiates[7]; benzodiazepines[8]. This poses the question: if so many drugs and toxins can be detected through the analysis of larvae, then can we not do the same with ignitable liquids? The results of research conducted during this study have answered that question. However, it may seem logical to ask: how will these findings benefit fire investigation?

According to the Australian Institute of Criminology, 64 percent of fires in 2001/02 to 2003/04 were considered deliberate[9]. In addition, the rate of arson has doubled every eight years since 1964[10]. These figures indicate that arson is an ever-increasing problem. If the method that I am proposing will help to reduce these figures, even slightly, then it will most definitely be useful. The method that is being presented will be particularly useful when a body is located a considerable period after the fire has ceased. Ignitable liquids characteristically evaporate over time[11]. Hence, the probability of standard fire debris providing a positive result for an ignitable liquid, if it is indeed present, would be low. The collection of larvae from a cadaver is therefore presented as a potential alternative substrate to fire debris, or perhaps it may also compliment fire debris in certain cases.

vae; developing a method for the sampling and storage of these samples and finally; assessing the method in a fieldwork situation.

The lack of research available in this area made developing a method for the detection of ignitable liquids challenging. A method was designed under the premise that small levels of petrol and kerosene would be detected hence, it was essential for the method to offer high sensitivity. With this in mind solid phase microextraction (SPME) was selected as the extraction method. SPME is a highly sensitive, solvent free extraction technique. It involves the use of an adsorbent bonded to a fibre. The sample is exposed to the fibre for a pre-determined period at a particular temperature. In that time the volatiles from the fibre are extracted and adsorb onto adsorption sites on the fibre. The fibre can then be injected into the port of a gas chromatograph-mass spectrometer (GC-MS) where the extracted volatiles can be analysed. Due to the countless number of ignitable liquids available, only petrol and kerosene were selected for this study as they are the most prevalent in fires[12].

The next major portion of experiments tested a variety of methods that could be used to analyse larval samples collected from burnt flesh. The methods tested were derived from those used for both fire debris and entomological evidence. The reason for selecting methods from both groups was that larvae collected from burnt flesh could be considered as both a type of fire debris and entomological evidence. Since fire debris is routinely collected in unlined paint tins, and stored at room temperature this method was adopted. Entomological samples on the other hand are routinely stored in vials with 70 to 95 percent ethanol and stored at room temperature. Hence, this technique was also trialled as well as storing the larval samples in vials in the freezer. The latter proved to give the best result. The other issue with the method being proposed was that all accepted entomotoxicology methods wash the larvae prior to analysis. This is viewed as an important step among the entomology community. By washing the larvae the results obtained can be shown to come from what the larvae has ingested, rather than being present merely on the surface of their bodies. Experiments were therefore carried out where the larvae were washed in a variety of liquids. Positive results for both petrol and kerosene were still obtained after washing. This indicated that the larvae were ingesting the ignitable liquids. Despite these results, the washing step was not adopted as part of the final method. This was due to the fact that in fire investigation a positive result for an ignitable liquid would be significant, irrespective of whether it is found to be ingested or merely on the surface of the larva. In addition, it is important to maximise the concentration of ignitable liquids present, rather than wash a portion of them away.

After considerable method development, the final method was proposed that gave optimum results during both the stages of extraction and analysis. The final set of experiments will test the proposed method at an external site, using neonatal piglets. This portion of the experimental work is important as it will allow the proposed method to be further scrutinised in more of a "real case" situation. The fieldwork will be carried out in a bushland area in Western Sydney, in each season of the next year.

There are several reasons for conducting these experiments seasonally. Firstly, it will be interesting to note if there is a seasonal difference in positive results for the ignitable liquids of interest. That is, in summer when many insects colonise the carcass rapidly, is the ignitable liquid detected for a shorter period compared to winter, when colonisation occurs much more slowly? It will also be interesting to note the differences in species that colonise the piglets in each season. Results from this study will be beneficial to both the entomology and fire community as there has not been such a study undertaken anywhere else in the world.

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The Detection of Ignitable Liquids in Blow Fly Larvae

Abstract

The analysis of fire debris can indicate the presence of an ignitable liquid however due to the volatility of ignitable liquids, the likelihood of detecting them diminishes over time. As an alternative to fire debris, the analysis of fly larvae for the detection of ignitable liquids is proposed when a scene contains burnt human remains.

Petrol and kerosene were able to be detected in larvae of the blow fly *Lucilia cuprina* (Wiedemann) (Diptera: Calliphoridae) that had been fed on meat burnt using these ignitable liquids. Four samples sets of meat, each with six replicates (twenty-four meat samples in total) were prepared. The first and second set was burnt using petrol and kerosene, respectively. The final two sets were control groups. The first of these was burnt without the use of an ignitable liquid and the final set remained unburnt. Six larvae were collected daily from each of the twenty-four meat samples for a period of five days. Once the adults had emerged, six adults and six puparia were also collected from each meat sample. All of the entomological samples collected were analysed using solid-phase microextraction gas chromatography-mass spectrometry (SPME GC-MS). From the sample set burnt with petrol, a high proportion of larvae tested positive over the five days they were analysed, but only a small proportion of puparia and adults tested positive. Conversely, from the sample set burnt with kerosene, a high proportion of larvae and adults, but only a small proportion of puparia, tested positive throughout the sampling period.

These findings demonstrate that larvae of the blow fly *Lucilia cuprina* can be used in a small-scale setting to detect both petrol and kerosene from burnt substrates for at least five days. Positive results for the ignitable liquids of interest were also obtained for a limited number of adult flies and puparia. Given these findings, there is scope for further research in this area.

Keywords: Forensic entomology; Entomotoxicology; Fire debris; Ignitable liquids; Solid-phase microextraction

1. Introduction

The investigation of a fire involves the determination of whether it was an accident, or ignited under suspicious circumstances [1]. An important aspect of fire investigation is the collection and chemical analysis of fire debris to establish whether materials present in this debris may have assisted in starting or accelerating the fire. The presence of an ignitable liquid can indicate this. Petrol and kerosene are the most prevalent ignitable liquids in casework [2,3]. They each comprise a complex mixture of

hydrocarbons. However, they differ greatly based on the boiling point ranges of these components and their volatilities [4]. These differences make it challenging to recover the residues from these ignitable liquids in fire debris. Numerous methods have been proposed to achieve this goal [5]. As technology has developed, so too have the methods and instruments employed for these analyses. Despite this, the current ASTM test method for ignitable liquid residues in extracts from fire debris states that the essential requirement for making a classification using this procedure is the matching of the sample chromatogram with a reference chromatogram, noting points of correlation [6]. That is, despite technological advances, standard methods rely upon the correlation of several volatile components to indicate the presence of an ignitable liquid. Unfortunately, these components diffuse rapidly in air at a fire scene [7]. There appears to be no estimate of the maximum time fire debris can be exposed to the external environment before the ignitable liquid residues becomes undetectable. Anecdotally, it has been suggested that reportable traces of ignitable liquids may evaporate in as little as a few hours [8]. It is clear that time remains the critical factor when attempting to recover fire debris. However, a quick recovery may not always be possible if, for example, the fire scene is located in a remote area, because it may take the investigator considerable time to arrive at the scene and recover evidence. According to the New South Wales Fire Brigade in Australia, of the 319 fires investigated in 2008/09, the cause of 90 fires remains undetermined [9]. This figure has remained relatively constant over the last five years [9-13]. Although there are many factors that contribute to the number of undetermined fires, devising alternative ways to analyse fire debris may help identify the cause of a fire and thus contribute to the reduction of this figure. For fire scenes involving burnt human remains, one such alternative is analysing fly larvae that may infest the remains.

Insects can be utilised as alternative specimens for qualitative toxicological analyses when a body is in an advanced state of decomposition, and conventional post-mortem samples, such as blood or urine, may not be available or do not provide reliable results [14,15]. In these situations, fly larvae are typically abundant [16-29]. Drugs and toxins that have been identified in this way include (but are not limited to) barbiturates [29], analgesics [17], benzodiazepines [23], cocaine [25], opiates [30] and, most recently, gunshot residue [31]. Methyl mercury [32], copper, iron, zinc and calcium have also been detected in adult flies [33,34]. These studies have focused on substances associated with the cause of death. The potential of utilising larvae in the detection of chemicals associated with the destruction of a cadaver (to conceal the cause of death) has been largely overlooked. Of particular interest are chemicals used to accelerate the spread of fire. Although the analysis of insects in case-work is a relatively new application, extraction techniques specific to fly larvae are not required as they can be analysed in the same manner as other samples [17,26]. In addition, it has been reported that results obtained from fly larvae exhibit less interference from decomposition by-products compared with other traditional specimens [18,23,25]. Furthermore, perhaps the most significant advantage of using insects as alternative toxicological specimens is that they extend the sampling window considerably. In

particular, puparia can not only withstand changing climatic conditions, but unlike the larvae and adults, they are immobile, and hence can be found close to human remains even after many years [19,26,35].

This preliminary study was the first of a series of experiments and explored a novel small-scale method to detect the presence of petrol and kerosene in fly larvae, adults and puparia using solid-phase microextraction gas chromatography-mass spectrometry.

2. Material and methods

2.1 Study Site

The experimental work was conducted at the University of Technology, Sydney (UTS), within a ventilated laboratory.

2.2 Food Source

Superficial sheep's meat with associated adipose tissue (lamb neck chops weighing between 80 and 120 g) was used, as the presence of adipose tissue increased and prolonged burning.

Meat was stored in 500 mL plastic containers, within a 5 L container. The larger container was sealed with cotton material and the base covered in sand (the smaller container rested on the surface of the sand). The sand acted as a medium in which the larvae could pupate and simplified their retrieval at the conclusion of the experiment, as it could be sifted. The sand was replaced after each experiment in order to remove any contaminants.

2.3 Preparation of Insect Samples

Lucilia cuprina flies were used in this study because they are commonly encountered in forensic cases in eastern Australia and there are established protocols for their rearing [36]. The specimens used were derived from a culture maintained by the Department of Medical Entomology, Westmead Hospital, Australia.

The experiment comprised four sample sets of meat, each with six replicates. The first set consisted of meat burnt with petrol, the second was burnt with kerosene, and the third was burnt without an ignitable liquid. The final sample set was unburnt. The third and fourth sample sets acted as burnt and unburnt control groups, respectively.

The ignitable liquid sample sets were prepared by adding 10 mL of the relevant ignitable liquid to the surface of the meat. The ignitable liquid was allowed to absorb for one minute prior to ignition. The meat was ignited using a match and allowed to burn until it self-extinguished. It was then left in a laboratory to ventilate for six hours. A Bunsen burner was used to achieve the same approximate

degree of burning in the samples of meat that did not have an ignitable liquid present. This sample set, together with the unburnt samples, was also ventilated for six hours. The ventilation period, where the meat remained undisturbed in the laboratory for six hours prior to the addition of larval samples, was selected to best represent the time required for first-instar larvae (first growth stage after hatching) to develop. Following ventilation, approximately 100 first-instar larvae of *L. cuprina* were transferred to each of the replicates and allowed to feed undisturbed for a minimum of 14 hours. Due to the preliminary nature of this experiment, larvae were stored under temperature-controlled laboratory conditions which were monitored with small data loggers (iButtons® Maxim Integrated Products Sunnyvale, CA) every 30 minutes.

Six larvae were collected from each of the twenty-four meat samples on a daily basis for five days. A sampling time of five days was selected as this was approximately the time normally taken before larvae of *L. cuprina* began to pupate under these conditions. The remaining larvae feeding on the meat samples were allowed to pupate. Once adults had emerged, six adults and six empty puparia from each meat sample were collected separately.

The larvae, adult and puparium samples were collected into 1 L metal cans. Prior to and following their use, the metal cans were placed into an oven for at least 24 hours at 110 ºC. Preliminary testing indicated that this was sufficient to remove any volatile contaminants. These vessels were selected as they are often used for casework purposes in New South Wales, Australia.

2.4 Chemical Analysis

Solid-phase microextraction (SPME) was used for the extraction process. Prior to extraction, a small hole was punched in the lid of each metal can to accommodate the insertion of the SPME fibre. A piece of adhesive tape was placed over this hole to minimise the escape of volatiles. The SPME fibre selected for these experiments was polydimethylsiloxane-divinylbenzene (PDMS-DVB). Prior to extraction with SPME, the metal can (housing the entomological samples of interest) was preheated in an oven to achieve a headspace (area above the sample) temperature of 90 ºC. Once this temperature had been achieved, the SPME fibre was inserted into the headspace and the tin was placed in an oven at 90 ºC for three minutes. Following adsorption, the fibre was inserted directly into the GC-MS inlet to thermally desorb.

The analysis was carried out on an Agilent 6890 series GC system, coupled to an Agilent 5973 network mass selective (MS) detector using a Zebron ZB – 5 ms 30 m × 0.25 mm ID × 0.25 µm 5% polysiloxene 95% polydimethylsiloxane column. The injector was maintained at 250 ºC and operated for 2 min in splitless mode. Helium was used as a carrier gas with a flow rate of 1.8 mL/min. The GC oven was programmed to maintain the initial temperature at 35 ºC for 2 min, ramped at 10 ºC/min to 190 ºC, then ramped at 70 ºC/min to 290 ºC, and finally held for 4 min. The total runtime was approximately 22.93 minutes.

2.5 Data Analysis

Total ion chromatograms (TIC) were produced for all samples tested. These chromatograms were evaluated as being positive for either petrol (P) or kerosene (K) through visual pattern matching against known reference chromatograms, according to the guidelines set out by the current ASTM method [6] (examples shown in Figures 1 and 2). However, if a sample differed from the reference chromatogram, the sample was classified as negative for an ignitable liquid (N). Furthermore, principal component analysis (PCA) was performed using Pirouette® 4.0 software (following mean centring and vector normalisation) for exploratory analysis of the data, and a multivariate analysis of variance (MANOVA) was conducted using SPSS® on selected principal components.

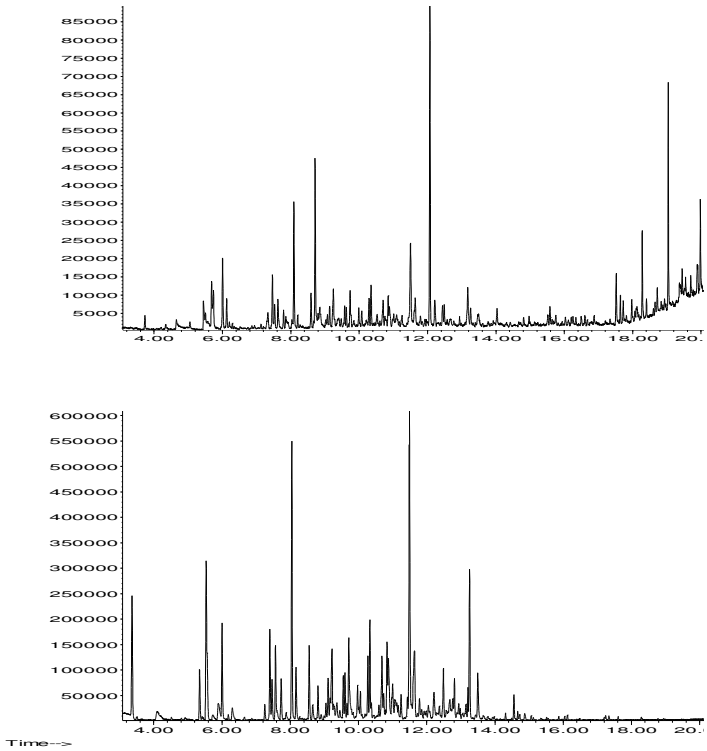

Fig. 1. Example of a chromatogram obtained from the volatile residues extracted (using SPME-GC-MS) from larvae feeding on meat burnt with petrol (top) and a petrol reference chromatogram prepared in the same way (bottom).

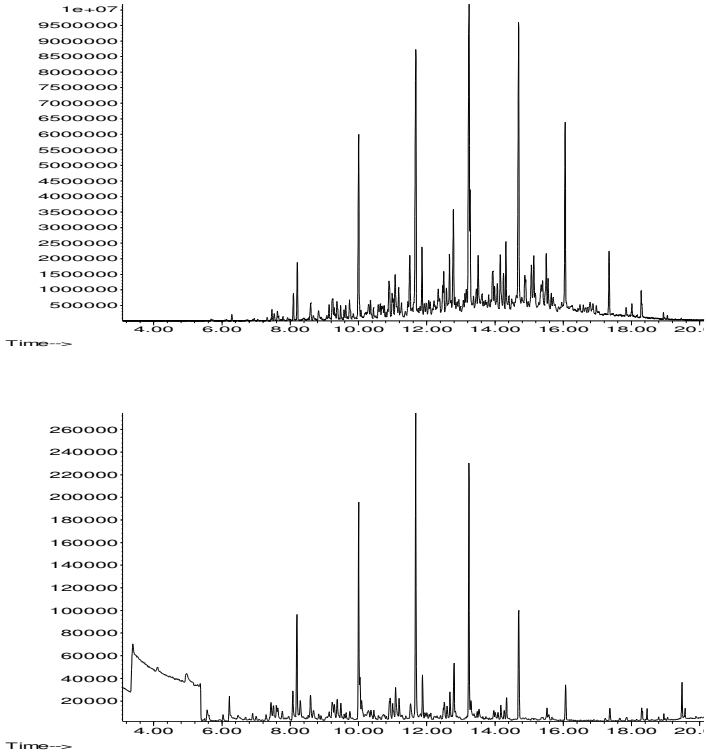

Fig. 2. Example of a chromatogram obtained from the volatile residues extracted (using SPME-GC-MS) from larvae feeding on meat burnt with kerosene (top) and a kerosene reference chromatogram prepared in the same way (bottom).

3. Results

The ambient temperature in the laboratories where the larvae were stored varied from approximately 20 to 25 ºC within the sampling time.

The replicates for each sample set were compared and gave comparable results (Table 1). All samples collected from the burnt and unburnt sample sets were negative for both of the ignitable liquids of interest. From the entomological samples collected from the meat burnt with petrol, 73.3% of larvae, 33.3% of adults and 33.3% of pupae tested positive. From the entomological samples collected from the meat burnt with kerosene, 100% of larvae, 100% of adults and 16.7% of pupae tested positive.

Sample Type	Petrol (%)	Kerosene (%)	Burnt (%)	Unburnt (%)
Larvae	73.3	100	0	0
Adults	33.3	100	0	0
Puparia	33.3	16.7	0	0

Table 1 Percentage of larvae, adults and puparia that was positive for an ignitable liquid in each of the individual sample sets.

The graphical representation (scores plot) of the first three principal components (representing the greatest variability in the data; shown in Figure 3) demonstrates that the petrol, kerosene and control groups can be distinguished from each other. The MANOVA calculation also found that there was a statistically significant difference between the four sample sets: $F(21,480)=10.47$, $p<0.001$, Pillai's Trace=0.942, partial eta squared=0.314.

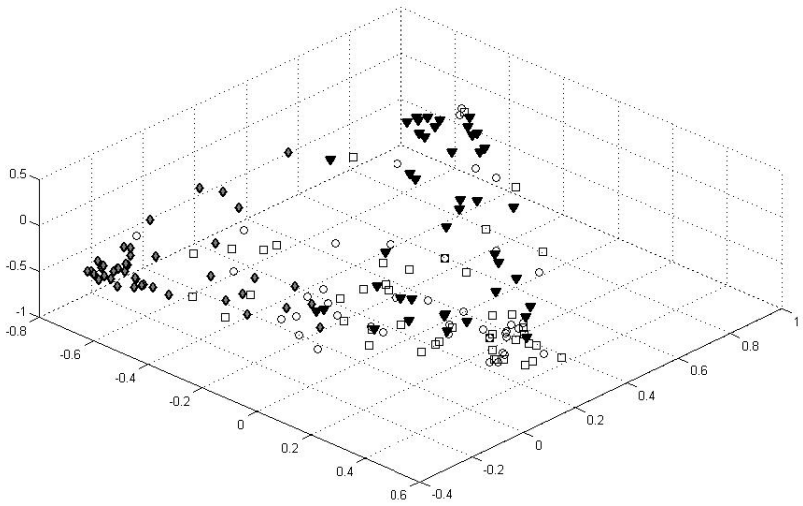

Fig. 3. The graphical representation (scores plot) of the first three principal components for all samples. The ◆ symbol represents kerosene samples, the ▼ symbol represents gasoline samples, the □ symbol represents unburnt samples and the ○ symbol represents burnt samples.

4. Discussion

There were no major differences observed in the replicates; however, it was noted that some of the chromatograms obtained from the petrol replicates could not be adequately matched with the reference chromatogram. That is, some of the peaks that comprise the distinctive petrol pattern were missing. This would suggest sample-to-sample variations exist between larvae since all of the replicate meat samples were prepared and stored in the same way. Whilst studying the accumulation of amitriptyline, other authors have also observed variations between larvae [27].

All larvae, puparia and adults derived from the burnt and unburnt control groups were negative for the ignitable liquids of interest. However, a number of volatile organic compounds were noted in these control groups that were also present in both the petrol and kerosene experimental and reference samples. These included 1,3,5-trimethylbenzene, 1,2,4-trimethylbenzene, 1,2,3-trimethylbenzene, 2,3-dihydro-1H-indene (indane) and eicosane. These compounds have also been reported in studies which examined the volatile organic components evolved from decaying remains [37,38,39]. Despite the presence of these compounds, a negative result for the ignitable liquids of interest could still be

concluded as the profiles obtained for the control groups did not exhibit the same distinctive patterns as petrol or kerosene. Furthermore, these compounds did not interfere with their analysis of petrol or kerosene. However, these findings do stress the importance of complete correlation between reference and sample profiles, as noting merely a few common components as an indication of a positive result could lead to misidentification.

The results obtained from the meat burnt with petrol indicate that the profiles from 73.3% of larval samples still exhibited the distinctive petrol pattern. Given the volatile nature of petrol, these positive findings are particularly noteworthy. Conversely, the higher boiling point of kerosene may help to explain its persistence and detection in all of the larval samples compared with petrol. Therefore, even after five days of exposure, petrol and kerosene could both be identified in many of the larval samples. This finding is also particularly worth noting given that time is a major limiting factor in fire debris analysis. Entomological samples have increased the sampling time to a minimum of five days. In addition, positive results for petrol and kerosene were also obtained from the adult and puparium samples. However, there were differences between the ignitable liquids here. Only two of the six adult samples analysed from the petrol sample set tested positive, while all six adult samples analysed from the kerosene sample set tested positive. However, only two of the six puparia samples analysed from the petrol sample set tested positive, while only one of the six puparia analysed from the kerosene sample set tested positive. It has been documented that post-feeding larvae expel all remaining food, begin to wander and finally pupate [40,41]. Given this fact, lower levels of ignitable liquids would be expected in the adult and puparium samples. It is not known whether the mechanism of metamorphosis may affect the level of ignitable liquids present in the puparium and whether this may also contribute to the lower levels observed in the adult and puparium samples.

It has been highlighted previously that both petrol and kerosene comprise volatile components that evaporate rapidly rather than remain in fire debris. With these factors in mind, the results obtained highlight the potential value in collecting and analysing entomological samples for casework applications, especially puparia. This type of sample, unlike the larvae and adults, is immobile, and hence can be found close to human remains even after many years [26]. This will allow for potential analysis of fire debris long after the fire occurred. In addition, these findings demonstrate an additional use for SPME in the field of fire investigation that has not previously been examined.

Statistical differences were noted between the petrol and kerosene sample sets using principal component analysis (PCA) and multivariate analysis of variance (MANOVA). This is important because it indicates that even though these ignitable liquids have several common components within their distinctive profiles, they can still be identified as separate sample sets. Conversely, the PCA results from the burnt and unburnt control groups are fairly diffuse and there is some overlap with the ignitable liquid groups. However, according to the MANOVA results these control groups were also considered

statistically different from each other. The diffuse nature of these groups in the PCA results may be attributed to two factors: (i) volatile organic compounds that are common to both the control and ignitable liquid groups; and (ii) some of these common compounds are present at low and/or inconsistent levels in the control samples. One of the challenges arising from examining multiple chromatograms is that the results are subject to interpretation by the operator. Performing PCA removes this bias by producing a scores plot. As a result, negative or contaminated samples can be easily identified as they are separated from their designated group.

5. Conclusion

This preliminary study has shown that petrol and kerosene can be successfully extracted and detected in the majority of samples using *L. cuprina* larvae, adults and puparia. Given the potential implications for casework, these findings must be further tested in an experiment that is closer to a realistic scenario. Further development of the methodology underlying this technique is therefore recommended.

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