AN INVESTIGATION OF A NON-DESTRUCTIVE SAMPLING METHOD FOR DETERMINATION OF MINIMUM POST-MORTEM INTERVAL ($_{MIN}$PMI) AT DIFFERENT TEMPERATURES USING DIPTERAN LARVAE.

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Abstract

The determination of the precise identification of forensically important Dipteran species are paramount to the establishment of minimum Post-Mortem Interval (minPMI). Insect specimens collected from a crime scene can be at multiple instars of their life cycle and due to the complexity of identification keys are difficult to identify at the early larval stages. This leads to larvae being predominantly reared to adult stages for their identification, which can be a time-consuming process. Which can be increasingly problematic if the specimens are in limited numbers.

This paper investigates the use of a non-destructive methodology to analyse the cuticular hydrocarbons (CHC) of forensically important flies, the final species focussed on being the Dipteran species in the UK, *Calliphora vicina* and *Lucilia sericata*. The non-destructive methodology was first established to determine whether the use would be comparable to current destructive methods used in CHC analysis, and whether the storage or collection method for the specimens affected the methods outcome. The second aim was to discover whether the non-destructive method could distinguish between the CHC profiles of the two fly species and in recognising these variations form an identification. Once distinctions between the species were made, could the method detect temperature changes to the profiles.

Using the non-destructive silica rub method 86,500 samples, were measured, sampled and analysed through oviposition to pupation, by gas chromatography – mass spectrometry (GC-MS). The observed results demonstrate that the individual species can be differentiated and that the temperature variables can also be detected within the species profiles. The capabilities for this technique to be used for identification is significant and shows great potential for developing for use at crime scenes. This

technique also shows sizeable capacity for use within the agricultural entomology discipline, with the ability to utilise the low-cost nonskilled sampling methodology out in the field.

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1.0 Introduction

1.1 Overview

This chapter presents a general outline of entomology and the multiple areas that this can incorporate. This includes a brief history of the discipline, an introduction to the biology of the three most forensically important fly species and the distinction between them. It demonstrates current methods for the interpretation of their life cycle, the decomposition timelines and factors that can affect them. To conclude the justification for the thesis is discussed.

1.2 Thesis background

The purpose of this research was to determine whether there was an alternative method to Isomorphen or Isomegalan diagrams, Accumulated Degree Hours (ADH) or Accumulated Degree Days (ADD), that could be incorporated at an actual crime scene, which could aid in the collection and processing of forensic entomology evidence by Scene of Crime Examiners (SCE). It is widely recognised that any evidence found at a crime scene needs to be able to be scrutinised at the highest level – namely a Crown Court. For this, police forces and SCE are required to follow best practice guidelines (ENFSI, 2022). An alternative method for the collection of entomological evidence once verified and protocols implemented, could potentially increase the accuracy of entomological evidence. As entomological evidence is time dependent due to an insects life cycle, this could be extremely valuable for SCE's to enable evidence to be collected

and processed without delays once at a crime scene and aid in the establishment of time since colonisation.

1.3 Entomology

Entomology is a branch of zoology and is the study of insects and arthropods and their relationship to other insects or organisms, the environment, and humans. Insects are some of the oldest inhabitants on earth, with millions of species of insects thus far identified. May (1988) recorded that this makes insects one of the most abundant animal categories in the world, this was reviewed by Stork (2018) who agreed but suggested that the number of obscure species could potentially be higher than originally estimated due to the advancement of DNA techniques used for their identification. Insects have been studied in earnest for centuries, with Aristotle in the 4th Century BC, starting the journey of entomologists today, by documenting and collating early insect anatomy information Weiss H B (1929). It is the categorisation and investigations of insects over centuries that has seen these arthropods become widely documented, therefore used worldwide for their known characteristics, habits, and life cycles.

1.4 Forensic entomology

Forensic entomology as Benecke (2001a) states is an important part of forensic science. It has gradually become introduced into numerous fields, from animal wildlife crime through to murder investigations (Anderson, 1999; Benecke, 2001a; Benecke, Josephi and

Zweihoff, 2004; Oliveira-Costa and de Mello-Patiu, 2004). Although not a true indication of the time since death (Adair, 2012), it is beneficial as an indication of the time when, physical or environmental factors permitting, a cadaver was populated by carrion species. Diptera are usually the first insects to colonize a corpse, whether animal or human, which is why their life cycle and behaviour is studied most when researching postmortem interval (PMI) (Anderson and Cervenka 2001; Chen et al., 2011).

For results to be used in crime scenes insects and larvae are usually taken from the scene and then reared through to determine species and matched to their known life span. The Tennessee Forensic Anthropology Centre established in 1981, as described by Williams, Rogers and Cassella (2019), was the first Human Taphonomy Facility (HTF) research facility that used human cadavers to research and interpret scenarios. This was increased to ten HTFs over the next forty years mainly in the USA with one in the Netherlands, but due to public objection and location issues, research in other countries and especially the UK is limited mostly to laboratory-based research. For this research pork meat would be used, although as Connor, Baigent and Hansen (2018) report, human cadavers should not be substituted by pigs, this was found to be for decomposition purposes and relating to the actual cadaver itself, but for general research it was suggested that the pig may be a suitable alternative. As Catts and Goff (1992) indicate, a pig is a suitable substitute for human matter, although this has implications on the recreation of crime scenes when the differing rearing medium is reported to have differing effects on the growth of Diptera under laboratory conditions (Clark, Evans and Wall 2006; Day and Wallman, 2006a; Ireland and Turner, 2006). Laboratory rearing conditions are also discussed by Weidner et al. (2020), where it is indicated that collection and rearing protocols are an issue in

laboratories, in crime analysis facilities. They determined that collection and rearing methods were found to be inadequate, with inexperience and insufficient understanding in correct handling and husbandry procedures. This ineffectiveness of potential evidence management could have severe implications in the evidential value and importance for any entomological development.

Forensic entomology is the use of entomology in a legal context. In this context it has been studied through various forms over centuries, which can be described through five strands of entomology as illustrated in table 1.1.

Table 1.1 Five categories of entomology strands

1	**Medical entomology**	**The study of insects to solve medical problems. Whether this is the use of insect larvae to eradicate infected or dead skin or tissue, or to determine the entomological causes of allergic reactions or infections. This can also include the investigations into the reduction or elimination in insects spreading of vector borne diseases. (**Service M W, 1980)
2	Veterinary entomology	Insects or arthropod that transmit diseases or infections are covered under this strand. These can infect wildlife, pets, farm livestock and poultry and cause catastrophic devastation to livelihoods, countries, and long-term havoc to worldwide trade. For cases of illegal poaching or hunting the determination of how, what, and why for any type of animal wildlife crime is vital. In hot, tropical, and subtropical countries myiasis can occur when flies lay their eggs in open sores, wounds, ears, noses, and any open orifice. This can also happen when people and animals are bitten by mosquitoes or ticks that are themselves infested with larvae. These examples can see veterinary, medical, and forensic entomology combine, dependent on the circumstances (Kettle, 1984).
3	Stored product entomology	Which is the infection of commodities that are being traded and transported worldwide which insects can cause disastrous destruction to (Hagstrum and Subramanyam (2006). This can lead to reduced seeds and crops to replant, therefore creating a vicious circle of loss and destruction (Parkin, 1956).
4	Structural or urban entomology	This being the destruction and/or demolition of items, property, and structures through wood eating insects. Insects can also infest buildings and structures which can lead to extreme medical complications with inhabitants of the buildings (Gordon, 2020; Rust et al., 2024).
5	Medico-legal entomology	Any crime that results in murder, neglect and/or abuse of the elderly or the young, would be under this strand. Where there are cases of neglect and therefore any open wounds, insects can then penetrate making any wounds or injuries, and therefore an individual's health, to deteriorate. This is not to be confused with myasis attributed to certain tropical regions to living individuals, mentioned in veterinary entomology. Medico-legal entomology would also include any criminal case where illicit drugs were involved – this could also bring veterinary entomology to the forefront if the drugs could be investigated due to any insects or arthropods and their environmental importance. This strand of entomology incorporates entomotoxicology. Which, as the name implies, is the studies toxins or of xenobiotics and their effects on living systems (Casarett and Doull,1975; Casarett and Doull, 1978; Casarett, Doull and Klaassen, 2008). Xenobiotics have been defined as any chemical that are foreign to the regular metabolism of any living being (Croom, 2012). This can be the intentional or unintentional introduction of chemicals or drugs into a body. This could also be linked to veterinary entomology, due to contaminants of water, food and other materials introduced into wildlife, pets, farm livestock and poultry. These contaminants could then be passed on to humans through the food chain or through infestation due to myiasis.

For the purpose of this research, it is the medio-legal entomology strand of forensic entomology (section 5 in table 1.1) that experimentations will be aligned to, but will be limited to, the infestation of deceased and decomposing cadaver models (pig) by necrophagous insects.

1.5 Carrion succession

The first insects to arrive when a body is killed or exposed, and the following carrion succession is widely documented, (Byrd and Castner, 2001; Gennard, 2012). From this previous documentation, table 1.2 lists the most common carrion succession data of insects that are of forensic significance in the UK.

Table 1.2 List of collated names for the most common forensically important carrion succession in the UK

	Family	Species	Common name
Blow flies	Calliphoridae	*Calliphora vicina*	Blue bottle
Blow flies	Calliphoridae	*Calliphora vomitoria*	Blue bottle
Blow flies	Calliphoridae	*Lucilia sericata*	Green bottle
Muscid flies	Muscidae	*Musca domestica*	House fly

This list is under constant revision as the understanding of insect succession on cadavers progresses due to many factors that can affect this succession. The key factors that can affect or impact any carrion succession can be found in table 1.3 but are not limited fully to these according to Byrd and Castner (2001). This list also links to table 1.4 when introducing the key stages of decomposition.

Table 1.3 Key factors known to affect carrion succession.

Geographical location	Although species are remarkably similar, each species has different species depending on where they are in the world.
Temperature	This is discussed further in this chapter.
Humidity	This is also discussed further in this chapter.
Seasonal differences	Some species have a prevalence for summer or winter months, this again is linked to temperature.
Time – day or night	Some species have a prevalence for daylight for colonisation, this is discussed later in this chapter.
Setting – rural or urban	Some species have a natural habitat as rural or urban, these due to humans and vegetation/food transportation can inadvertently transfer species to areas not usually inhabited by them. This can also occur when a body is moved from one setting to another.
Cadaver setting – above ground, buried, wrapped, concealed or in/under water.	This impacts on the chance of colonisation of a body. It will also introduce other variable to the body and the decomposition process.

1.5.1 Diptera

As shown in table 1.2 within the insects most strongly associated with first colonisation and decomposition of cadavers are Dipterans. This, in the UK, is predominantly the Calliphoridae family, also referred to as blowflies. These can be blowflies with a metallic blue or green colour thorax and abdomen, giving them the commonly recognised names of bluebottles and greenbottles. The bluebottles are by majority the Calliphora species and greenbottles primarily the species Lucilia. Within minutes of a corpse being open to colonisation by insects, the adult female flies will begin to lay eggs, or oviposition. For flies, oviposition is predominantly reported to occur during hours of daylight (Greenberg (1990; Zulaikha and Zuha, 2016), as they are mostly inactive during the night (Erzinçlioğlu, 1996). This was investigated by Barnes, Grace and Bulling (2015) for England, whose results supported this observation. Consequently, if a cadaver is disposed of during the night, this may delay when oviposition transpires. Female adult blowflies can lay one to two hundred eggs during each oviposition and during their lifetime can lay several thousand eggs. Oviposition usually occurs around any open orifice of a cadaver, being the mouth, eyes, nose, and ears. This can also happen in any open wounds if a violent assault has occurred to a body. These orifices, once oviposition has taken place, is what the hatching eggs find as a moist protected arena for their growth and development through their life cycle.

The life cycle of the Diptera order has the larvae develop through to adults with a total transformation, referred to as holometabolous metamorphosis. During this metamorphosis, the egg through to emergence as an adult fly goes through three main evolving stages, defined as instars. During these instars, the fly larvae go through a

moulting process, or ecdysis, growing significantly before ecdysis through to the next instar. The larvae feed with mouthparts that are hook-like and necessarily secrete enzymes that help with digesting food. The larvae breathe through posterior spiracles which are slit-like structures at the posterior end of their forms (figure 1.1). The variations in these posterior spiracles and the shape of the internal larval head called the cephalopharyngeal skeleton are what entomologists can use to help them identify species. (Barrett, Yuan and Garraway, 2022)

Figure 1.1 Image of third instar spiracles of a *Calliphora vicina* (Authors own)

Naturally, the larvae increase in size as they progress from first to third instar with the quantity of required food increasing. When the larvae reach the third instar it is at this stage that their feeding stops. They are then in a post feeding, prepupal stage and start to migrate away from a cadaver, this is in preparation for their pupation stage. Here they can travel up to ten metres away from a body (Amendt *et al.*, 2007). Once they have migrated to a suitable location that is damp, dark, and cool their mobility then reduces, and the larvae then begin their pupation. This is where their larval exoskeleton body contracts and the colour begins to darken, and the puparium, a hardened outer casing, is formed. It is

within this puparium that the metamorphosis occurs of the pupae into the adult fly. The puparium then shown signs of eclosion, this is where the adult fly then slowly emerges from the pupa casing. At this stage, the adult fly can begin the colonisation process again or fly away to another site.

1.5.1.1 Calliphora vomitoria (C. vomitoria)

C. vomitoria are larger flies and are black/blue in colour with sheen colouration to their abdomen, with the colour of the base of their wings where they join the body, the basicosta, is black. The head is mainly black, although their orange hairs give them a look of having orange cheeks, and they are covered mostly in black bristly hairs, see figure 1.3.

Figure 1.2 Image of an adult *Calliphora vomitoria* (copyright D R Skingsley)

Figure 1.3 Enlarged image of an adult *Calliphora vomitoria* head (taken from figure 1.2 - copyright D R Skingsley) – showing cheek (a) and bristle (b) black colouration.

1.5.1.2 Calliphora vicina (C. vicina)

C. vicina again are a large fly and have a noticeable black with a silvery grey/blue sheen colouration to their abdomen, with the colour of the base of their wings where they join the body, the basicosta, is yellow/brown and their anterior spiracle is orange. The lower part of the face is black with black hairs, the upper part, the cheeks, are orange in colour (figure 1.5), and they have distinct rows of bristles on their thorax.

Figure 1.4 Image of an adult *Calliphora vicina* (copyright D R Skingsley)

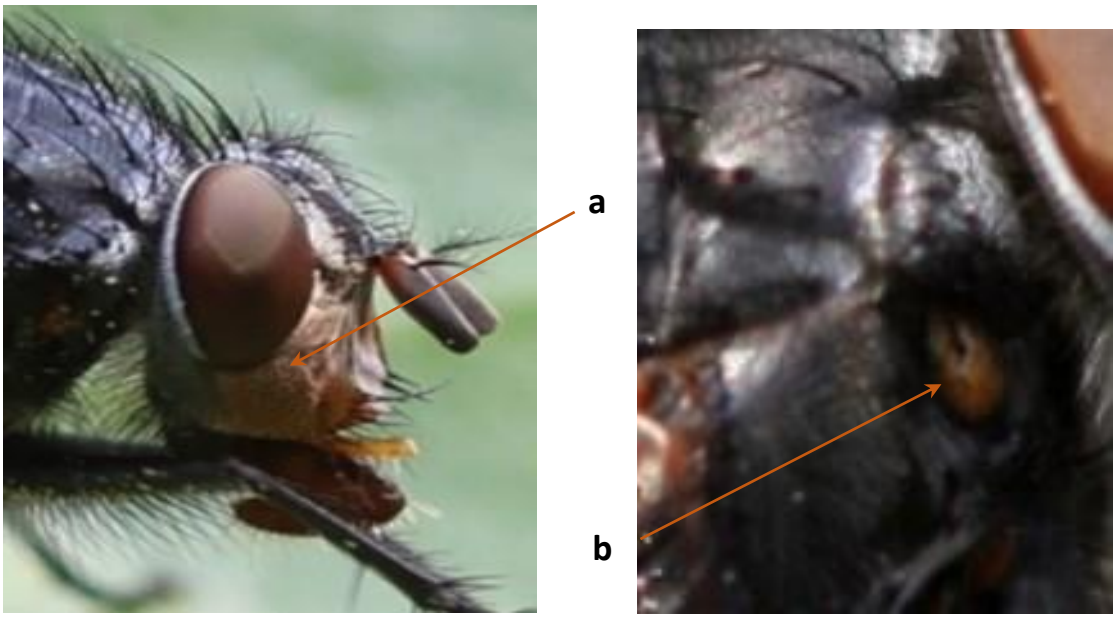


Figure 1.5 Enlarged image of *Calliphora vicina* head – showing cheek and bristle colouration (a) and thorax showing orange anterior spiracle (b) (copyright D R Skingsley)

1.5.1.3 Lucilia sericata (L. sericata)

L. sericata are a metallic green colour on their thorax and abdomen. They have distinct ridges that run across their thorax, which gives the impression that the thorax is made up of three sections. The colour of the base of their wings where they join the body, the basicosta, is yellow/orange and they have a distinctive three bristle formation in the centre of their thorax, termed post suture acrostichal bristles.

Figure 1.6 Image of an adult *Lucilia sericata* (copyright D R Skingsley)

1.5.1.4 Musca domestica (M. domestica)

The *M. domestica* is a smaller fly, dark grey/black in colour with stripes running down their thorax. They have shorter hairs that cover their whole body, so are less bristly than the previously mentioned species. Their abdomen is a grey/yellow colour, and males have

a yellow/orange/brown colour to the underside of their abdomen, compared to the females of the species. The *M. domestica* are also not a solid food feeder, they prefer a liquid diet, and their mouthparts are softer and adapted for their liquid food intake.

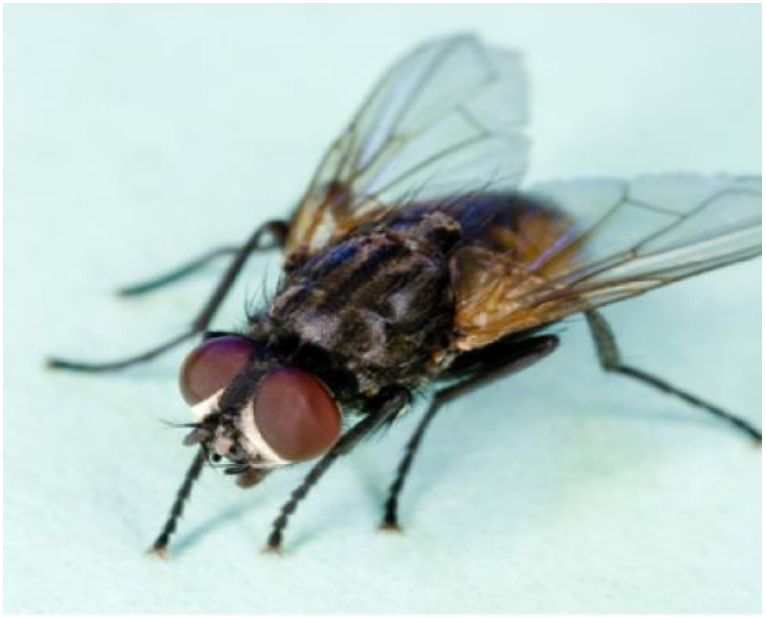


Figure 1.7 Image of an adult *Musca domestica* (Authors own)

1.6 Decomposition timeline

Initially Payne (1965) described decomposition as six stages - fresh, bloated, active decay, advanced decay, dry, and remains. He then reduced this to five stages for cadavers that had been protected from insect activity, these were - fresh, bloating and decomposition, flaccidity and dehydration, mummy, and desiccation and disintegration. Gennard (2012) initially gives three distinct phases for decomposition, autolysis, putrefaction, and bone diagenesis. Goff (1993) evaluates numerous researchers who claim that the decomposition stages are anything from one to nine stages. When investigated these all encompass the five most recognised stages as shown in table 1.4.

Table 1.4 The five stages of decomposition that bodies got through when left exposed for carrion to inhabit. This can be affected by but not limited to a number of factors including temperature, humidity and body size. (Authors own)

Stage	**Timeline**	**Information**	**Carrion succession**
Fresh	(0-3 Days)	Commences at the point of death and ends when bloating is evident. The breakdown of proteins and carbohydrate molecules occurs during this stage.	Blowflies, flesh flies, muscid flies, Hymenoptera (wasps), ants, crane flies.
Bloated	(4-10 days)	Putrefaction occurs during this stage. Gases produced from metabolic activities of the anaerobic bacteria cause the corpse to inflate.	Adult and larval blowflies, flesh flies, muscid flies, rove beetles, hister beetles, carrion beetles, ants, assassin bugs, Hymenoptera (wasps).
Decay	(8-20 days)	Begins when the abdominal wall is broken, allowing gases to escape and the corpse to deflate.	Adult and larval blowflies, rove and hister beetles, carrion beetles, dermestid beetles, ants, scarab beetles, muscid flies, cockroaches.
Post-decay	(18-30 days)	In dry habitats, remains consist of dry skin, cartilage, and bones. In wet habitats, a wet viscous material, termed byproducts of decomposition, are found around corpse.	Dermestid and hister beetles, mites, fungus beetles, springtails, fungus gnats, fruit flies, cheese skipper flies, phorid flies.
Dry	(30 plus days)	Bones, hair, dry skin remain. Odour, that of normal soil and litter.	Dermestid beetles, ants, cheese skipper flies, sow bugs, soldier flies, ground beetles.

1.6.1 Factors affecting decomposition

These stages are all dependent on numerous variables, whether the cadaver is inside or outside and effected by any other environmental weathering or temperature, the size of a cadaver, whether the cadaver has been kept away from insect activity. The presence of any other wounds or injuries would give a cadaver more orifices for natural bacterial and insect invasion to increase the progression of decomposition.

Although (Dadour *et al.*, 2001) suggested that temperature has no determinate influence on the growth rate of larvae; if it could be found to have a morphological or chemical

effect on the larvae then this would give Forensic Entomologists another facet to their evidence analysis.

1.6.1.1 Temperature

An insect's temperature is greatly influenced by the temperature of the environment they exist in, they are poikilothermic (cold-blooded), and as such, their life cycle development is correlated to temperature. This is reported by (Gennard, 2016), who suggests that this is not only fluctuations of the ambient air temperature at a crime scene but can also be temperature increases due to maggot masses, transportational temperature changes due to incorrect transportation containers, also rearing and storage temperatures. Generally, the warmer the temperature (up to some tolerance point of the insect), the faster development occurs. The faster insects develop on a corpse, the faster decomposition will occur. A body left in the open on a hot summer day may be reduced to bones in a little over a week, whereas a corpse left out in the late autumn or winter may show little change for months. In addition to temperature and humidity dynamics, this can also be attributed to increased number of fly and insect species and insect population numbers. Entomologists have studied the development of several species of carrion-feeding flies, determining their thermal requirements for completing each stage of development, from egg to adult. Some of this temperature data has already been reviewed and collated by Bambaradeniya, Magni and Dadour (2023a) to understand the methods used and pre-determined protocols that were followed. They suggest that a regulated protocol would be beneficial to enable data to be consistently accumulated and that this data should be amassed into a comprehensive database to enable entomologists around the globe to

have this information for convenience when determining PMI in current and future crime scenes. However, this is not a complete set and there is still much work to be done with other species to create comprehensive records for more countries. (Zhang et al. 2019; Wang et al., 2020; Bravo-Pena, Galián and Romera, 2021) As demonstrated in figures 1.9 & 1.10 where Grassberger and Reiter (2001) had their minimum temperatures for their investigation of *L. sericata* as 15^{o}C, yet at this temperature some of the larvae did not emerge as adults. This is noticed more within the smaller species of colonisers, so when observing crime scenes where temperatures are lower this could give a false indication of life cycles. A body that is deposited at a time when the air temperature is below 10°C will generally not be colonised by flies until the temperature rises. Therefore, this would potentially give a false timeline for $_{min}$PMI determinations.

When substantial numbers of second and third larval instars are feeding in a concentrated maggot mass, this can cause an exception to the temperature developmental time. The activity of several hundred fly larvae in a small area as reported by Heaton, Moffatt and Simmons (2014) can raise the temperature within this mass up to 2.5^{o}C to 14°C above the ambient temperature and fly larval development may proceed at a much faster pace than might be expected at a given temperature. This was also investigated by Kotzé, Villet and Weldon (2016) who reported a higher temperature above ambient of 18.7°C. Although, in research completed by Swiger (2007) it was observed that larvae were able to regulate to the best temperature for growth, despite using an additional temperature heater. If a maggot mass is present on a body, a forensic entomologist will usually record the temperature within the mass to help to adjust any calculations to determine the PMI. Fouche, Hedouin and Charabidze (2018) suggest that the behavioural effects of larvae during a maggot mass could be attributed to signals left by the larvae which indicate that

they recognise and migrate to their own species. They also suggest that this could be ascribed to the cuticular layer leaving traces as the larvae move along surfaces. This would give additional biological support that cuticular hydrocarbons being species specific.

1.6.2 Calculation of PMI

To determine the PMI or in an entomology perspective the $_{min}$PMI, the objective is to determine when the cadaver was first colonised. This is achieved by first identifying the first or oldest coloniser at the remains, or the oldest life cycle of that coloniser. This needs to consider several aspects relating to a crime scene. When a victim was last seen alive, this would give a Post-Mortem Maximum ($_{max}$PMI) period, the period of insect activity (PIA), also observations of the insect development would also give the time since colonisation (TOC). All these factors together would estimate a $_{min}$PMI timeline, illustrated in Figure 1.8.

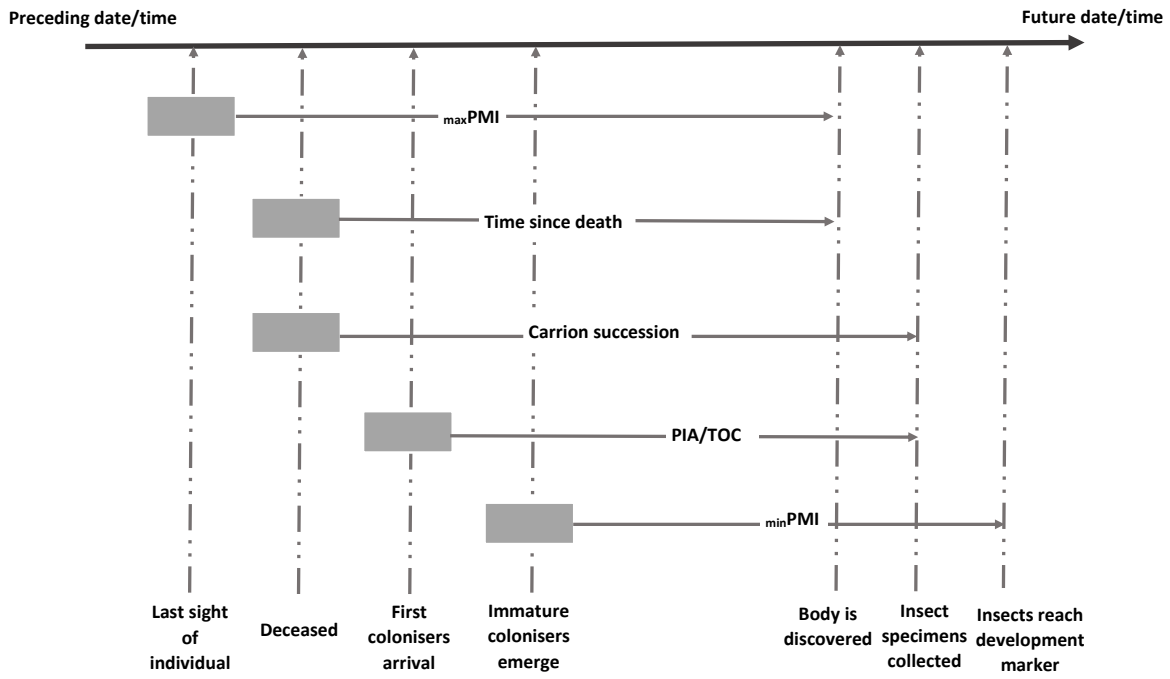


Figure 1.8 An estimated timeline summary for determining $_{min}$PMI. This demonstrates the key points in the PMI timeline and the importance of discovering the colonising species and its life cycle. (Authors own)

1.6.2.1 Isomegalen and Isomorphen diagrams

Isomegalen and Isomorphen diagrams as illustrated in figures 1.9 & 1.10 are currently used in the determining of PMI data within the laboratory, although these as Grassberger and Reiter (2001) and Amendt et al. (2011) indicate are only suitable if temperature is reasonably constant. This was supported by Richards (2001) who suggested that caution be taken when using these diagrams due to the differences found with fluctuations in temperature.

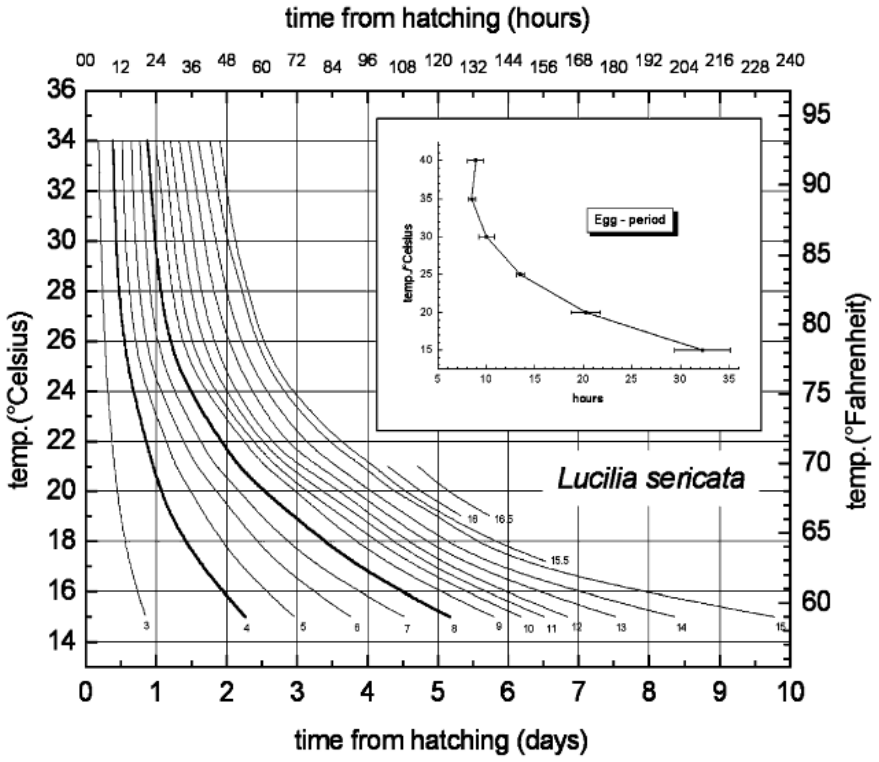


Figure 1.9 Isomegalen diagram indicating egg hatching to peak feeding for *L. sericata* – taken from Grassberger & Reiter (2001)

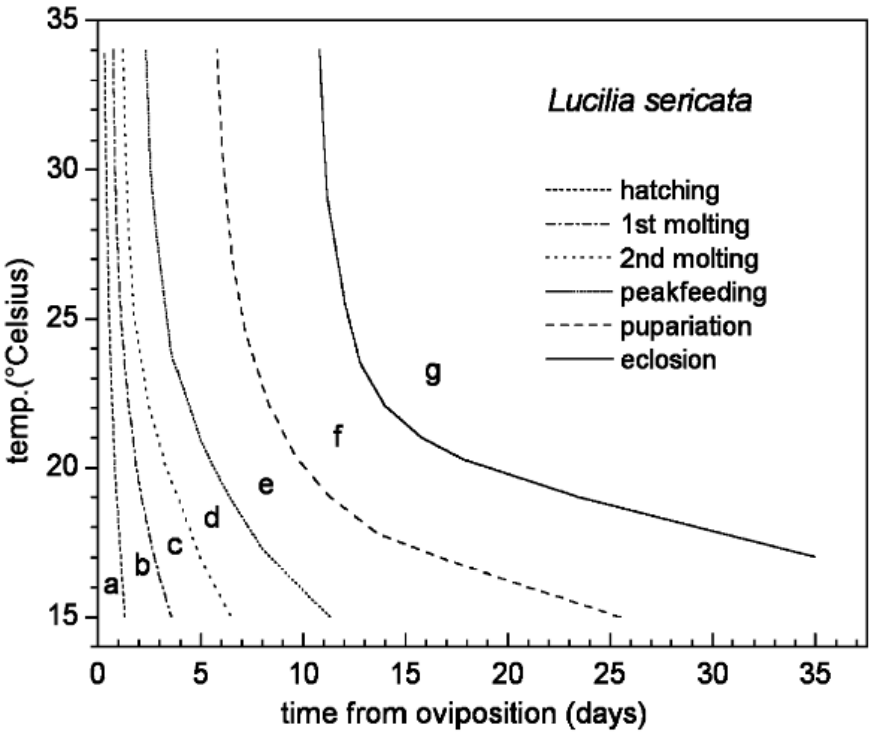


Figure 1.10 Isomorphen diagram representing oviposition to eclosion for *L. sericata* – taken from Grassberg & Reiter (2001)

As previously mentioned in table 1.3, it is widely recognised that there are factors that can affect larvae growth and colonisation, all these factors can also have a severe impact on any data that is collated into these Isomegalan and Isomorphen diagrams. It is therefore acknowledged that caution should be used when working with these diagrams. Ireland and Turner (2006) and Heaton, Moffatt and Simmons (2014) discuss the increase in temperature due to the larval massing, this needs factoring into any analysis of growth data with these diagrams. Also, as Greenberg (1991) suggests that if larvae are starved of a food source, this could lead to slower growth rates, while Richards, Rowlinson and Hall (2013) state that having a food source with an increased rate of decomposition can cause the larvae to develop much slower, so although the food source is available, this has the same impact on growth as if there were no food source at all. Reibe, Doetinchem and Madea (2010) advocate that the major problem in the application of any growth data to criminal investigations is that there is not sufficient data in existence for fluctuating temperatures. If 'real-time' temperature fluctuations were to be followed, then these diagrams could possibly become redundant in some cases due to their inaccuracies. Amendt et al. (2011) also note that these diagrams are only prevalent for the measurements of the larval stages, which limits the data to three stages of the fly's life cycle.

1.6.2.2 Accumulated Degree Hours (ADH) and Accumulated Degree Days (ADD)

When interpreting entomology evidence at a crime scene, it is important, as already mentioned to consider thermal or temperature insect developmental times and

conditions. These are identified and using accumulated degree-hours (ADH) or accumulated degree-days (ADD) calculations, aid in the determination of PMI.

ADH = Time (hours) X (Average temp. – Minimum development threshold temperature)

ADD = Time (days) X (Average temp. – Minimum development threshold temperature)

These calculations are both subject to corrections for the difference between recorded temperature at the crime scene and the recorded temperature from a meteorological station closest to the crime scene. Gennard (2012)

It has been widely researched for the timescale of the life cycles of geographical specific species. (Kamal 1958; Greenberg and Tantawi, 1993; Anderson, 2000; Byrd and Allen, 2001; Grassberger and Reiter, 2001; Marchenko, 2001; Grassberger and Reiter, 2002; Warren and Anderson, 2013) Although, due to changes in worldwide climate and weather conditions, it would be prevalent to update data to ensure any changes are revised for accuracy. Temperature differences to a recognised developmental timescale and recorded temperature data will change the timeline, which in turn will alter what the $_{min}$PMI will be calculated to. In addition, there is always some threshold temperature below which no development takes place, and below which adult flies are inactive. For most species, this threshold temperature is between 6-10°C (Higley and Haskell, 2001), although this has since been collated by Gennard (2012) to include base temperatures as low as 1^{o}C (Donovan *et al.*, 2006), this demonstrates why developmental data requires updating on a consistent basis to ensure correct information when performing these calculations to determine $_{min}$PMI.

1.7 Thesis Rationale

Apart from initial macroscopic and basic microscopy, before the fixing of a specimen, this research has found that all other current physical sampling methods discussed in chapter 2, result in the destruction of any entomology specimen. This was only found to not be the case if specimens were required for a forensic entomologist expert to be employed for species determination. Out of all the methodology investigated before this research began it became apparent that the Cuticular Hydrocarbon (CHC) analysis was showing to be the method that was increasing in significance. Amendt et al. (2007) reported of the importance of specimen collection and preservation at the crime scene was also determined to be of huge importance. With numerous papers increasing the significance of educating anyone attending a crime scene and for creating protocols for this process to improve the integrity of any evidence collected and analysed. (Lord and Burger, 1983; Catts and Haskell, 1990; Catts and Goff, 1992; Dadour et al., 2001; Amendt Jens et al., 2015)

Therefore, it is the CHC analysis through Gas Chromatography Mass Spectrometry (GC-MS) that will be an influence for the purposes of this investigation, which is combined with a non-destructive collection and sampling technique. This will enable non-skilled operatives to be able to collect and sample before sending the resulting samples to an analytical facility for results that again will be aimed at non-skilled interpretation. Temperature differences of any entomology evidence will also be investigated, with the aim of easier interpretation and understanding of differences in results that are obtained. This will undoubtedly result in forensic entomology evidence analysis become more accessible, understandable, and affordable.

2.0 Current processes in forensic entomology

2.1 Overview

This chapter investigates the current methodology and analytical processes utilised in the examination of forensic entomology evidence. It also demonstrates the structure of the dipteran cuticle layer and the destructive methods of cuticular hydrocarbon analysis that is currently utilised for forensic entomology evidence analysis.

2.2 Introduction

The core objective for forensic entomologists at a crime scene is to identify any eggs, larvae, pupae, or adult specimens to determine the species. As discussed previously in chapter 1, it is the known life cycles of these species that will enable the $_{min}$PMI to be established. Any erroneous identification could lead to incorrect interpretation of species data and therefore inaccurate $_{min}$PMI calculations.

The only drawback to this is that the identification of any insect samples at a crime scene is onerous and therefore requires time and extremely skilled and expensive insect specialists utilising classification keys. This, as Walter and Winterton (2006) report, is becoming a scarce profession so therefore more expensive. If insects are found in the earlier instars of their life cycle they can be challenging as these keys generally, are not comprehensive enough for the taxonomy of anything other than adult flies. Walter and Winterton (2006) also detailed that this is due to the lack of taxonomy experience and the

skills gap due to the records for any insect species being outdated and new keys not being researched due to the lack of specialism.

This would culminate in the protracted process of samples being reared through to adults to conduct the identification process becoming a more niche practice and consequently more expensive.

2.3 Microscopy

Insect samples have been examined using stereo microscopy since the 19th century and as Chick (2016) describes, this is an ideal medium for observing the morphology of any individual insect order due to requiring relatively low magnification. It is also an extremely valuable method for identifying infinitesimal characteristics that distinguish different species of the same families. This has been used mainly for adult fly specimens although techniques and equipment has allowed this to become a more powerful tool in morphological identification of species and therefore the evolution of new species. (Friedrich *et al.*, 2014)

In recent years this has been expanded to observing eggs, larvae, and pupae casings, but this has seen the microscopy methods for morphology advanced extensively. (Wipfler *et al.*, 2016). Queiroz, Mello and Lima (1997) found clear similarities between Diptera species, when comparisons of full life cycles morphological examinations have been completed, whilst Oliveira, Mello and Queiroz (2007) found that some species were distinctively different through their larvae stages making morphological identification extremely problematical. The investigations of Bunchu et al. (2012) are again a morphological identification and comparison for species identification, which relies on the

specimen being in good condition. Their research also aimed to determine any aging difference to support determination of PMI. Szpila, Pape and Rusinek (2008) discuss the importance of microscopy whilst also recognising the limitations, specifically with the early instars of the species. This can be impacted by any preparation methods for identification, which could potentially intensify any imperfections of the specimens, or obscure key features therefore hindering the identification of any specific morphological differences between species. Szpila, Pape and Rusinek (2008) also appreciate that any temperature variations occurring at a crime scene could cause fluctuations in the development times of the instars. Therefore, the short moulting times and the diminutive size of the first and second instar giving the identification period an extremely inaccurate timescale.

The branch of microscopy used determines whether the sample needs to be euthanised, general optical microscopy can be done with no euthanasia, but preparing for identification samples of a specimen requires for the sample to be euthanised and prepared for slide fixing. If this was used for a larval sample, euthanising for slide fixing would leave no sample for rearing through to identification. This was researched by Sukontason et al. (2004), in relation to fly eggs, where although they were using light microscopy, their samples were prepared in potassium permanganate to visualise the fly eggs to determine species. The eggs were euthanised from the preparation method, so no rearing opportunities were possible, with their findings showing that keeping the eggs for further analysis was not possible due to the degradation caused by the process.

2.3.1 Scanning Electron Microscope (SEM)

An SEM is a far more powerful research aid for microscopy, which has the capabilities to view smaller samples at larger magnifications without the constrictions of a small field of view of general light microscopy. This, using a beam of electrons, creates a signal from its interaction with the specimen sample. The signal is amassed though imaging and diagnostic detectors, to allow the image of the specimen to be recorded in contrast to light microscopy is in nanometres rather than millimetres (Mohammed and Abdullah, 2008).

On completing morphological examination of Diptera eggs using SEM, Mendonça et al. (2008) were able to discern fundamental characteristic differences between the different species of flies. Although this was found to be conflicting by Greenberg and Singh (1995), as they state that confusion can occur with species identification, suggesting that similarities may also exist among species of another genus. On these occasions this would lead species determination to revert to measurements being used. Although the improvements in resolution through instrumentation enhancements could be a contributing factor to these conflicting findings. Measurements, as previously discussed in chapter 1, can be affected by many external factors, this could therefore prove problematic and challenging for speedy species determination. Shaheen and Fathy (2008) suggest that the larval morphological development is affected by drug ingestion from cadavers. This is further discussed in the entomotoxicology subchapter 2.4.

Similarly, to the preparation of samples in light microscopy, samples for SEM also require preparation for analysis, by gold coating and fixing to a stub, this required the samples to be killed beforehand. Although due to the nature of the SEM analysis chamber requiring

the sample to be in a vacuum, this would lead to euthanasia of the sample, if the preparation was not required.

2.4 Deoxyribonucleic Acid (DNA)

DNA analysis is already a widely used analytical method for investigation of any bodily fluid sample found at a crime scene. (Gill, Jeffreys and Werrett, 1985; Rudin and Inman, 2001; Butler, 2005; Roewer, 2013; de Boer *et al.*, 2018) It can be crucial for aiding the discovery of a victim's identification, if a visual identification is not possible, or if a body has been moved from a crime scene leaving body fluids. (Njau *et al.*, 2016)

Using entomological specimens from crime scenes to determine unidentifiable victims has also been utilised in recent years. The collection and preservation of any entomology sample is of paramount importance, as Linville, Hayes and Wells (2004) state that certain preservation methods can decrease the possibility of DNA collection. Even through severe trauma or burning, de Lourdes Chávez-Briones et al. (2013) details that it is still possible to amplify short tandem repeats (STR) determined from entomology specimens found on severely burned cadaver, although without a credible DNA source could only be matched to a known family member. This would suggest that this is only suitable for identification purposes and not determination of a PMI. DNA can also be helpful for potentially ascertaining the detection for an alleged perpetrator, principally if multiple specimens of body fluids are left at a crime scene. (Jolicoeur, du Québec and Wilfrid-Derome, 2013; Kamodyová *et al.*, 2013; Magalhães *et al.*, 2015) This has also been researched for use with entomological specimens, in the first instance, for any DNA information pertaining to a cadaver that any entomological sample has been feeding on. (Linville, Hayes and Wells,

2004; Purwanti, Yudianto and Erfan Kusuma, 2020; Cantu, Bucheli and Houston, 2022). Durdle (2020) elucidate that this can contaminate the samples, therefore leading to incorrect convictions and miscarriages of justice.

At the other extreme, it can also be the case that the insects or larvae do not feed on a cadaver, therefore, if migrated away from a body, could have fed on other biological samples in the interim, consequently becoming irrelevant for DNA analysis if not related to a certain cadaver. This could lead to incorrect identification and miscarriage of justice. Durdle, John Mitchell and van Oorschot (2011) detailed that DNA could be determined from fly vomit and excrement, which is a positive for victim identification where other means of identification cannot be completed. Although this could also lead to contamination of scenes with flies bringing in DNA from other sources, leading to additional material being left at a scene, which could lead to misidentification of a victim as well as a perpetrator if an attack was suspected. Due to the flies migrating away from a scene loss of this evidence would reduce the probability of victim identification being made, this could also potentially lead to misinterpretation of the DNA evidence when investigating for a suspect too.

Although Wells and Škaro (2008) advocate that species determination is only obtainable from entomology specimens, they also suggest that this, with rigorous validation for methodology and analysis, could become a heavily used tool. This is supported by GilArriortua et al. (2015) who suggest that classification identification can be amplified between closely linked species by focusing analysis observations to the Cytochrome c oxidase I (COI) barcode and Cyt-b mitochondrial loci. Yang et al. (2022) also suggest that observations of the COI barcode prove extremely valuable for species determination but also suggest that ITS2 genes prove to be a more precise identification method using the

mitochondrial and nuclear DNA. Wells and Stevens (2007) advise that although a powerful tool for the future of forensic entomology, caution should be taken when analysing specimens due to pseudogenes. These are genetic loci containing a sequence comparable to a functioning gene, but with qualities implying that it is no longer operational, which would lead to misinterpretation of results and therefore a misidentification of species. Wells and Williams (2007) also suggest that using DNA analysis for species determination, that restraint should be exercised due to geographical differences to species, this is also supported by Mahat N A and Jayaprakash P T (2013). Chen et al. (2004) also suggest that individual primers would need to be developed to enable full species-specific identification, which would be time consuming. A review completed by Ren, Shang and Guo (2021) suggest that advances in technology of whole genome sequencing could improve the importance and breadth of entomological evidence. This is supported by Wells and Škaro (2023) who show that entomology DNA evidence can not only determine the insect sample but also the secondary identification of any human DNA within the insect itself.

Takayama et al. (2023) investigated the distinction between crime scene samples and fly artifacts utilising mitochondrial DNA (mtDNA), this also identified the specific species. As with microscopy evidence, any DNA evidence obtained from the excrement from flies would be completed without the killing of the insect, although there would potentially, in these cases, not be the actual fly to complete identification as they may have left the scene. Any DNA evidence taken from larvae at a crime scene would involve the euthanasia of the samples, removing the possibility for rearing to adult for identification.

2.5 Infrared Spectroscopy (IR Spectroscopy)

Infrared spectroscopy is employed in the analysis and interpretation of organic compounds. It is the covalent bond within these compounds that excite at certain frequencies within the infrared range. These variations are the rotation and vibration of the covalent bonds, that for each individual compound excite at different wavelengths. (Griffiths, 1983; Griffiths and Haseth, 2007; Baker *et al.*, 2014) The chemical composition of cuticular lipids of insects and the function of the cuticular layers have been investigated for their difference through species, phase development, weathering, and temperature changes for decades.

Wigglesworth (1947; Lockey, 1985; Blomquist, Nelson and De Renobales, 1987; Blomquist and Bagnères, 2010; Gołębiowski *et al.*, 2011; Chung and Carroll, 2015; Duarte *et al.*, 2019), discuss the complexity of the construction of hydrocarbons and convey that these can, dependent on the insect species, contain more than one hundred components. This Gibbs and Crowe (1991) and Kaneko et al. (2018) observe that this lipid layer can be affected by change from an optimum temperature, which they suggest is at which point any the cuticular lipids melt. This was during their research which saw Fourier-transform infrared spectroscopy (FTIR) paired with attenuated total reflection (ATR) to increase the effectiveness of the analysis with the aim of deciphering the cuticle structural information. The results showed a distinct difference between ordered and disordered hydrocarbons, which indicated that the more structured the hydrocarbon the better the frequencies of the peaks that were observed. This is aligned with the packing behaviour of these saturated and unsaturated hydrocarbons, which has significant implications for

their physical properties and therefore their melting points and separation values. (Blomquist and Bagnères, 2010)

As already previously discussed with any methods there are limitations to what can be interpreted from evidence, this is also the case with FTIR. Although this has been found to determine CHC they also determined that the lipid layers can interfere with each other, this could potentially impact the results determined. Durak, Ciak and Durak (2022), suggest caution is needed when completing FTIR analysis as the CHC chemical compositions could be subject to changes due to changes in environmental issues, namely temperature. Other factors that could limit the results obtained from FTIR are water, this was discussed by Durak and Depciuch (2020), who show that the water molecule vibrations severely impact on sample results and interpretations, which when looking at larvae found at a crime scene, could be a severe impediment in misidentification. Pickering et al. (2015) investigated drying larvae samples before sampling and determined that the additional process did not impact on the spectrum determined and the accuracy of the spectrums for the samples.

2.6 Entomotoxicology

Although chemical analysis was already used in relation to entomology, it was Pounder (1991) who first put the term entomo-toxicology into the research vocabulary, this was later maintained by Goff and Lord (1994). Many entomological investigations have been undertaken with drugs of abuse, alcohol and pesticides being the main toxicological components identified. (Goff et al., 1991; George et al., 2009; Muskan et al., 2020; Al-Shuraym et al., 2021; Bessa et al., 2021; Galil et al., 2021; Preußer et al., 2021). However,

Pounder (1991) suggests that this is only beneficial for qualitative and not quantitative consideration due to limitations in research in this area.

Introna, Pietro and Lee (2001) also discusses the limitations of drugs of abuse investigations, due to the differing chemical structures of the different drugs and the metabolism of these substances, in the victim and in the larvae themselves. This was supported by Sadler et al. (1995) who suggested that to determine drug concentrations to an optimum, that only larvae still consuming from a cadaver should be sampled. Byrd and Peace (2012) also suggest that this will also vary dependent on whether larvae have fed on skeletal muscle or organ tissue.

When discussing toxicology in relation to entomology, it is also pertinent to understand the consequences of any drug ingestion on the development of an insect, throughout its life cycle. For morphological identification under SEM, as Shaheen and Fathy (2008) discussed, the developmental formation of larvae was impeded by drug ingestion. This was also noted by Yasmeen and Amir (2023) who observed morphological as well as developmental deficiencies of larvae through contact with Imidacloprid, an insecticide developed to simulate nicotine. Research conducted by Chick (2014) established an apparent effect of nicotine when observing cadaver colonisers, this would again show the impact of chemicals or drugs on the determination of $_{min}$PMI. This was considered by Goff, Omori and Goodbrod (1989; Goff *et al.*, 1991; Bourel *et al.*, 2001) revealed that morphine was expelled from larvae before transformation to the pupae stage, although traces were still determined with the pupae, this was at a reduced concentration. This was also found with Bourel et al. (1999); Carvalho, Linhares and Trigo, (2001); Ishak et al., (2019); Boulkenafet et al., (2020); Malejko et al., (2020); Sari et al., (2023). While Goff et al. (1997) observed that there was no difference in the concentrations to dosage, but still

noted that the developmental rates were adversely shorter than expected, again impacting the determination of $_{min}$PMI. Tracqui et al. (2004) appeared to contradict most entomotoxicological research and comment that any compound identification that can be conducted on any entomological specimen taken from a crime scene can also be identified from the actual cadaver itself, which would deem the use of entomological samples superfluous. This of course, would not be the case if a cadaver had been completely consumed by insects and scavengers or if the body had been moved. (Nolte, Pinder and Lord, 1992)

When observing the research completed in this area, this would suggest that analytical methods, as they are already used by forensic entomologists, could also prove valuable in determining individual chemical properties of the actual larvae itself at differing instars, like this study to establish baseline characteristics of the larvae irrespective of drug induced morphological changes. Which would again give a more reliable method of identification than measurements alone to support the determination of $_{min}$PMI.

Again, it is noted, that with any chemical analysis, that the sample is euthanised in the preparation, so therefore there is no potential for further rearing for identification.

2.7 Gas Chromatography – Mass Spectrometry (GS-MS)

2.7.1 Gas Chromatography

Gas chromatography is an analytical technique for the separation of smaller volatile and semi-volatile organic molecules/components (VOC's). It has been used in industry in many forms to identify compound components, encompassing substances such as

steroids, hormones, fatty acids, alcohols and aromatics and hydrocarbons. This makes this form of analysis a very common and trusted technique throughout research and industry.

It is a separation technique that is used to separate the chemical components of a sample mixture and then detect them to determine their presence or absence and/or how much is present. GC detectors are limited in the information that they give; this is usually two-dimensional giving the retention time on the analytical column and the detector response. Identification is based on comparison of the retention time of the peaks in a sample to those from standards of known compounds, analysed using the same method. This can be directed to a valve that has the capability to split the effluent to a GC detector or can be linked to a mass spectrometer.

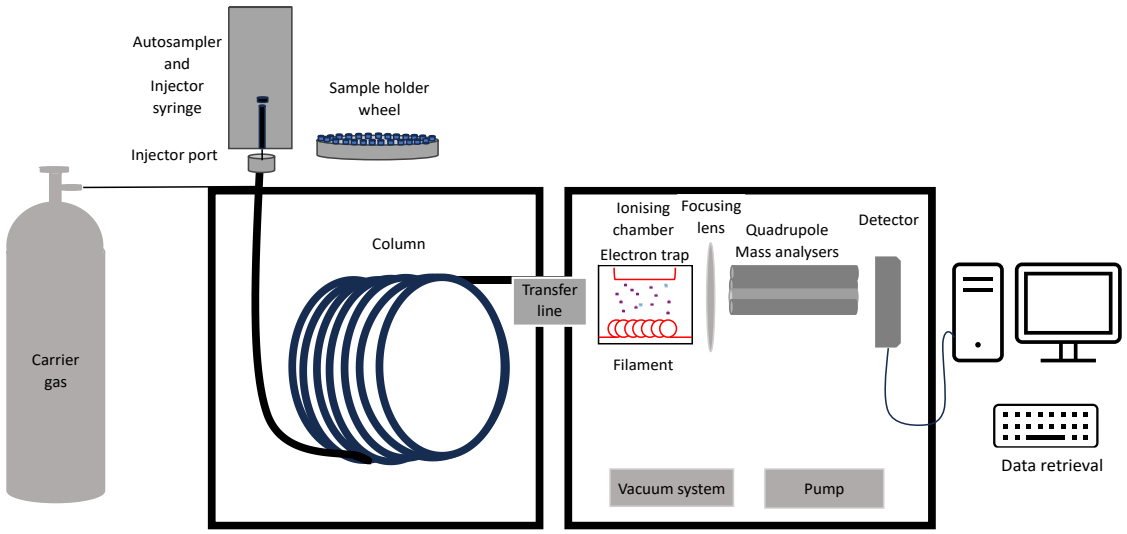


Figure 2.1 A basic schematic of a GC-MS running system. The sample would be injected through the column by means of the carrier gas. The components would then be transferred through the electron trap and ionising chamber and focused onto the mass analyser through to the detector. (Authors own)

2.7.2 Mass Spectrometry

Mass spectrometry (MS) is an analytical technique that can be utilised to determine the molecular weight and elemental composition of a particle. In a basic description the MS works similarly to the GC by having the element flowing through the device to separate these off at each different mass. This all occurs under vacuum conditions to enable the moving ions to travel freely and to remove the possibility of the ions colliding with air molecules, therefore reducing the noise created due to additional volatile compounds in the air.

The MS first ionises the vapourised sample eluted from the GC by electron ionisation. This involves electrons passing between a heated coil and plate, these electrons moving vigorously collide with the sample to force electrons out to form molecular ions. The molecular ions are then fragmentated, these ions are themselves fragmentated and the process continues. These particles are then accelerated through several separately charged plates containing holes in their centre. This has the ions with larger charge accelerated more than ions with a smaller charge and these then travel through the holes on to the quadrupole mass analyser.

Again, similarly to the GC, the ions are then separated through the quadrupole analyser, comprising of four parallel rods arranged around a central axis for the ions to travel through. These rods are configured so that opposing rods have the same direct charge voltages flow through them and additionally have radio frequency voltages passed through them. These have opposite polarities, which has the effect of creating a fluctuating magnetic field within the quadrupole analyser. It is this fluctuating magnetic field that will deflect ions through the quadrupole based on its mass and charge. Lighter

ions will be deflected more than heavier ions, and greater charged ions will be deflected more than a smaller charged ion. This signifies differently charged ions will be deflected through the analyser dependent on their mass to charge ratio. Therefore, those ions too large or small will strike the quadrupoles and not travel directly through the analyser to move on to the detector. Ions that travel through the quadrupole analyser without interference from rod collision are then passed through to the detector, this is where the mass to charge ratio is analysed through the ions hitting the detector.

From this the mass spectrum is produced with a plot of the ion abundance/relative intensity versus the mass to charge ratio (m/z). (Shown in figure 2.2)

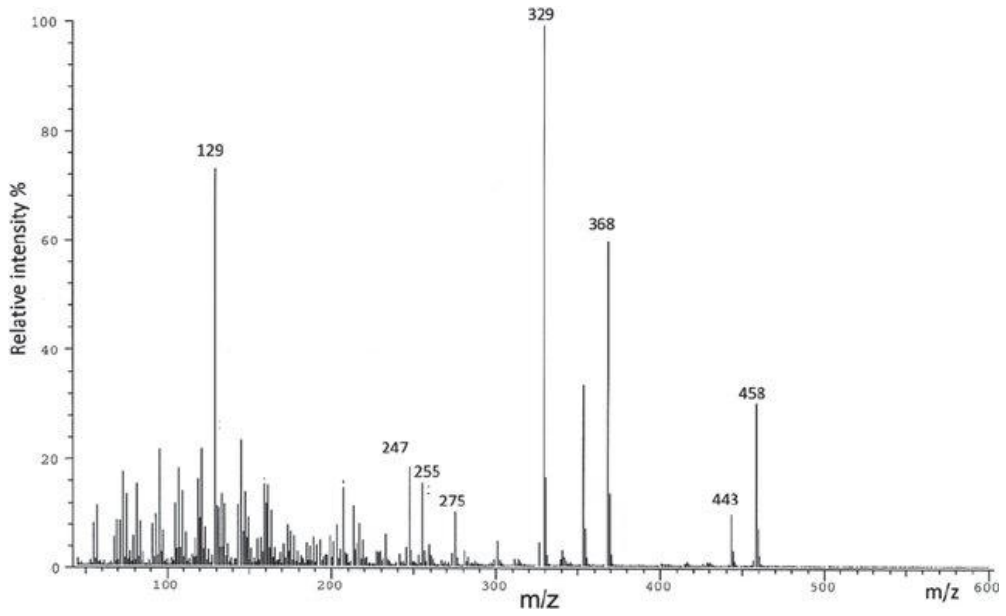


Figure 2.2 Mass spectrum of heneicosane that was found in the Calliphora vicina samples, discussed in subchapter 2.5.3. (A saturated C21 straight chain hydrocarbon – a white wax)

GC-MS is a fully tried and tested analytical tool for quality assurance in many fields and industries and is also widely accepted as an outstanding discovery instrument in analytical investigations. (Krone *et al.*, 2010; Seeley and Lynch, 2023)

2.7.3 Cuticular hydrocarbons (CHC)

The variances between different insect orders have been studied for centuries. Gilby (1964) investigated the differences of the lipids in insects with the aim of determining the metabolism of these within the insect's system, although the variation between different insects was also found to be ascertainable. More recent periods have focused on CHCs as a practice to be able to differentiate between the individual insect species and categories. (Baker and Nelson, 1981; Sutton and Steck, 1994; Haverty *et al.*, 1997; Page *et al.*, 1997; Brown *et al.*, 2000; Chapman, Espelie and Peck, 2000; Lavine and Vora, 2005; Roux, Gers and Legal, 2006) The cuticle of the Diptera family is a compliant, hygroscopic layer that inhibits desiccation and the invasion of micro-organisms. This outer lipoprotein layer of the insect cuticle is comprised of hydrocarbons, waxes, glycerides, phospholipids, glycolipids, fatty acids, and alcohols, which is exhibited in figure 2.3.

The analysis of insect hydrocarbons has also been a rapidly developing tool, showing that each individual insect hydrocarbon configuration is specific to their species, sex, group, and class (Singer., 1998; Hannah E. Moore, Adam and Drijfhout, 2013) if this would be utilised to determine larvae age then this would be additional data to support any entomology evidence as suggested by Roux, Gers and Legal (2008).

In recent years this is starting to become a newly researched area. (Xu, G. Y. Ye, *et al.*, 2014; Moore *et al.*, 2016; Victoria Bernhardt *et al.*, 2017; Sharma *et al.*, 2021). Moore (2013) noted that CHC interpretations were prone to variations due to the specimen age, which has further been researched to show that these distinct changes to CHC profiles are identifiable Although Braga et al. (2016) suggest these differences are not in the CHCs placement in the samples profile, but to the abundance of the CHC.

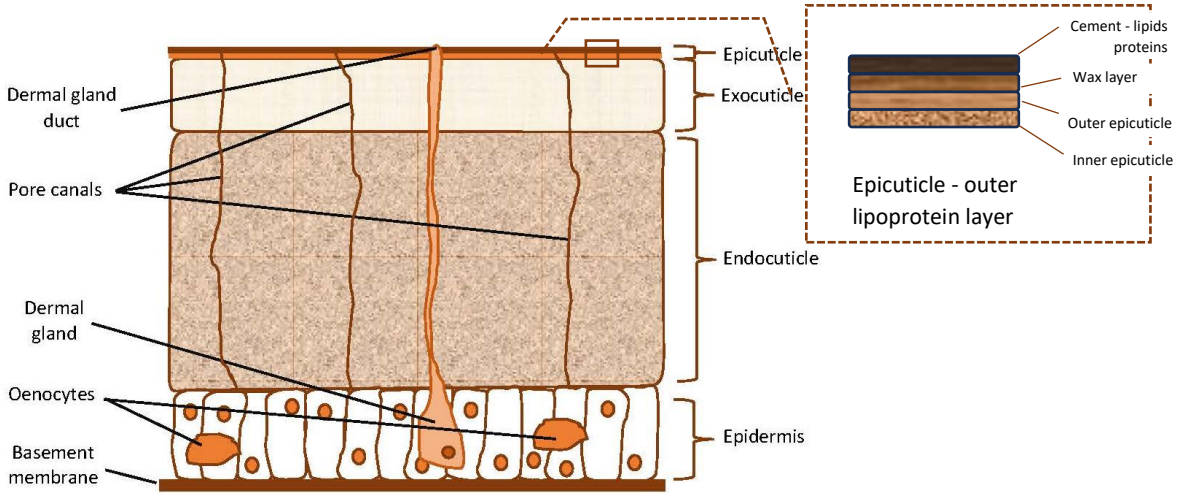


Figure 2.3 A diagram of the structure of dipteran cuticle, including an illustration of the outer lipoprotein layer – authors own

It has been suggested by Mendonça et al. (2014) that like other forensic disciplines that baseline databases are collated, so that documentation of species can be developed and expanded on as research progresses. This is currently being undertaken at Cranfield University using the destructive methodology of CHC determination. (Cranfield University, 2019). Current forensic databases are accumulated from human samples or specimens and can be quite controversial and have ethical or legal ramifications. Levitt (2007). Any entomological data collected from human specimens would be incidental to this due to the removal of the presence of human cells within the resulting samples during any analytical processes.

Cody, Sparkman and Moore (2022) suggest that adding the chromatograms of CHC's of known entomological samples would be beneficial, similarly to the current chemical and drugs know chromatograms. This would give GC-MS libraries known databases to search any unknown entomological specimens. A caveat that Cody, Sparkman and Moore (2022)

report for observing would be that with this as there would be variations among different labs and equipment, this could lead to shifts in peak position or shape and variations in retention times and the patterns of key peaks that can be obtained from the specimen to enable identification.

Again, with all the methods in this chapter, apart from initial morphological observations and low-level forms of analysis, these methods have all been found to be completely destructive of the samples. This would leave no further possibility for further rearing opportunities for identification, or for any further investigation, especially when the specimen has been treated for its analysis which would impede any further analysis. Hence the requirement for a non-destructive method.

3.0 Method development for the recovery of cuticular hydrocarbons from living specimens

3.1 Overview

This chapter follows the examination of current destructive methodology utilised within the forensic entomology field and investigates whether a non-destructive methodology could be developed. Utilising the life cycles of the fly as discusses in chapter one and the cuticular hydrocarbons discussed in chapter two, this chapter further expands this to determine whether differences can be determined without disrupting the life cycle of the specimen. This chapter also discusses the identification, storage and husbandry methods that will be used for this research.

3.2 Introduction

The aim of this aspect of the investigation was to determine from current methods whether there was a difference in CHC determination, dependent on the collection and preservation of forensically important Diptera larvae found at a crime scene. Furthermore, could a non-destructive, low-cost methodology be implemented that could be used by personnel with no entomology expertise, to help to determine specific species, in the aim of determining a $_{min}$PMI. Erzinçllioğlu (1986) indicated, that someone who is not an experienced entomologist cannot collect evidence from a crime scene that will yield as much information as the experienced entomologist. If the development of a method that could potentially increase the probability of accuracy for entomological

evidence, once implemented, this would increase the likelihood of the evidence becoming stronger verification for a criminal case.

3.3 Fly husbandry

The decomposition room of the Science Centre at Staffordshire University, Leek Road, Stoke-on-Trent. ST4 2DF (Latitude 53.008717, Longitude -2.175701) was used for the study. This ensured that the method variables for the study could be contained and controlled. This also ensured that there was no external contamination from unidentified wild flies to impact the experiment or the species identification.

3.3.1 Environments

Large BugDorm-4 Insect Rearing Cage (47.5 x 47.5 x 47.5cm) shown in figure 3.1 were used for the initial experiment, to give the flies a large living space, this ensured that there was enough space for the flies to have a healthy mating competition environment, while also safeguarded that the flies were not too overcrowded ensuring that their wings were not damaged allowing for an enhanced rearing capacity. These cages were individually labelled, to ensure no cross-culture movement while working and enabling multiple environments to be running simultaneously.

Figure 3.1 Rearing BugDorm environment individually labelled which were used for each of the separate species. (Author own image.)

Larger BugDorm-4D netted environments (47.5 x 47.5 x 93cm) shown in figure 3.2 were used for the temperature variable experiment, to further increase the living space for the flies. This ensured that there was enough space for the flies to have a healthy mating competition environment, while also safeguarded that the flies were not too overcrowded to ensure that their wings were not damaged allowing for an enhanced rearing capacity as previously stated. This enabled multiple oviposition pouch environments to be placed simultaneously to optimise the oviposition opportunities. This was to safeguard that there were enough larvae to run consecutive day sampling with consistent sample numbers, while also facilitating using the live larvae only once for the experimentation. This was to maintain that the CHC's were only sampled once per sample, eliminating the risk of influencing any CHC changes through the instars.

Once sampled, all the larvae were placed into separate containers and environments to enable them to continue their life cycle without interfering with the successive days sampling.

Figure 3.2 The larger Bugdorm rearing enclosure used during the experimentation that allow space for multiple oviposition pouches to be used. (Author own image.)

Each of the environments contained two drip feeding bottles containing a 10% sugar solution (Figure 3.3). These were situated with the necks positioned in sugar solution-soaked cotton wool within petri dishes. This ensured consistent and constant flow for the flies to feed on to ensure no pooling therefore reducing the risk of drowning. This was changed twice a week, more frequently during hotter temperatures, to ensure the solution did not dry out or become putrid.

Figure 3.3 Image of one of the water bottles feeding into cotton wool, used for consistent feeding. (Authors own image.)

The environments also contained two petri dishes holding milk powder, a protein supplement extremely important to the female flies. Protein aids the development of the tubular component of their ovaries - the ovarioles, subsequently supporting egg formation.

When oviposition was required small portions of porcine liver were used. This was to ensure that the body substitute was as fresh as possible to reduce the risk of development differences, through any decomposition during the rearing process for the period of the research. The sizes of the liver used for all the rearing setups were tailored to the instar stages. This as determined by Richards, Rowlinson and Hall (2013), would aid in the reduction of any inaccurate postmortem interval determination. Maintaining that the early instar stages were less biologically adapted to feed on fully decomposed material in comparison to third instar larvae that would be more naturally developed on substantially decomposed materials. This guided how the oviposition medium was controlled, to ensure that the liver was fresher in line with the instar stages. This can be

seen in table 3.1 where smaller quantities were used for 1st instar larvae and the quantity increases as the larvae developed through 2nd and 3rd instar and their growth increased. The quantity of liver for the third instar larvae was kept at a constant along with the second instar larvae to ensure that the larvae would keep feeding until ready to migrate away without starving to keep their energy levels at an optimum, which if not ensured could potentially restrict their size and their pupation and metamorphosis.

Table 3.1 The weights of liver that were determined through the methodology for oviposition and feeding for each life stage

Life stage	Quantity
Eggs	10-15 grams
1st Instar	25-40 grams
2nd Instar	50-60 grams
3rd Instar – until migration	50-60 grams

The liver was placed into small aluminium pouches (Figure 3.4). These pouches were squares of aluminium foil (150cm x 150cm) folded to create small open top boxes, into this was placed small pieces of absorbent paper soaked in water, to help create and maintain a humid environment, this was also to ensure that the liver also remained moist once it was placed on top. The top of the pouch was then loosely scrunched to recreate an orifice to give the flies the impression of their conventional oviposition setting on a cadaver.

(a) (b)

Figure 3.4 Aluminium pouch preparation. (a) Aluminium foil folded to cube containing saturated paper towel (b) prepared pouch containing 50g porcine liver. (Authors own images.)

In line with Byrd and Castner (2001) these environments were then placed into a container containing vermiculite, as shown in figure 3.5. The vermiculite was sourced from Garners Garden Centre, Cemetery Road, Silverdale, Newcastle under Lyme, Staffordshire, ST5 6SH. This helped to facilitate the larvae to migrate away from the feeding site and to have a suitable medium to be able to burrow into the vermiculite to pupate safely.

Figure 3.5 Prepared aluminium pouch sitting in vermiculite, ready for placing into the netted environment. (Authors own image.)

The vermiculite also functioned as an absorbent substrate during the oviposition and 1st instar, which helped to control the feeding environment from 'flooding', and potentially limiting the survival of the larvae (Authement, Higley and Hoback, 2022). Singh and Greenberg (1994) found that there was a 100% mortality rate for any aged larvae when submerged for five days, which was supported by Souza and Keppler (2009). Whereas Reigada et al. (2011) found this to be below 30%, but for three days submergence, with Carmo, Astúa and Vasconcelos (2022) finding that the emergence rate of larvae submerged was two days.

3.3.2 Calliphora vomitoria

An initial colony of *Calliphora vomitoria* were originally sourced from Blades Biological Ltd, their identification was checked visually using a Nikon SMZ745 stereo microscope and the Naturalists handbook (Erzinçlioğlu, 1996) on arrival. These numbers were then

enhanced with locally caught wild flies that were identified by the same means, before placing with their appropriate species in their separate netted environments.

3.3.3 Calliphora vicina

Calliphora vicina were locally caught as wild flies, these were then identified using a Nikon SMZ745 stereo microscope and the Naturalists handbook (Erzinçlioğlu, 1996) on arrival, before placing with their appropriate species in their separate netted environments. These were then reared to increase the numbers adequate for oviposition purposes.

3.3.4 Lucilia sericata

An initial colony of *Lucilia sericata* were originally sourced from Blades Biological Ltd, their identification was checked visually using a Nikon SMZ745 stereo microscope and the Naturalists handbook (Erzinçlioğlu, 1996) on arrival. These numbers were then enhanced with locally caught wild flies that were identified by the same means, before placing with their appropriate species in their separate netted environments.

3.3.5 Musca domestica

An initial colony of *Musca domestica* were again originally sourced from Blades Biological Ltd, their identification was checked visually using a Nikon SMZ745 stereo microscope and a muscid identification key (Crosskey and Lane, 1993) on arrival. These numbers were then enhanced with locally caught wild flies that were identified by the same

means, before placing with their appropriate species in their separate netted environments.

3.3.6 Temperature

Initially the research was aiming to mimic the genuine temperatures of a set one-year period, to ensure that observations of full temperature variables were trialled to substantially determine the behaviours and changes in life cycles of the flies. Weather data was first collected from Keele University Weather Station, School of Geography, Geology, and the Environment, during the initial investigation into this study.

Due to the level of variation in environmental factors the temperature study was brought in vitro to enable a better understanding of temperature as a factor in cuticular composition determination. From this it was decided that a small study was to be run on the oviposition and the eclosion of any eggs, to determine the effect of the extreme temperatures observed from the temperature data collected from Keele Weather Station (Latitude 53.004353, Longitude -2.267430). This would ensure that known localised temperatures would be relatable to the research base, as suggested by Donovan et al. (2006).

The temperature data that was observed demonstrated a vast expanse of variation to temperatures that had been recorded in the area during a whole calendar year. It was therefore determined that from the deviations of the temperatures that -10^{o}C, 5^{o}C and 30^{o}C would be used, as these would encompass the observed extreme temperatures that were recorded.

During the initial study, the room temperature was observed to be 20^{o}C, therefore it was decided to include this temperature as a midway point of the observed temperature ranges. This was initially used to rear the larvae to 3rd instar for the initial analysis to determine the chemical fingerprint of each of the three species. Whilst determining the initial study temperature the lighting of the decomposition room was also observed, this was noted as a 14:10 hours photoperiod and was monitored throughout this initial study.

3.3.7 Humidity

The relative humidity for all specimens throughout this temperature for the experimentation was kept at 70-80%rh. This was to ensure that all samples were fully hydrated to reduce any additional risk of desiccation of any of the instar stages particularly the eggs and 1st instar larvae.

All temperature and humidity data for the research experimentation was collected and logged using an EL-USB-2-LCD, EasyLog USB, from Lascar Electronics Ltd. The datalogger had an LCD display which allowed easy observations of the temperature and humidity, but the data was regularly downloaded and then exported to an Excel spreadsheet. This was checked and saved to ensure that all temperature and humidity parameters were consistent, specifically during the nighttime when away from the experiment. This also allowed for observations and documentation of any anomalies that could potentially affect any of the rearing variables that were being experimented.

3.4 Sampling techniques

One of the aims of this research was to determine whether there was an alternative methodology that would be suitable for CHC analysis and whether there was a non-destructive protocol that would be able to be applied when specimen sizes were of a negligeable amount. This was researched to establish a method that could be widely used at crime scenes by any non-skilled personnel, which would also be an inexpensive and timesaving technique. This would also allow for specimens to be used for other experimentation, this being entomotoxicological, chemical or in the case of rarer species, specialist specimen determination if not already ascertained by CHC analysis.

When determining a methodology for the analysis of forensic entomology samples, it was decided that how the specimens were prepared and stored to be a valuable inclusion. This would be beneficial to confirm whether any results would be expected for samples processed contrarily to the suggested way of this researched procedure. The research protocol being investigated has been found not to deteriorate or euthanise the samples, more than their natural life cycle development which is extremely advantageous for evidence collection as shown in tables 3.2 and 3.3. Any degradation that would reduce the condition of entomology sample collected would impede any analysis or identification possibility for any entomological evidence.

The techniques for the larvae killing, and storage for this research were initially based on conditions of samples considering extreme environmental settings and those most extensively used in current forensic practice, (Gennard, 2012). This current practice was then combined and expanded to include killing methods also reported by D. H. Choe,

Ramírez and Tsutsui (2012). This would establish whether the killing or storage methods affected the analysis of the larvae samples for the purposes of this research.

3.4.1 Dead larvae

For this the larvae were killed in three ways.

1. Samples were killed by placing into a beaker of boiling deionised water before removal, these were then frozen.
2. Samples were killed by placing directly into a freezer.
3. Samples were killed by soaking in 1ml of hexane, HPLC grade. This was purchased from Fisher Scientific.

These processes were to simulate previous research, to represent a control and to replicate specimens collected from different crime scene conditions. Although it is researched that colonisation is reduced in frozen scenarios, insects can colonise before freezing occurs to a cadaver, and Day and Wallman (2006) reported that liver tissue that has been frozen and thawed does not hinder insect colonisation. Payne (1965) found this when placing frozen pig carcasses out for his research in the summer. This is similar in relevance if a corpse is killed, left for a period before the environmental conditions fall below freezing temperatures, or as Gunn (2019) describes before being concealed in a freezer, in the aim of delaying disposal or pausing decomposition for an inaccurate time of death. This was the case reported by Zugibe and Costello (1993) where a victim had been killed before storing in a freezer for over two years before then being disposed of.

3.4.2 Live larvae

The live samples were originally sampled straight from their husbandry habitat, described in subchapter 3.1.1. The larvae were then measured as described in subchapter 3.4.2.1, with the addition of a transparent guide track to move along to ensure that the larvae were consistent in their movement over the scale and that they could be measured at their full length. These were then placed into either a vial containing the silica gel to complete the rubbing method or into a vial containing hexane for the soaking method.

Observations after the initial sample run showed that contamination from the porcine material and rearing pouches was being transferred along with the larvae to the sampling methods. This was amended to include a sample wash in deionised water before drying then processing through the methods. The larvae were then measured as mentioned above, they were then placed into either a vial containing the silica gel to complete the rubbing method or into a vial containing hexane for the soaking method.

3.4.3 Hexane soaking

The prepared larvae were first soaked in 1ml hexane, HPLC grade, purchased from Fisher Scientific. (D. H. Choe, Ramírez and Tsutsui, 2012) The larvae were soaked for 10 minutes before removal; this was in line with D. H. Choe, Ramírez and Tsutsui (2012) and Moore (2013)

This soaking method was used as it is currently widely used for CHC analysis using flies, larvae, and pupae. (Bernhardt et al., 2017; Moore et al., 2013, 2014, 2016; Xu et al., 2014)

It was also used in a comparison study completed by D. H. Choe, Ramírez and Tsutsui (2012).

3.4.4 Silica rubbing

The silica rubbing method that was used for this study was modified from a previous species identification method used. (D.H. Choe, Ramírez and Tsutsui, 2012)For this 0.15g of a 70–230 mesh silica gel was weighed into a 4g glass vial both obtained from Fisher Scientific Ltd, as shown in figure 3.6. This was ascertained as a vial that would be large enough to be used for all the sample sizes for all the instar sizes allowing all the surface areas to be fully rubbed/agitated with the silica gel. Once sampled and measured the specimen were placed into the vial and the lid replaced.

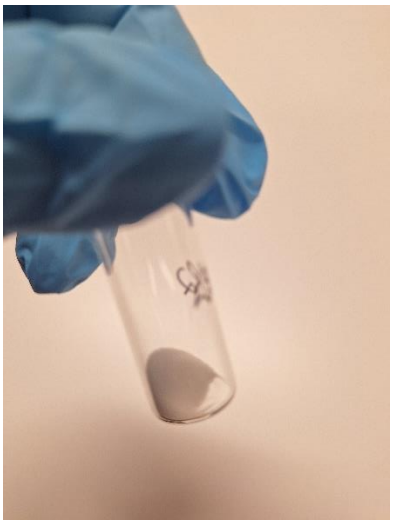


Figure 3.6 A sample glass vial containing the 0.015g of silica gel ready for addition of measured samples. (Authors own image.)

The vial was then agitated on a Stuart vortex machine at 2200 rpm for 30 seconds, which was not harmful to the larvae with no observed increase in their mortality rate (table 3.2)

to ensure that the specimens were fully rubbed with the silica to allow for the cuticular hydrocarbons to be sampled.

The speed of the vortex was experimented with the larvae beforehand to ensure that there was no apparent mortality increase due to the agitation, and this was determined to allow the eggs and larvae to continue their life cycle with no increase in mortality rates, as shown in tables 3.2 and 3.3.

Table 3.2 The percentage survival rate controls for the non-agitated samples for comparison to the agitated control samples found in table 3.3.

Sample	Species	Instar stage	% survival
Control no agitation	*C.vicina*	Eggs	96
Control no agitation	*C.vicina*	1st instar	97
Control no agitation	*C.vicina*	2nd instar	100
Control no agitation	*C.vicina*	3rd instar	100
Control no agitation	*C.vicina*	Prepupae	100
Control no agitation	*C.vicina*	Pupae	100
Control no agitation	*L. sericata*	Eggs	94
Control no agitation	*L. sericata*	1st instar	93
Control no agitation	*L. sericata*	2nd instar	100
Control no agitation	*L. sericata*	3rd instar	100
Control no agitation	*L. sericata*	Prepupae	100
Control no agitation	*L. sericata*	Pupae	100
Control no agitation	*M. domestica*	Eggs	92
Control no agitation	*M. domestica*	1st instar	90
Control no agitation	*M. domestica*	2nd instar	100
Control no agitation	*M. domestica*	3rd instar	100
Control no agitation	*M. domestica*	Prepupae	100
Control no agitation	*M. domestica*	Pupae	100

Table 3.3 The percentage survival rate controls for the agitated samples for comparison to the non-agitated control samples found in table 3.2.

Sample	Species	Instar stage	% survival
Agitation	*C.vicina*	Eggs	98
Agitation	*C.vicina*	1st instar	100
Agitation	*C.vicina*	2nd instar	100
Agitation	*C.vicina*	3rd instar	100
Agitation	*C.vicina*	Prepupae	100
Agitation	*C.vicina*	Pupae	100
Agitation	*L. sericata*	Eggs	96
Agitation	*L. sericata*	1st instar	93
Agitation	*L. sericata*	2nd instar	100
Agitation	*L. sericata*	3rd instar	100
Agitation	*L. sericata*	Prepupae	100
Agitation	*L. sericata*	Pupae	100
Agitation	*M. domestica*	Eggs	96
Agitation	*M. domestica*	1st instar	93
Agitation	*M. domestica*	2nd instar	100
Agitation	*M. domestica*	3rd instar	100
Agitation	*M. domestica*	Prepupae	100
Agitation	*M. domestica*	Pupae	100

3.4.5 Column chromatography

Once the silica rub, and the hexane soak methods were complete, the larvae were removed from their vial and returned to the separate species rearing environments for continuation of their life cycle. The silica and hexane were then ready to be fed through the chromatography column filter system.

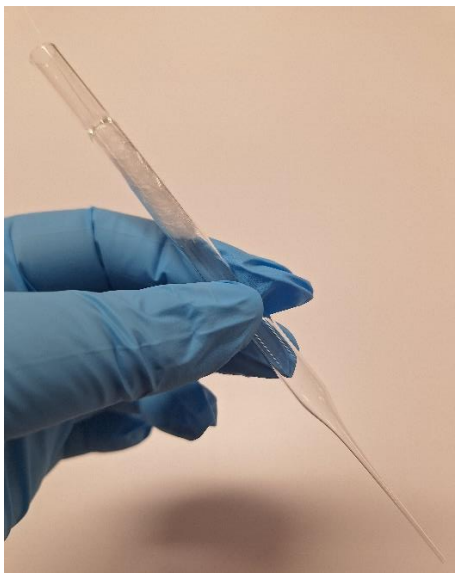


Figure 3.7 A Pasteur pipette containing glass wool, creating the small sample size chromatography column. (Authors own image.)

As seen in figure 3.7, this filter system consisted of a soda lime glass Pasteur pipette plugged with glass wool both purchased from Fisher Scientific Ltd. For the hexane soak method, 0.15g of the silica gel was placed above the glass wool to add an additional filter process to the filtration system. This aided the separation of polar and non-polar components contained in the sample-soaked hexane solution and would be a consistent process for the silica rub system. The 1ml of hexane used for the soaking was then pipetted through the column and once all the hexane was filtered and through to the vials, the Polypropylene lids with Silicone/PTFE sampling septa were secured.

The 0.15g of silica gel from the rub method was also poured into the glass wool column, this was then followed by 1ml of hexane to wash the silica gel through the glass wool filter column. This again aided in the separation of polar and non-polar components within the sample and to ensure that any physical residue in the hexane would be collected by the column so that only the hexane was collected in the clean 2ml sampling vials below. Once all the hexane was filtered and through to the vials, the Polypropylene

lids with Silicone/PTFE sampling septa were secured, these items were all purchased from Fisher Scientific Ltd.

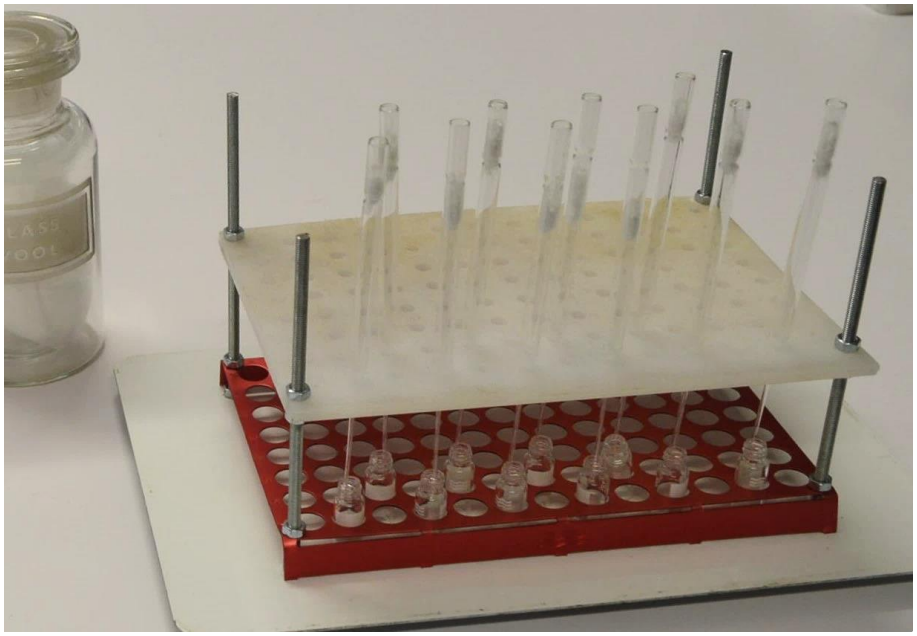


Figure 3.8 The chromatography column filter system with constructed stand to support multiple samples (Copyright Dr C Halsall.) (Authors own image.)

This process was initially only prepared one sample at a time, due to the filter column being constructed using small Pasteur pipettes, holding these individually proved awkward and time consuming. The methodology of Moore (2013) was to hold the pipette in a clamp, which proved to be time consuming and problematic, as they were prone to breaking due to the fragility of the pipette. To enable multiple samples to be processed sequentially a pipette stand was constructed from a metal base for rigidity, a stand just above this for the fixed vial spaces and two plastic boards to hold the pipettes. The upper plastic board was drilled to hold the shaft of the pipette, while the lower plastic board was drilled to hold the shoulder of the pipette to protect the fragile tip. Plastic cutting boards were used to give a small degree of flexibility to hold the delicate Pasteur pipettes.

The four corners were fixed with threaded metal rod to allow for the layers, separated with bolts to be used at differing heights. This guaranteed that the pipettes were stable when the silica gel and the hexane was being put through them and that the pipettes were equally spaced apart to eliminate the risk of cross contamination of the samples during the experimentation. (Figure 3.8). The items in this column process were purchased from Fisher Scientific Ltd and Sainsbury's Home were the source of the Large Plastic Chopping Boards.

3.4.6 Ageing samples

For the sampling of the larvae throughout the research, the samples were observed using a Nikon SMZ445 stereoscopic zoom microscope. This model of microscope gave the advantage of being able to make observations of the oviposition medium during the initial stages of the experimentation, to ensure that any oviposition was not overlooked without disrupting the sample environment. This was found to be a common occurrence, due to the flies hiding in the corners and flaps of the aluminium pouch as well as any fissures and recesses in the liver. If oviposition was unnoticed and only observed at a later instar stage, this environment was taken out of the fly enclosure and placed in another species-specific netted cage for development through until pupation. These were then returned to the experimentation enclosure to allow to hatch through to begin the process again.

The microscope was also used for examining the posterior spiracles for the ageing of the different instars of the experimentation, to safeguard correct identification of the 1st, 2^{nd,} and 3rd instar of each species. Observations were also made on the condition of the

samples, to guarantee that any of the storage or killing processes did not damage the cuticle of the sample. If there was any damage to the cuticle observed, the specimen was replaced with another within the sample set (n) to ensure that the sampling process was not impacted by any false results or anomalies, due to the analysis of anything deeper than the cuticle of the specimen.

Once the samples were treated to their killing and storage process, they were measured immediately, as discussed in subchapter 3.6.1.2. This was to ensure that the measurements were all consistently processed and to ensure that no degradation or shrinkage occurred after the processes. Although it is documented that the preservation medium can influence the measurements of a sample, (Adams and Hall, 2003; Day and Wallman, 2008) this was not the case as preservation chemicals were being used on this occasion.

3.4.7 Sample size determination

During the study it was established that due to the size differential between the individual instars and the independent species, it was effective to have varying sample sizes for each sample preparation that was completed. As D. H. Choe, Ramírez and Tsutsui (2012); Moore, (2013); Sharma et al., (2021) all suggest, the size of larvae sample size need to be adapted to the instar sizes to ensure that there is a high enough concentration of CHC to be detected. Multiple sample sets were trialled and due to the surface area coverage of the samples, for homogeny and reliability between all samples, it was determined that the most effective sample sizes per vial were as recorded in table 3.4.

Table 3.4 Determined samples sizes for each species.

Species	**Instar**	**Sample size (n)**
Calliphora vomitoria	Egg	50
Calliphora vomitoria	1st	30
Calliphora vomitoria	2nd	20
Calliphora vomitoria	3rd	10
Calliphora vomitoria	Prepupae	10
Calliphora vomitoria	Pupae	10
Calliphora vicina	Egg	50
Calliphora vicina	1st	30
Calliphora vicina	2nd	20
Calliphora vicina	3rd	10
Calliphora vicina	Prepupae	10
Calliphora vicina	Pupae	10
Lucilia sericata	Egg	50
Lucilia sericata	1st	30
Lucilia sericata	2nd	30
Lucilia sericata	3rd	10
Lucilia sericata	Prepupae	10
Lucilia sericata	Pupae	10
Musca domestica	Egg	50
Musca domestica	1st	30
Musca domestica	2nd	30
Musca domestica	3rd	20
Musca domestica	Prepupae	20
Musca domestica	Pupae	20

These individual sample sizes were determined the most effective for both the hexane soaking and the silica rubbing methods.

In currently used methodology, Moore, Adam and Drijfhout, (2013); Moore, (2013); Moore, Adam and Drijfhout, (2014); Moore et al., (2016) and Braga et al. (2016) subjected their resulting hexane wash to a nitrogen drying process to concentrate the components.

This additional process was not used on this occasion due to amalgamating the two methods to incorporate the silica rub methodology processed by D. H. Choe, Ramírez and Tsutsui (2012).

Which proved to be as effective at obtaining enough sample for subsequent analysis, hence removing a time-consuming step.

3.5 Analytical techniques

As previously mentioned, chemical analysis methods are already being used in entomotoxicology cases, with drugs of abuse and alcohol being the main toxicological components to be identified. (Goff *et al.*, 1991; George *et al.*, 2009; Muskan *et al.*, 2020; Al-Shuraym *et al.*, 2021; Bessa *et al.*, 2021; Galil *et al.*, 2021; Preußer *et al.*, 2021). These analysis methods are also increasingly being used by forensic entomologists. The analysis sensitivity when analysing smaller samples, could prove invaluable in determining individual chemical property fingerprints of larvae at differing sizes and instar stages. This would fundamentally give a more reliable and robust method of identification than measurements, ADD/ADH calculations or Isomorphen/Isomegalen diagrams alone. The measurement methods as previously discussed, need to be used with caution for their accuracy for PMI as they can be influenced by so many external factors, these can include, but are not limited to, temperature, food supplement, shortage of food, drug, or chemical interaction. It was found that the most abundantly utilised analytical technique throughout the research explored to be Gas Chromatography – Mass Spectrometry, the use of which was used for this experimentation. (Moore, Adam and Drijfhout, 2014; Moore *et al.*, 2016; Victoria Bernhardt *et al.*, 2017; Sharma *et al.*, 2021).

3.5.1 Gas Chromatography – Mass Spectrometry (GC-MS)

As this research was being aligned with previous studies completed by Moore (2013) and D. H. Choe, Ramírez and Tsutsui (2012) the analytical equipment programming had to be modified due to the configuration of the GC-MS that was used by Moore, Adam and Drijfhout (2013) not being available in the Analytical Suite of the Science Centre at Staffordshire University. Therefore, the D. H. Choe, Ramírez and Tsutsui (2012) method for the GC-MS set up was used. This included using the splitless method for the injection of the sample into the GC. With the size of the sample that the experiment used, this would relate to Grob (1994) who summarised that a splitless injector transfers all the sample to the column, which is used for trace analysis, whereas the split injector transfers only a small portion of the sample to the column and is used for concentrated specimens. However, there was not enough temperature detail available from the D. H. Choe, Ramírez and Tsutsui (2012) paper, so for the methodology the holding temperature was determined from their resulting chromatograms.

The chemical analysis for all the method development phase sample sizes and room temperature samples were completed on the Clarus 500 Gas Chromatograph using a Perkin Elmer Elite – 5MS 30 meter 0.25mm, fused silica capillary. This was paired with the Perkin Elmer 500 Mass Spectrometer using electron ionisation (EI) with quadrupole mass analyser. This used Turbomass software for data collection and evaluation.

The initial oven temperature was programmed at 100^{o}C to be held for 1 minute, this was then ramped up to 300^{o}C at 15^{o} per minute, which was then held for 10 minutes. The samples were injected into the GC using the autosampler at a sampling rate of 1.56250

pts/s using capillary injection port B using a splitless method followed by FID detection. The resulting run time for each sample was 24.33 minutes.

3.6 Method comparison

For the analysis of the methods, the measurements of all larvae were taken, this is aligned with methodology currently used as discussed in subchapter 3.4.1.2, this data would then be available to be interpreted through the Isomorphen or Isomegalen diagrams demonstrated in figures 1.9 and 1.10 in chapter 2. For this research these will not be used, as the main aim was to determine whether the sampling experimentation methodology could be beneficial for the field.

The larvae were reared through to 3rd instar for each of the species and then subjected to the killing and storage methods. The 3rd instar larvae were used for this initially to ensure that the larvae were of a reasonable size for observations to be made. This also allowed for the surface area of each of the species to be large enough for both the silica rub and soak methods. Initially this study focussed on the three most forensically important flies for forensic entomology that had been researched in the UK, *C. vomitoria*, *L. sericata* and the *M. domestica.*

Due to the timing of the study and the availability of the species of fly that were available for purchase and the localised wild flies that were caught and identified to replenish and increase stocks, it was decided to repeat this initial study replacing the *C. vomitoria* for the *C. vicina*. This would allow for continuous and consistent comparisons for the whole of the research.

3.6.1 Results

3.6.1.1 Temperature

The data determined from the variations of the initial localised minimum and maximum observed temperatures of -10°C, 5°C and 30°C were trialled, the results are shown in table 3.5.

Table 3.5 Measurements in millimetres taken of larvae for the extreme temperature investigation.

	Control (20°C) eggs	Control (20°C) 1st instar	10°C eggs	5°C eggs	30°C eggs
Mean	1	3	1	1	1
s. d	0.000	0.516	0.000	0.000	0.000
n	50	30	50	50	50
Mean	1	2	1	1	1
s. d	0.000	0.422	0.000	0.000	0.000
n	50	30	50	50	50
Mean	1	1	1	1	1
s. d	0.000	0.422	0.000	0.000	0.000
n	50	30	50	50	50

Species Key	
C. vicina	
L. sericata	
M. domestica	

The fly husbandry was followed, and any resulting oviposition was then placed into the three extreme temperature conditions, with the control sample kept at the 20°C room temperature. It was observed that the eggs placed into incubators at the three extreme temperatures showed no sign of hatching, while those samples kept at the room temperature 20°C hatched to their 1st instar stage. The total number of eggs and larvae observed for this trial was 690, the larvae from the 20°C enclosure were observed for survival rates and this was observed to be 100%.

The extreme temperatures were observed for an additional 30 days with still no sign of hatching, although signs of severe desiccation were observed, leading to this trial to be terminated.

Another trial was undertaken, this time to include the oviposition possibilities at the extreme temperatures being investigated. The results for the controls kept at 20^{o}C were equivalent to table 3.5 but for the flies placed into the extreme temperatures, these had a 0% survival rate for the flies, and no oviposition was witnessed. This would support the discussion of temperature importance as already discussed in sub chapter 1.4.1.1 and strengthen the case for more temperature investigations to be conducted for all forensically important fly species. The total number of eggs and larvae observed for this trial was 780, this was for the flies kept at room temperature, there were no eggs or larvae to analyse at the extreme teperatures due to no oviposition occurrence.

3.6.1.2 Larvae measurements for method comparison

To measure the larvae a geometric micrometre was used, this was aligned to a model devised by Villet (2007). This was drawn on to a white tile for ease of observation, directing the larvae and for ease of keeping the samples contamination free. A transparent plastic ruler and transparent guide track was aligned with a baseline that was calibrated with a Moore & Wright ABSolute digitronic calliper, with an accuracy of ±0.03 mm. This method was used instead of using the actual callipers due to the methodology using live larvae, which were too active to be able to measure in the calliper jaws and were easier to align and measure to a fixed scale. The number of larvae observed and measured for this trial was 6,300.

Table 3.6 The mean measurements and 1 s.d. of the 3rd instar larvae lengths for each of the species and the killing/storage methods.

	Con	BF	F	R	RL	S	SL
Mean	15	15	14	14	15	14	15
s.d.	0.450	0.509	0.761	0.479	0.490	0.498	0.504
Mean	12	12	11	11	12	11	12
s.d.	0.430	0.498	0.450	0.466	0.507	0.466	0.498
Mean	11	11	10	10	11	10	11
s.d.	0.479	0.490	0.407	0.466	0.498	0.498	0.466

Species Key	
C. vicina	
L. sericata	
M. domestica	

BF	Boil & frozen	RL	Rub live larvae
F	Frozen	S	Soak method after boil
R	Rub method after boil	SL	Soak live larvae
Con	Control	s.d.	1 Standard deviation

When measuring the larvae for each of the killing and storage methods, it was immediately observed that the larvae lengths were slightly different (Figure 3.10). Day and Wallman (2008) concluded that all species do not respond the same to storage methods, although this was in terms of preservation methods with chemicals, this was also observed with killing and storage methods during this initial study.

The larvae killed in boiling water before freezing had a difference of 1mm between their length and that of the samples that were placed directly into the freezer. It was observed that the larvae when not killed with boiling water before placing into the freezer were prone to slight shrinkage, this was due to the larvae nestling together to try to keep their centre mass warmer. This is obviously not an ideal occurrence when the length of the larvae is important when using measurements for calculations of $_{min}$PMI. It was also noted

that the larvae lengths were slightly larger with the live larvae that was sampled, the frozen and then soaked or silica rubbed larvae appeared to shrivel and curl slightly.

This was identified to be similar observations made by Heaton (2014) if the larvae were left for short periods after killing before measuring. This would support the importance of measuring and sampling as quickly as possible to ensure accuracy of any measurements taken, again this would support a non-destructive crime scene method of sample analysis before the specimen goes through ecdysis or become damaged in any way.

Adams and Hall (2003) noted results that differed from Tantawi and Greenberg (1993) with larvae that had been killed in boiling water, or hot water killed (HWK) before storage. The temperature of the boiling water that was used for this study was not taken, although the larvae lengths do not appear to be considerably affected. This is demonstrated by the similarity to the measurements for the live larvae that was also sampled for the hexane soak and silica rub methods.

All these results, measurements wise, would indicate that using the live larvae provide comparable data to the methods suggested as best practice in forensic entomology by Amendt et al. (2007).

3.6.1.3 Method comparisons – GC chromatograms

When observing the chromatograms for all the samples it was observed that, although the GC and resulting chromatogram recorded the top ten peaks for all the samples, but it was noticeable that there were key peaks from each of the species. These are highlighted in figures 3.9, 3.10, 3.11, 3.12, although for the boil and frozen in figure 3.9 and the frozen larvae in figure 3.10, the profiles were lacking in a distinctive profile pattern and

strong composition content for the retention times. This, due to the nature of the silica rub method, would indicate that there may not be a suitable cuticular surface for the hydrocarbon sampling to be rubbed with the silica gel.

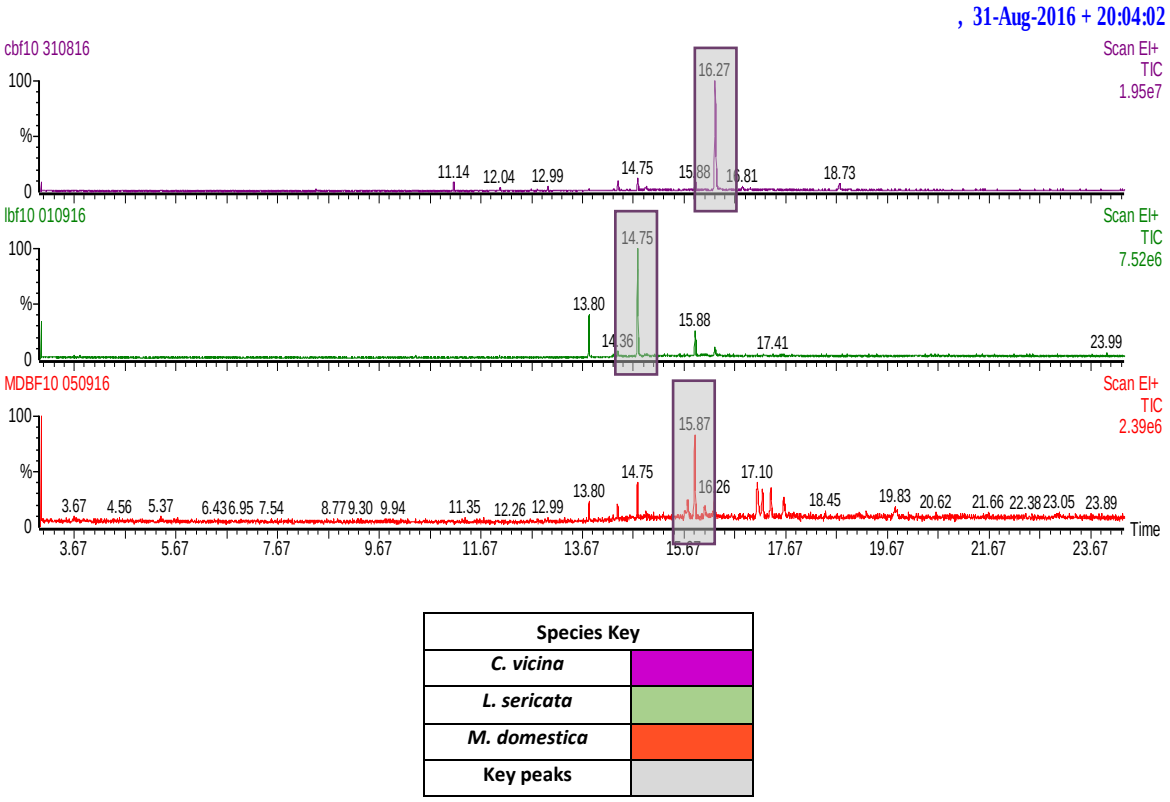
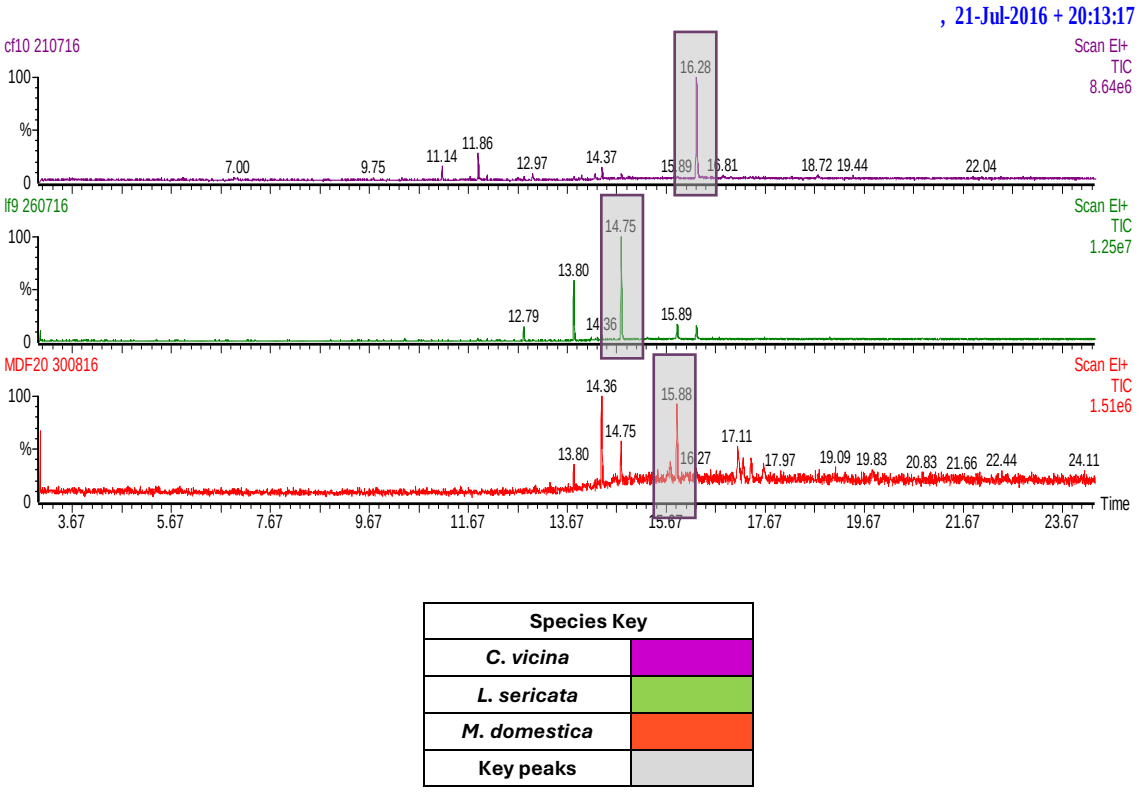


Figure 3.9 A comparison of three chromatograms for the boiled and frozen larvae for the initial three species

The nature of the cuticular surface of the larvae, although resilient to certain environmental parameters to enable the larvae to survive, could be damaged or hardened through the process, deeming the surface too rigid for the cuticular hydrocarbons to be rubbed against. Although it is known that most insect larvae can survive lower temperature fluctuations, Layne and Kuharsky (2001) state that *Eurosta solidaginis* (Diptera: Tephritidae) can withstand storage at extreme frozen temperatures,

although this is reliant on how the lower temperature affects the water content of the insect stage.

Villazana and Alyokhin (2019) report that holding *Hermetia illucens* (Diptera: Stratiomyidae) at freezing temperatures for longer than 60 minutes can cause thermotropic impairment to their cell tissue. This could impact the cuticular hydrocarbon surface for any sampling, which would indeed reduce the compositional content for analysis.

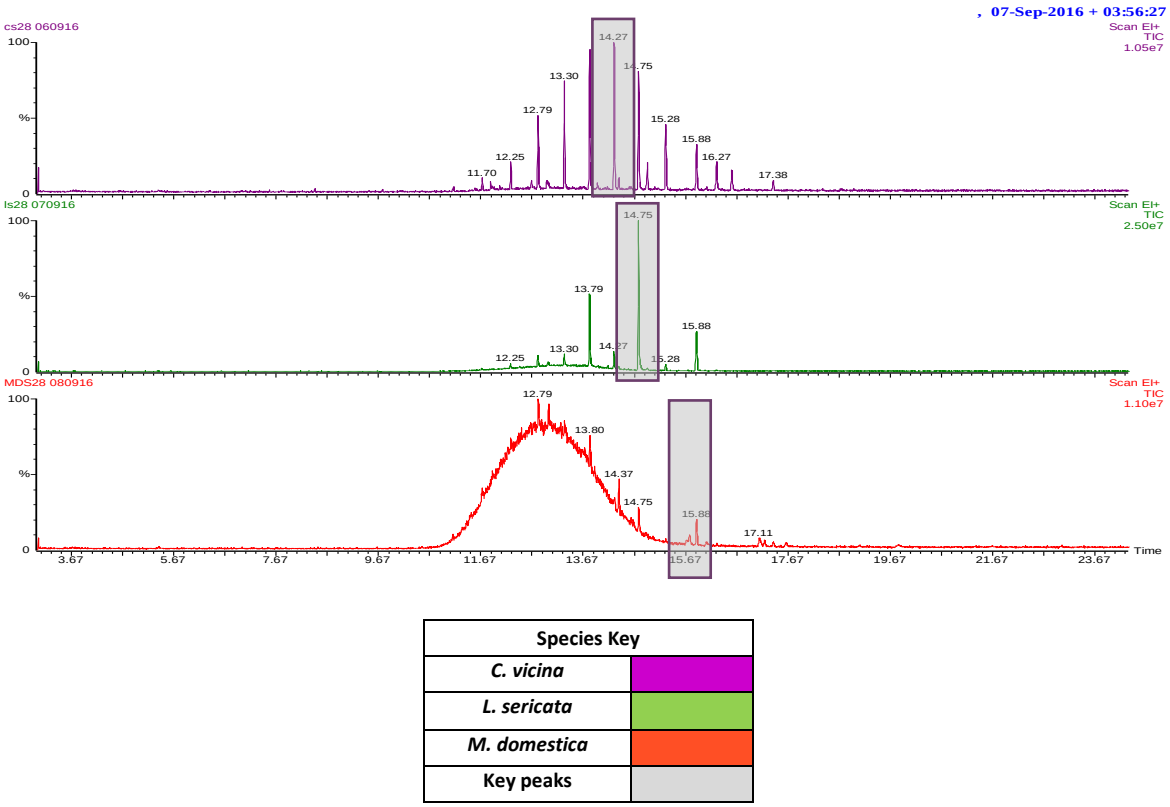


Species Key	
C. vicina	
L. sericata	
M. domestica	
Key peaks	

Figure 3.10 A comparison of three chromatograms for the frozen larvae for the initial three species

It can still be observed that the key peak that is highlighted in grey for all the chromatograms are still observed in figures 3.11 and 3.12. This would indicate that these CHCs were still adequate after the storage methods for the silica to have rubbed these for

analysis, although these were not enough to present a clear and identifiable pattern for quick and straightforward species identification.



Species Key	
C. vicina	
L. sericata	
M. domestica	
Key peaks	

Figure 3.11 A comparison of three chromatograms for the hexane soak method for the initial three species

The clear notable differences between the boiled/frozen, frozen and the rub and soak methods are that a pattern of key peaks is more observable with the soak and rub methodology. Given that these were completed with live larvae, this would indicate that live larvae were a better sample to be processing and that the storage methods degraded the CHC's too much for their full profile pattern to be identifiable.

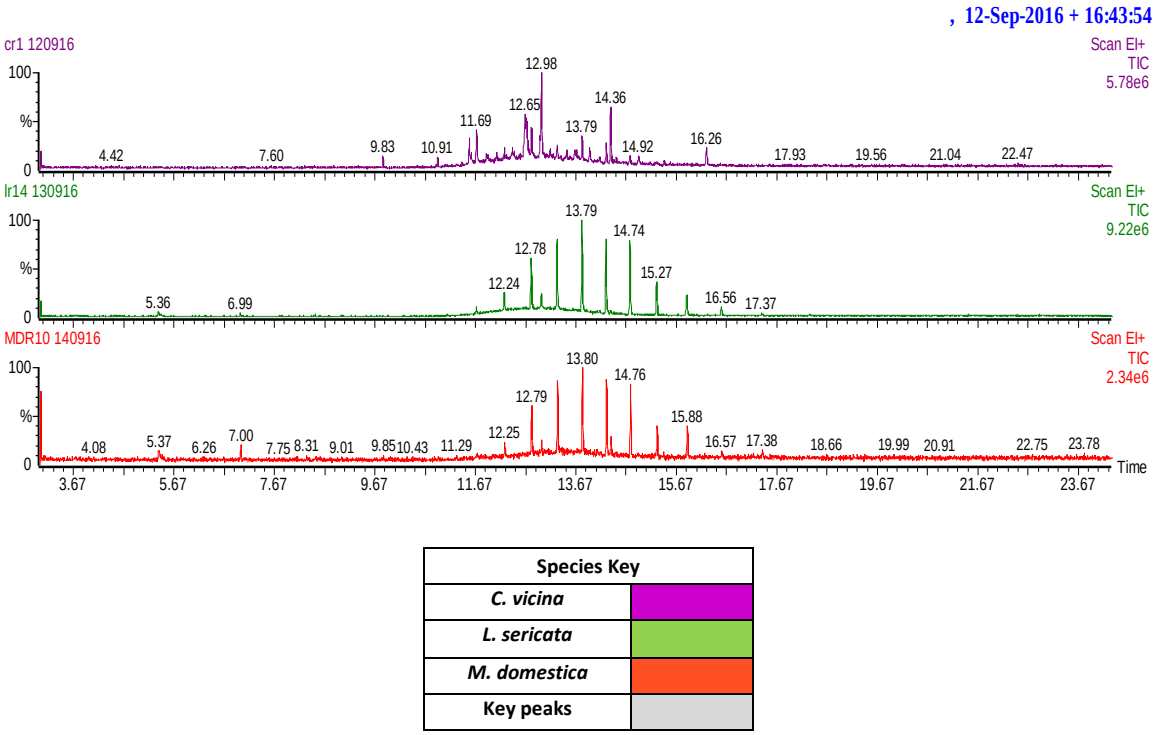


Figure 3.12 A comparison of three chromatograms for the silica rub method for the initial three species.

An initial visual observation was made of the retention times of the distinct species, once these were visually compared, the top ten peaks of absorbance, identified by the Turbomass software, for each method and each species were input onto an Excel spreadsheet. Standard deviation of the retention time was then calculated for the top ten peaks identified by the GC for each of the methods for each of the species used. The Standard error of the mean (SEM) was also calculated, this was completed to signify the samples mean deviation for the distribution of the samples, to aid in the visualisation of the variable of the sample mean as suggested by Ennos and Johnson (2018). This was calculated x2 to align with a 95% confidence interval as suggested by Streiner (1996) and Altman and Bland (2005).

When primarily scrutinising the data, compiled in figures 3.13, 3.14 & 3.15, it was noticed that there are clear distinctions between the methodologies with the peak patterns. It

was observed when comparing these results from the boiled/frozen and the frozen samples for all the species have the greatest variable from the sample population mean. This also shows the greatest variation within the peak patterns for the retention times, considering that the boil/freeze and freeze methods were also subjected to the rub method.

When comparing the rub and soak methods for the *C. vicina* these also show a comparable similarity to each other and their SEM gives a good indication that the variable of the sampling is closest to the population mean. The error bars also indicate that there is no statistical difference between the samples. This would indicate that the methods are allowing for similar consistency in the identification of the samples, inferring that the CHC sampling would be of a consistent nature.

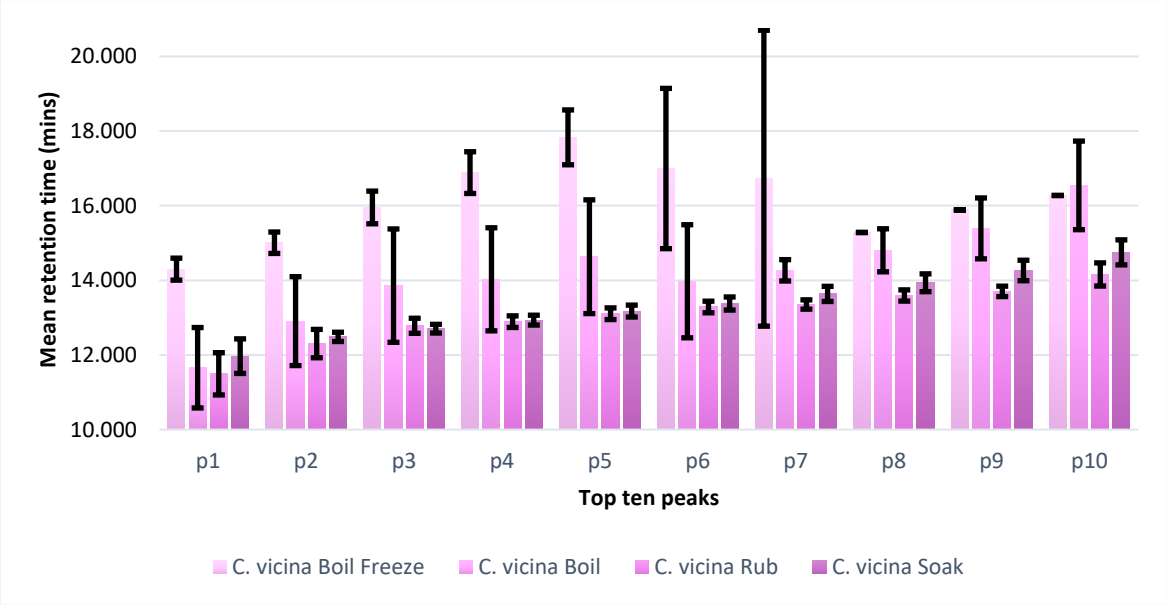


Figure 3.13 Method comparison for 3rd instar *C. vicina* for the top ten peaks with 2x standard error mean (SEM) bars

On analysis of the *L. sericata* samples it was noted that like the *C. vicina,* there were consistencies with the observations for the top ten peaks. The error bars illustrate that there are no differences between the boil/freeze and freeze methods due to their proximity to each other and to the samples mean. It is also noticed that the boil/freeze method only provided seven peaks for the comparison, this would indicate that the process had degraded the samples to much for any further peaks to be detected. Although there is overlapping with the error bars for the first two peaks, these are then separate for all other peaks, this would indicate that there are differences with the boil/freeze and freeze and the silica rub and soak methods. Similarly, showing a clear split between the methods using freezing and those not using freezing.

It is recognised that although there are differences between the methods, there is a clear trend for the comparison of the peak pattern, this would indicate that the sample is consistent with the surface sampling.

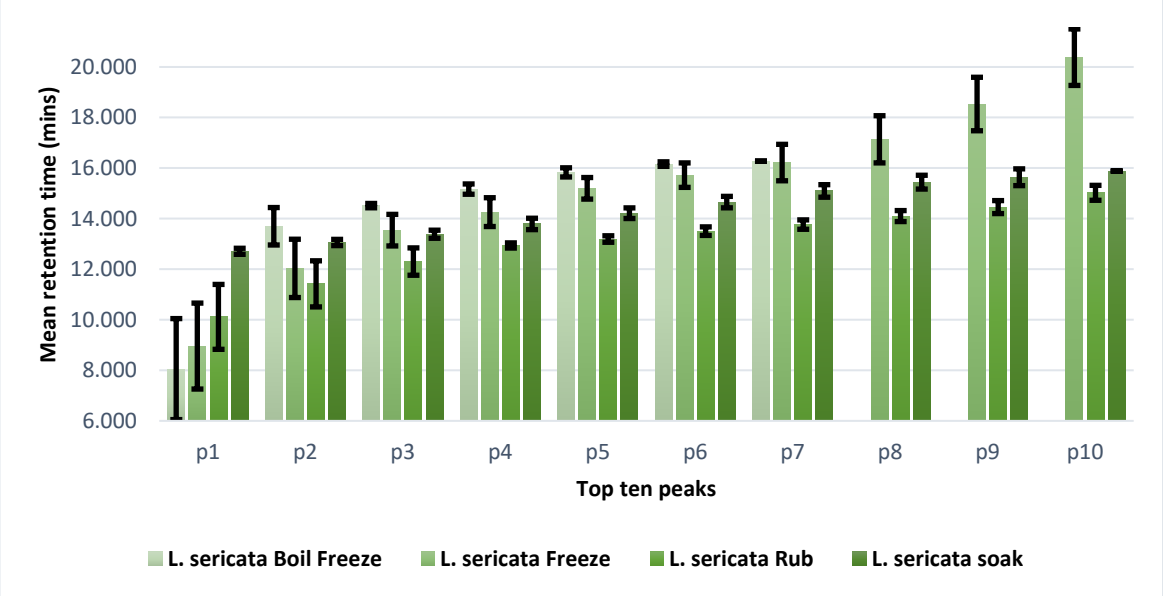


Figure 3.14 Method comparison for 3rd instar *L. sericata* for the top ten peaks with 2x standard error mean (SEM) bars

On analysis of the *M. domestica* method comparisons it becomes obvious that the retention time peaks are noticeably higher for all the storage methods and sampling methods. Which would indicate that a greater abundance of components being identified from these samples than observed with the *C. vicina* and *L. sericata* species. This would correlate to the larger sample size being used for the *M. domestica*, indicating that the methodology is comparable for a smaller species sample. The boil/freeze and free methodology standard error indicates that there is a statistically significant difference to peak 1 sample. Although this is conflicting for the other nine sample peaks as these overlap and no statistically significant differences can be inferred. The rub and soak methods show no statistical differences for their differentiation.

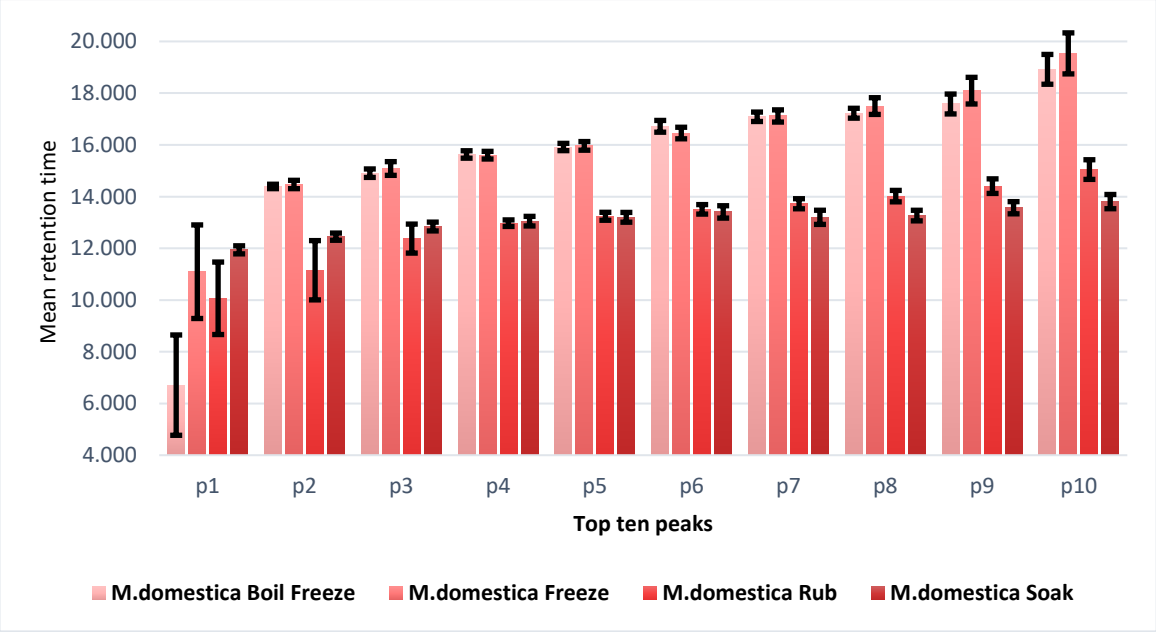


Figure 3.15 Method comparison for 3rd instar *M. domestica* for the top ten peaks with 2x standard error mean (SEM) bars

3.6.2 Discussion

The aim of this initial investigation was to determine whether storage factors impacted on larvae to be analysed and to identify the key top retention times peaks by their abundance and observe whether the pattern to these was consistent for individual species, therefore enabling the distinction between the species.

On comparison for all the storage methodology for all the species it is noted that the boil/freeze and the freeze methods all show inconsistencies with all the species, but also with all the other methods. These show no consistency that would merit further work on this occasion.

When observing the chromatograms for the *M. domestica* it was found that the chromatograms were subject to baseline humps, as seen in figure 3.11. This could be attributed to several factors. Column bleed is often mentioned during interpretations like this especially when temperature ramps are being used (Bauwens, Pantó and Purcaro, 2021). Septum bleed is also mentioned as a contributory factor for baseline humps or drifts(Kumar and Sharma, 2022). English (2022) states that column bleed and septa bleed are relatively the same, these would show different chromatogram patterns including bumps. Baseline humps or drifts can also be attributed to unresolved complex mixtures (UCM), this is usually related to samples of hydrocarbons that have become degraded (White *et al.*, 2013). This could be the case with samples that have been frozen as degradation can occur with biological samples more readily, especially when including any additional decomposition factors such as putrefaction of the larvae itself.

Due to the small proportions of the *M. domestica* samples paired along with the storage methods, this could be influential with the baseline humps that were observed especially

with the frozen method. The *M. domestica* was also challenging for rearing in the laboratory, which also proved problematic when trying to catch more of the species to supplement the bought in culture. *M. domestica* are also determined to be a less influential species due to it being a later arriving succession species.

After observing the three most commonly seen forensically important species of fly in the UK, it was determined to concentrate on the two most abundant species seen associated with cadavers to be sampled for the next study in this investigation. These were established to be the *C. vicina* and *L. sericata*. *C. vicina* are more adaptable to colder temperatures than the *C. vomitoria* and therefore availability of the *C. vicina* was greater due to the timings of the experimental fly trapping, which was another reason for moving forward with the *C. vicina*. This allowed for the two Diptera species to be compared more comprehensively throughout their life cycle for the second objective of this research.

The silica rub method was chosen for the completion of the study to determine whether non-destructive sampling the external CHC's would be sufficient to determine species of a sample without soaking the specimen in hexane. The current methodology, which is a completely destructive method due to the nature of soaking larvae in hexane, could potentially mean internal hydrocarbons are sampled in conjunction with the CHC's. This would not happen with the silica rub method.

After the initial phase of the research was completed, it was determined that the method of silica rubbing was a comparatively operational method for this application. This was observed by the similarity of retention time peak patterns, as a method that could be simply implemented to be fully used at a crime scene without destroying any of the small sample size that may be available, and for the specific species selection this was found to be the preferential method.

3.6.3 Proposed method protocol

From the results of the experimentation, it was determined that the specimen collection to results should follow the experimentally validated protocol establish in figure 3.16.

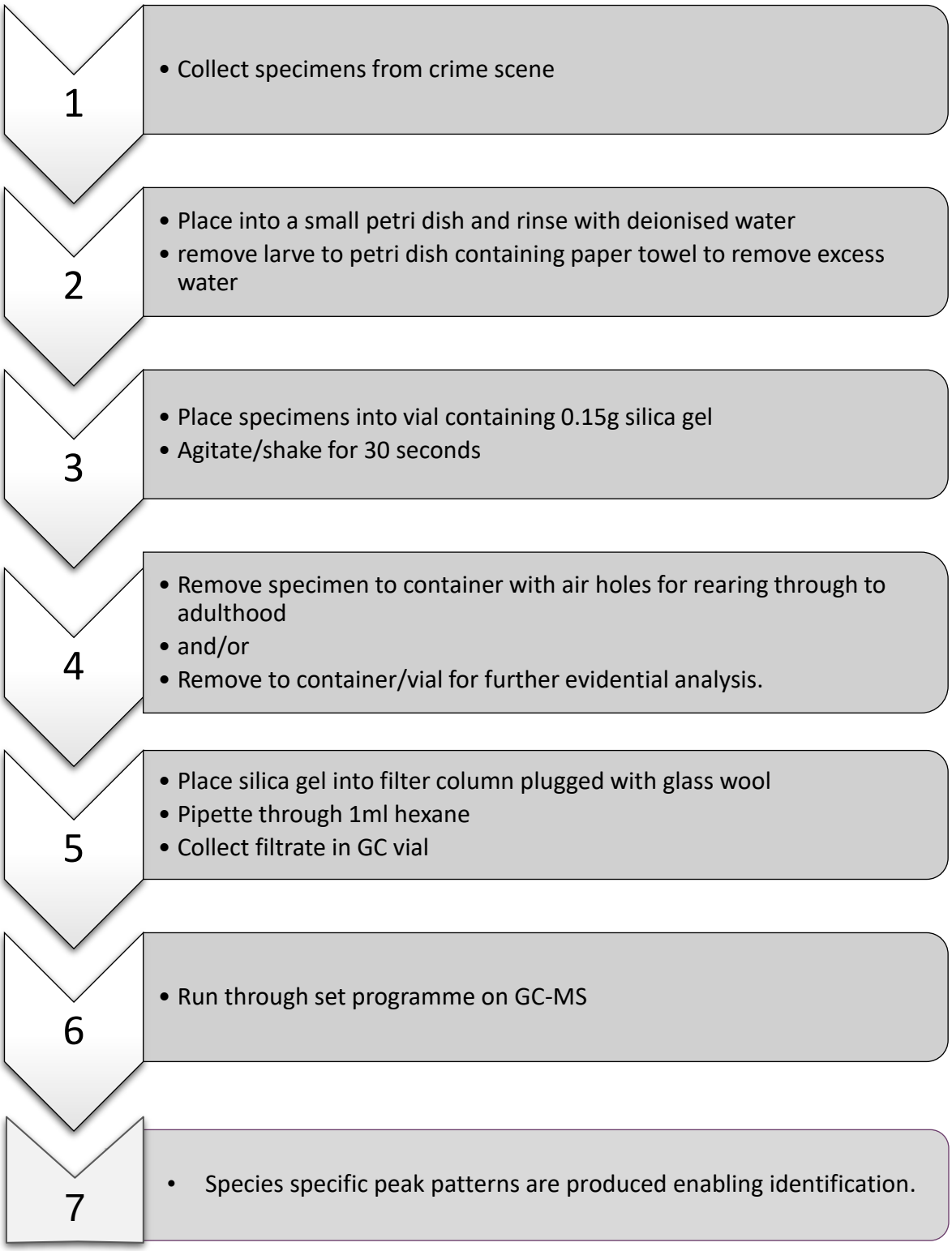


Figure 3.16 Flowchart of experimentally proposed protocol

4.0 Temperature investigation

4.1 Overview

This chapter will expand on utilising the non-destructive sampling method developed in chapter three, in conjunction with Gas Chromatography-Mass Spectrometry (GC-MS) again. The collation and visualisation method also utilised in chapter three for the chromatograms will also be discussed and expanded on to further visualise key retention time peaks.

4.2 Introduction

With differences in species larval identification found using the non-destructive method, the next objective was to determine whether these differences would be observed when at different temperatures. Given that it is widely known that larval development can be affected by temperature changes (Grassberger and Reiter, 2001; Martin Grassberger and Reiter, 2002; M Grassberger and Reiter, 2002; Ames and Turner, 2003; Warren and Anderson, 2013; Matuszewski and Mądra-Bielewicz, 2016; Ody, Bulling and Barnes, 2017; Duarte *et al.*, 2019; Bravo-Pena, Galián and Romera, 2021; Hu *et al.*, 2023), the usual length measurements and timescale would be expected to differ over the instar stages of the growth against the known lifecycle period.

Although not an entirely new technique for identification between different insects, D. H. Choe, Ramírez and Tsutsui (2012) introduced the silica rub method as a destructive method. Along with this they investigated an SPME fibre method, which Turillazzi, Sledge

and Moneti (1998) also investigated, but as a non-destructive method. The silica rub method originally utilised for ecology was then amended for this trial as a non-destructive method and is unique for forensic entomology purposes. The methodology adapted for ease of use within the field would be a vast improvement to currently used sampling methods, as any specimen sampled would still be available for further analysis or rearing through to adult. With the potential to be expanded to be used for all morphospecies at crime scenes. CHC analysis of forensically important flies similarly has already been widely used within the laboratory but, as it relies on specimens to collected at the scene and stored in preservatives, is a completely destructive method.

The aim of this investigation was to determine, employing the non-destructive methodology, whether the individual species of two forensically important flies at different temperatures could still be determined. If so, would these temperature changes show any variations from the spectral profile patterns determined in the methods development of this technique in chapter 3. If variations were observed this would potentially influence any $_{min}$PMI determination.

4.3 Experimental setup

4.3.1 Methodology

As determined from the chapter 3 study, this temperature investigation was being conducted using two of the most forensically important fly species *C. vicina* and *L. sericata*. The methodology for sampling the *C. vicina* and *L. sericata* instar stages for this study was also investigated in chapter 3. This was the modified silica rub technique

demonstrated by D. H. Choe, Ramírez and Tsutsui (2012). This temperature study also integrated the sample number size (n) that was determined for each instar stage from data collected in chapter 3, which can be found in table 3.4.

To give a full representation of the whole life cycle for each of the species it was determined that samples would be taken and analysed for each day of each species life cycle. This was determined to be day 0 which was the first occurrence of oviposition through to the first day of pupation, for each species at each of the three temperatures being investigated.

4.3.2 Temperature

At the outset of the project planning a temperature range set that simulated an average year in the UK was planned, this was proposed through a full model of annual temperatures. The data for this was collected from the Keele University Weather Station. However, the comprehensive data was determined to be too variable to mimic and to control in this study. Therefore, to allow control of the temperatures the following temperatures were selected based on the established literature on larval growth for this study. Considering the results of the minimum and maximum extreme temperatures study, the final temperatures that were used for the remaining study were selected as 10^{o}C, 20^{o}C and 25^{o}C. The lower 10^{o}C threshold was chosen as the lower eclosion temperature of *L. sericata* determined by Greenberg (1991). Although the lower eclosion temperature for *C. vicina* has been determined by Kamal (1958), to be 6^{o}C, it was decided for consistency of the evaluation of the species at each of the temperatures that this would be kept constant at the lower eclosion rate for *L. sericata*. 20^{o}C apart from being

the room temperature of the working environment. Additionally, it was also one of the most consistent temperatures within the weather station data set collected. Therefore, it was determined to be a reliable mid temperature for the study. As the aim was to determine the differences between three set temperatures, it was crucial that a higher temperature was selected to encapsulate the variable extremes found in the local area temperature data. As the highest temperature observed was 32 ^{o}C, this was determined to be too high to replicate, due to the observations of desiccation of all egg specimens at the 30 ^{o}C in the extreme temperature study. The next highest temperature that had been observed with the most occurrences within the local weather data was 25^{o}C. It was therefore determined that this temperature would be beneficial for investigating on this occasion for the higher threshold. This would also be relevant when relating to localised observations, when determining $_{min}$PMI, again linking to current weather records to allow for additional corrections if temperatures when entomological evidence is found are different from recorded data.

This temperature investigation part of the study was completed using a Sanyo MIR-154 refrigerated incubator with a temperature consistency of ± 0.5°C.

The incubators were set with a 14:10 hr photoperiod lighting, to mimic the lighting that was observed in the decomposition room for the initial method comparison study. This corresponds to other research in larval development in a laboratory, where photoperiods are used to ensure that comparable data is collected to resemble variables of the fly species environmental behaviour. (Stoffolano and Matthysse, 1967; Saunders, 1973, 2021; Evans and Krafsur, 1990; Bauer, Bauer and Tomberlin, 2020; Carneiro *et al.*, 2021). It has been established that photoperiods have triggered larval diapause, so ensuring that the photoperiod is consistent ensures that the development rates of the larvae and, as

important, the adult flies is paramount (Mcwatters and Saunders, 1998). This is in accordance with their natural habitats as much a feasibly possible to ensure that this eliminates a variable that can affect the growth rates and therefore would impact on the determination of $_{min}$PMI.

4.3.3 Humidity

The relative humidity for all specimens throughout these two temperatures for the experimentation was kept at 70-80% rh, as discussed in section 4.2.1. This was to ensure that all samples were fully hydrated to reduce any additional risk of desiccation of any of the instar stages particularly the eggs and 1st instar larvae. To ensure that the humidity was consistent at 70-80%rh as with the initial study, containers of deionised water were placed into the incubator to give the additional moisture to maintain the balance with the temperature.

The humidity was monitored on a consistent basis each day to ensure that the parameters for each temperature study was not extremely lower or higher than the desired 70-80%rh during the experimentation. This is consistent with Bambaradeniya, Magni and Dadour (2023) who state the importance of keeping accurate temperature, humidity, and photo period conditions consistent with the conditions of a crime scene. Faucherre, Cherix and Wyss (1999) also stress the importance of correct rearing conditions in line with real life cases, to ensure accuracy of comparative results. In this case the conditions of the incubator were kept in equivalence with the weather data collected. This was geographically comparable to the average of the UK humidity for that period. Again, as discussed in chapter 3, this would be pertinent to being representative

for actual cases when relating the environmental conditions to actual crime scenes. The accuracy of these environmental variable is extremely important when correlating entomological data for $_{min}$PMI determination (Haskell, Lord and Byrd, 2001; Amendt *et al.*, 2007; Gennard, 2012).

4.3.4 Gas Chromatography – Mass Spectrometry (GC-MS)

Due to the enhancement of the analytical equipment at the university, there was an analytical equipment change in between the first method investigation discussed in chapter 3 and this temperature study. The Clarus 500 Gas Chromatograph using a Perkin Elmer Elite – 5MS 30 meter 0.25mm, fused silica capillary paired with the Perkin Elmer 500 Mass Spectrometer using electron ionisation (EI) with quadrupole mass analyser was upgraded. This was to a Perkin Elmer Gas Chromatography Clarus 690 with a Supelco SLB – 5MS 30 meter 0.32mm fused silica capillary. This was paired with the Perkin Elmer Mass Spectrometer Clarus SQ 8T using electron ionisation (EI) with a quadrupole mass analyser. This used Turbomass software for data collection and evaluation. For consistency for this investigation the 20^{o}C temperature that was completed during that investigation was repeated along with the 10^{o}C and 25^{o}C temperature investigations.

The initial oven temperature was programmed at 100^{o}C to be held for 1 minute, this was then increased up to 300^{o}C at 15^{o}C per minute, which was then maintained for 10 minutes. The samples were then injected into the GC using the autosampler at a sampling rate of 1.56250 pts/s using capillary injection port B using a splitless method followed by FID detection. The resulting run time for each sample was 24.33 minutes.

4.4 Results

4.4.1 10°C temperature

Samples were taken daily for both species and clear differences between the life cycles to pupation were observed between the *C. vicina* and the *L. sericata* samples. The timescale was observed from day one of oviposition – with this documented as day 0, and to day one of pupation for this study as it was the observations of differences to the instar stages to pupation that was the objective for this investigation. The lower temperature had an impact on all the stages of the fly species development, extending the life cycle from oviposition to pupation of the *C. vicina* from 15 days to 27 days, as shown in table 4.1. The *L. sericata* life cycle to pupation was extended from 20 days to 34 days. These were in comparison to the previous study in chapter 3 where the method development samples were all kept at 20°C. This data was extended to encapsulate from oviposition to pupation as seen in table 4.2 to enable this temperature study to have comparable data across all instar stages.

Table 4.1 Growth stages of the *C. vicina* at 10°C

C. vicina 10°C	Egg	1st Instar	2nd Instar	3rd Instar	Prepupae	Pupation
	n=50	n=30	n-20	n=10	n=10	n=10
	Day 0-4	Day 5-10	Day 11-16	Day 17-21	Day 22-26	Day 27
Mean of instar	1.00	4.22	9.94	13.84	14.10	12.20
s.d.	0.00	1.82	1.60	0.75	0.74	0.40
Total sample count	7500	5400	3600	1500	1500	300

It is widely researched that the life cycle timelines and the larval growth rates of diverse species are different to each other, and as already discussed in chapter 1, temperature has a marked impact on these timelines and growth rates. (Davies and Ratcliffe, 1994; Wells and Kurahashi, 1994; Byrd and Allen, 2001; Wells *et al.*, 2015). Further research has been completed on the determination of these life cycles at specific temperatures and the duration of the cycles (Kamal, 1958; Ash and Greenberg, 1975; Reiter, 1984; Greenberg and Tantawi, 1993; Anderson, 2000; Grassberger and Reiter, 2001; Martin Grassberger and Reiter, 2002a; M Grassberger and Reiter, 2002b). With a several of these focussing on the *C. vicina* and the *L. sericata* similarly to this research, which is important to recognise when developing new methodology in relation to these variables. Although it is also vitally important to understand the geographical and environmental differences that can also have a bearing on the growth and development rates of these species too. So, completing these studies at regular intervals in different regions will only help to increase the importance of resulting data of these forensically important species.

Table 4.2 Growth stages of the *L. sericata* at 10°C

L. sericata 10°C	Egg	1st Instar	2nd Instar	3rd Instar	Prepupae	Pupation
	n=50	n=30	n=30	n=10	n=10	n=10
	Day 0-5	Day 6-10	Day 11-16	Day 17-22	Day 23-33	Day 34
Mean of instar	1.00	4.13	10.03	11.97	11.48	10.20
s.d.	0.00	1.88	1.64	0.54	0.54	0.41
Total sample count	9000	5400	5400	1800	3300	300

The retention times for all the chromatograms produced for the 10°C temperature study were collated and from these the total number of occurrences for these were calculated.

Once calculated these retention time peaks were ranked in order of their occurrence and the percentage of the occurrence of the peaks was calculated. The occurrence percentage was then calculated by multiplying the number or chromatograms produced by the number of days. This figure was then divided into the occurrence total and multiplied by one hundred, this then produced the percentage occurrence within the total sample results for the 10°C temperature set. The resulting percentage occurrences were then plotted in an Excel X-Y scatter chart against the retention times in minutes, see figures 4.1 and 4.2. Principal component analysis (PCA) was investigated to analyse the results further, but on observing the results they were visualised in such a similar manner that it was decided to stay with the initial method. This would keep the results in line with the essence of the research for low skilled personnel to be able to replicate the processes for their purposes.

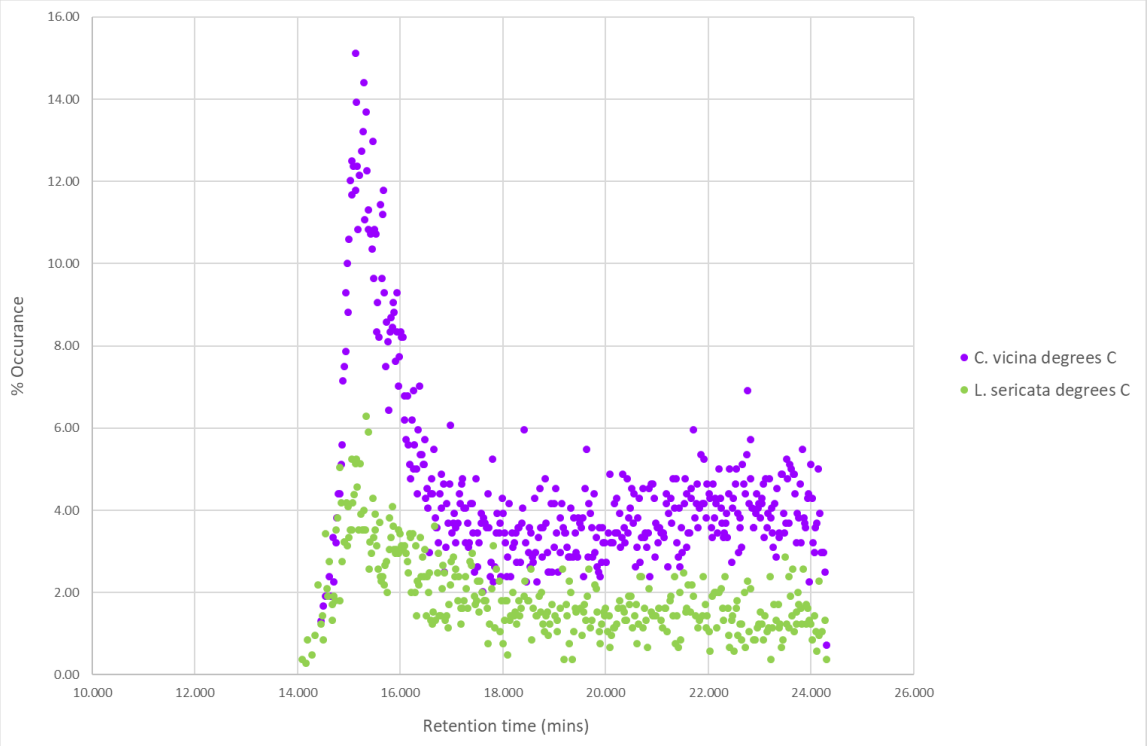


Figure 4.1 The retention times for C. vicina and L. sericata comparison at 10°C

Figure 4.1 indicates that there is an increase in the occurrence percentage for retention times of both species. This is shown by the peak spiking between 14.000 mins and 16.000 mins, although it is observed that there is significant difference to the shift between the species. The resulting percentage peaks for the remainder of the retention times up to the end of the GC-MS temperature programme at 24.330 mins appear to show no other distinctive percentage occurrence peak differences.

Due to the distinct increase in the peak percentages, it was decided that for the purposes of this comparison study to focus on the distinct apex of peak occurrences between 14.000 and 16.000 mins and to exclude the data around this specific retention time peak period for the remainder of the temperatures. To further concentrate on this key area of 14.000 mins to 16.000 mins, the data needed to be normalised, to visualise the key differences and to be able to compare these key retention times more accurately. Once normalised a threshold of 0.8% of the most abundant retention time peak occurrences were then plotted. This would have the overall effect of concentrating the peak occurrences percentages, therefore focussing the area of interest and the retention time peaks within this range.

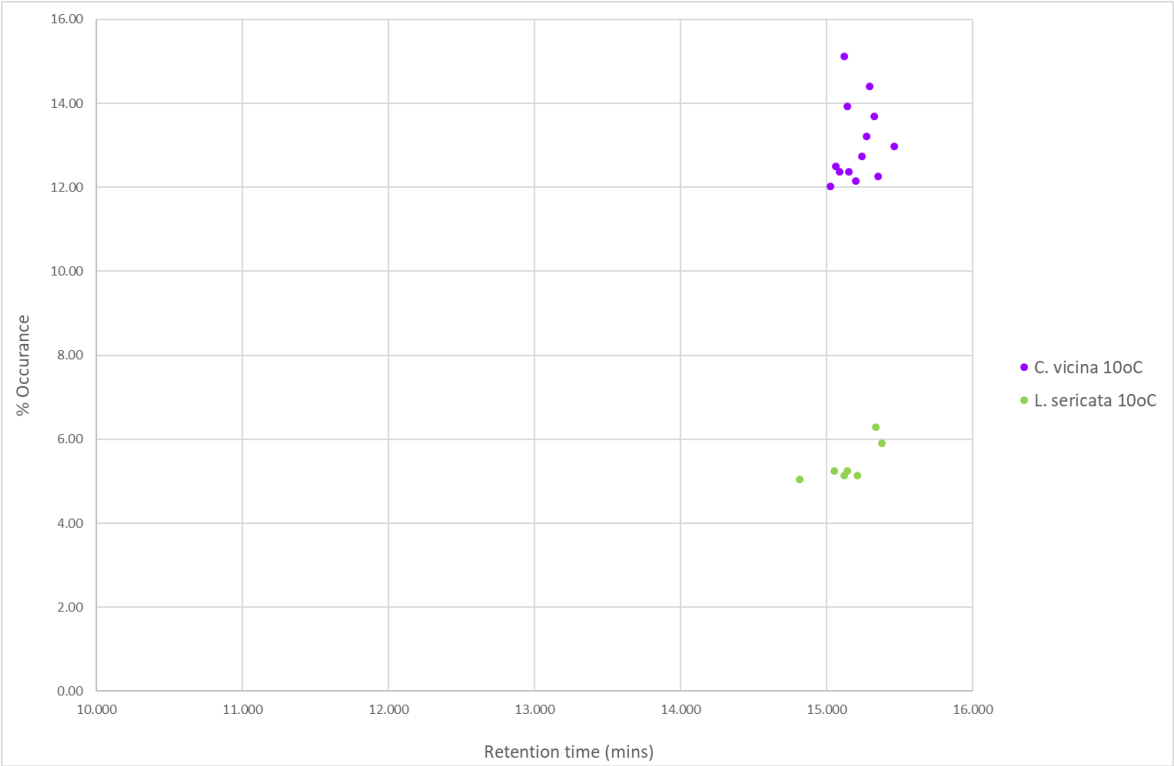


Figure 4.2 A comparison of the top % occurrences for C. vicina and L. sericata at 10°C

When comparing the focussed key peak range for the normalised data for the key retention times for 10°C it was noticed that although key peaks for both the species showed trends of similarities, there were key peak differences observed, as shown in table 4.3. Peak 15.124 was observed in both chromatograms for the key peaks of both species, for the *C. vicina* this was the most abundance peak occurrence that was observed, whilst for the *L. sericata*, it was the joint fourth peak observed.

Table 4.3 The key retention time occurrences to 0.8% threshold for *C. vicina* and *L. sericata* at 10°C

Occurrence Ranking	*C. vicina*	*L. sericata*	Occurrence Ranking
1	15.124	15.337	1
2	15.295	15.378	2
3	15.141	15.053	3
4	15.329	15.145	3
5	15.274	15.124	4
6	15.462	15.212	4
7	15.241	14.816	5
8	15.062		
9	15.091		
9	15.153		
10	15.353		
11	15.199		
12	15.028		

This comparison was expanded to include the complete data for the species peak data for 10°C, and 15.124 was one of seven peaks that were observed in both species, although found in differing ratios. The additional six peaks also found within the area of interest of the normalised data but were of a limited occurrence, were 14.503, 14.620, 14.707, 14.816, 15.537, 15.858. All other peaks were individual to the species samples, which demonstrates clear distinctions can be observed from using the silica rub methodology between *C. vicina* and *L. sericata* at 10°C.

4.4.2 20°C temperature

Samples were taken daily for both *C. vicina* and *L. sericata* species, and measurements were taken (as with the study in chapter 3), for all the instar samples. It was observed that there was a five-day difference between the species for pupation, with pupation for the *C. vicina* occurring at 15 days and pupation for the *L. sericata* occurring at 20 days.

The timescale was observed from day 1 of oviposition, this was documented as day 0, and then to day one of pupation for this study, as it was the observations of differences to the instar stages to pupation that was the objective for this investigation.

Table 4.4 Growth stages of the *C. vicina* at 20°C

	Egg	1st Instar	2nd Instar	3rd Instar	Prepupae	Pupation
C. vicina 20°C	n=50	n=30	n-20	n=10	n=10	n=10
	Day 0	Day 1-3	Day 4-6	Day 7-11	Day 12-14	Day 15
Mean of instar	1.00	4.21	10.27	14.88	14.07	12.57
s.d.	182.83	165.39	109.04	0.34	0.59	52.84
Sample count	1500	2700	1800	1500	900	300

Table 4.5 Growth stages of the *L. sericata* at 20°C

	Egg	1st Instar	2nd Instar	3rd Instar	Prepupae	Pupation
L. sericata 20°C	n=50	n=30	n=30	n=10	n=10	n=10
	Day 0	Day 1-3	Day 4-7	Day 8-14	Day 15-19	Day 20
Mean of instar	1.00	4.21	7.84	11.13	11.25	10.33
s.d.	0.00	1.19	1.20	0.86	0.67	0.48
Sample count	1500	2700	900	2100	1500	300

The process for the chromatograms was undertaken as with the 10°C data in section 4.2.1. The occurrence percentage was then calculated as in the previous section to obtain the percentage occurrence for the 20°C temperature set. The resulting percentage occurrences were then plotted in an Excel X-Y scatter chart against the retention times in

minutes, see figures 4.3 and 4.4. This method in comparison to PCA analysis was utilised as discussed in subchapter 4.4.1.

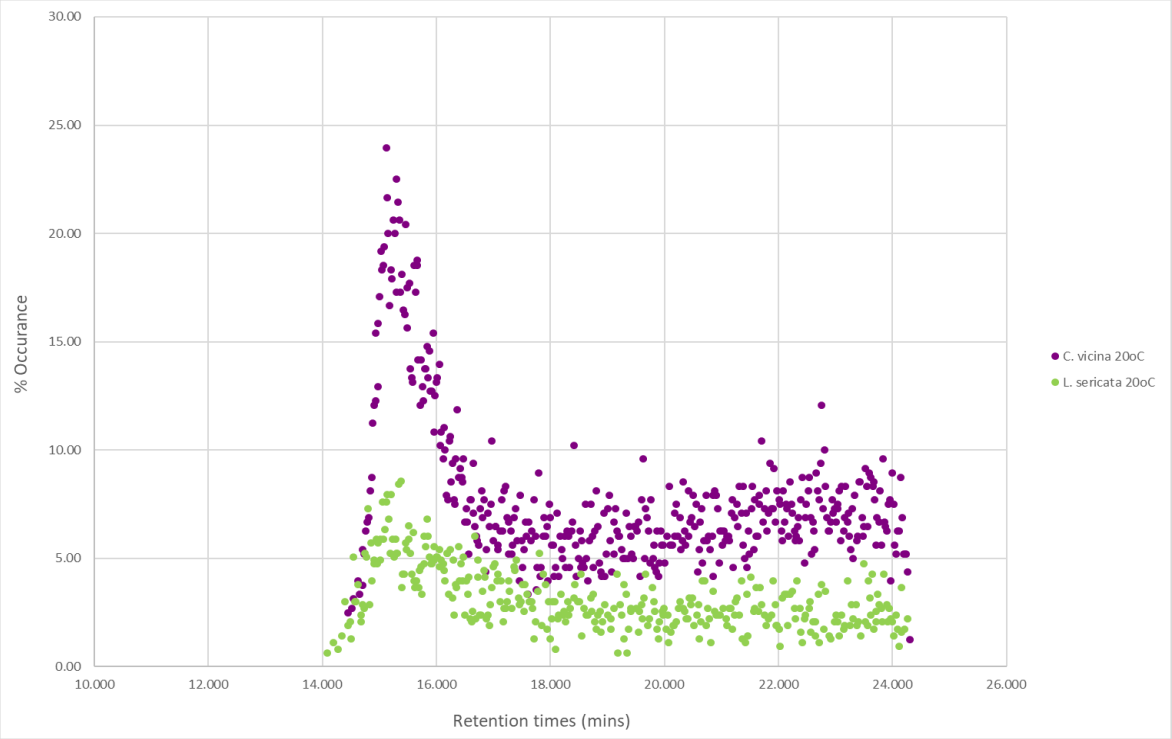


Figure 4.3 The retention times for *C. vicina* and *L. sericata* comparison at 20°C

Figure 4.3 illustrates, similarly to the retention times for 10°C study, that there appears to be an increase in the occurrence percentage for retention times of both species. This is shown by the peak spiking between 14.000 mins and 16.000 mins, although it is again noted that there is significant difference to the shift between the species. The resulting percentage peaks for the remainder of the retention times up to the end of the GC-MS temperature programme at 24.330 mins appear to show no other distinctive percentage occurrence peak differences.

Again, for the same reason as with the 10°C temperature study, it was decided to focus on the distinct apex of peak occurrences between 14.000 and 16.000 mins and to exclude

the data around this specific retention time peak period for the remainder of the temperatures. To further concentrate on this key area of 14.000 mins to 16.000 mins, the data, as with the 10°C study, was normalised to visualise the key differences and to be able to compare these key retention times more accurately. Once normalised a threshold of 0.8% of the most abundant retention time peak occurrences were then plotted. This would achieve the overall effect of concentrating the peak occurrences percentages, therefore focussing the area of interest and the retention time peaks within this range.

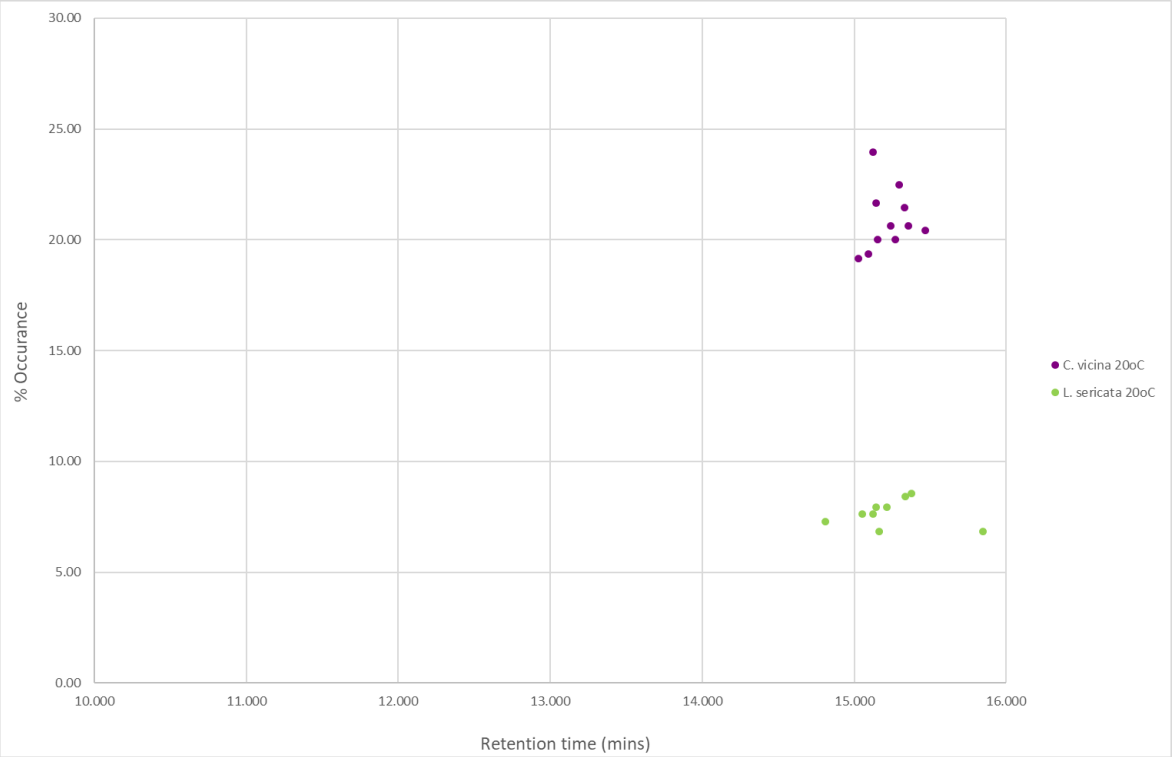


Figure 4.4 A comparison of the top % occurrences for *C. vicina* and *L. sericata* at 20°C

When comparing the focussed key peak range for the normalised data of the key retention times for 20°C it was observed that although key peaks for both the species as with the 10°C study, showed trends of similarities, there were key peak differences observed, as shown in table 4.6. Peak 15.124 was once again observed in both

chromatograms for the key peaks of both species, for the *C. vicina* this was once again the most abundance peak occurrence that was observed, whilst for the *L. sericata*, it was once more the joint fourth peak observed. This shows that the consistency of the sampling method in the detection of key observable peaks.

Table 4.6 The key retention time occurrences to 0.8% threshold for C. vicina and L. sericata at 20°C

Occurrence Ranking	*C. vicina*	*L. sericata*	Occurrence Ranking
1	15.124	15.378	1
2	15.295	15.337	2
3	15.141	15.145	3
4	15.329	15.212	3
5	15.241	15.053	4
5	15.353	15.124	4
6	15.466	14.807	5
7	15.153	15.162	6
7	15.270	15.846	6
8	15.091		
9	15.028		

This comparison was expanded to include the complete data for the species peak data for 20°C, and as in the 10°C study 15.124 was one of seven peaks that were observed in both species, although found in differing ratios. These peaks are within the area of interest of the normalised data but were of a limited occurrence, were 14.707, 14.978, 15.295, 15.395, 15.537 and 15.858, all other peaks were found to be individual to the species samples. This, again, is important as it indicates that clear differences can be observed from using the silica rub methodology between *C. vicina* and *L. sericata* at 20°C.

4.4.3 25°C temperature

As with the 10° and 20° temperatures samples were taken daily for both species and clear differences between the life cycles to pupation were observed between the *C. vicina* and the *L. sericata* samples. The timescale was observed from day one of oviposition – with this documented as day 0, and to day one of pupation for this study as it was the observations of differences to the instar stages to pupation that was the objective for this investigation. The higher temperature had an impact on all the stages of the fly species development, reducing the life cycle to pupation of the *C. vicina* from 15 days to 10 days. The *L. sericata* life cycle to pupation was reduced from 20 days to 7 days.

Table 4.7 Growth stages of the *C. vicina* at 25°C

C. vicina 25°C	Egg	1st Instar	2nd Instar	3rd Instar	Prepupae	Pupation
	n=50	n=30	n-20	n=10	n=10	n=10
	Day 0	Day 1	Day 2-3	Day 4-7	Day 8-9	Day 10
Mean of instar	1.00	2.00	6.13	12.68	12.92	12.00
s.d.	0.00	0.00	1.35	2.12	0.85	0.00
Sample count	1500	900	1200	1200	600	300

Table 4.8 Growth stages of the *L. sericata* at 25°C

L. sericata 25°C	Egg	1st Instar	2nd Instar	3rd Instar	Prepupae	Pupation
	n=50	n=30	n=30	n=10	n=10	n=10
	Day 0	Day 1	Day 2-3	Day 4-5	Day 6	Day 7
Mean of instar	1.00	3.33	7.03	10.62	10.70	10.17
s.d.	0.00	0.48	0.80	1.28	0.47	0.38
Sample count	1500	900	1800	600	300	300

The process for the chromatograms was undertaken as with the 10°C and 20°C data in section 4.2.1 and 4.2.2 and from this the percentage occurrence was once again calculated for the 25°C temperature set. The resulting percentage occurrences were then plotted in an X-Y scatter chart against the retention times in minutes, see figures 4.5 and 4.6. This method in comparison to PCA analysis was utilised as discussed in subchapters 4.4.1 and 4.4.2.

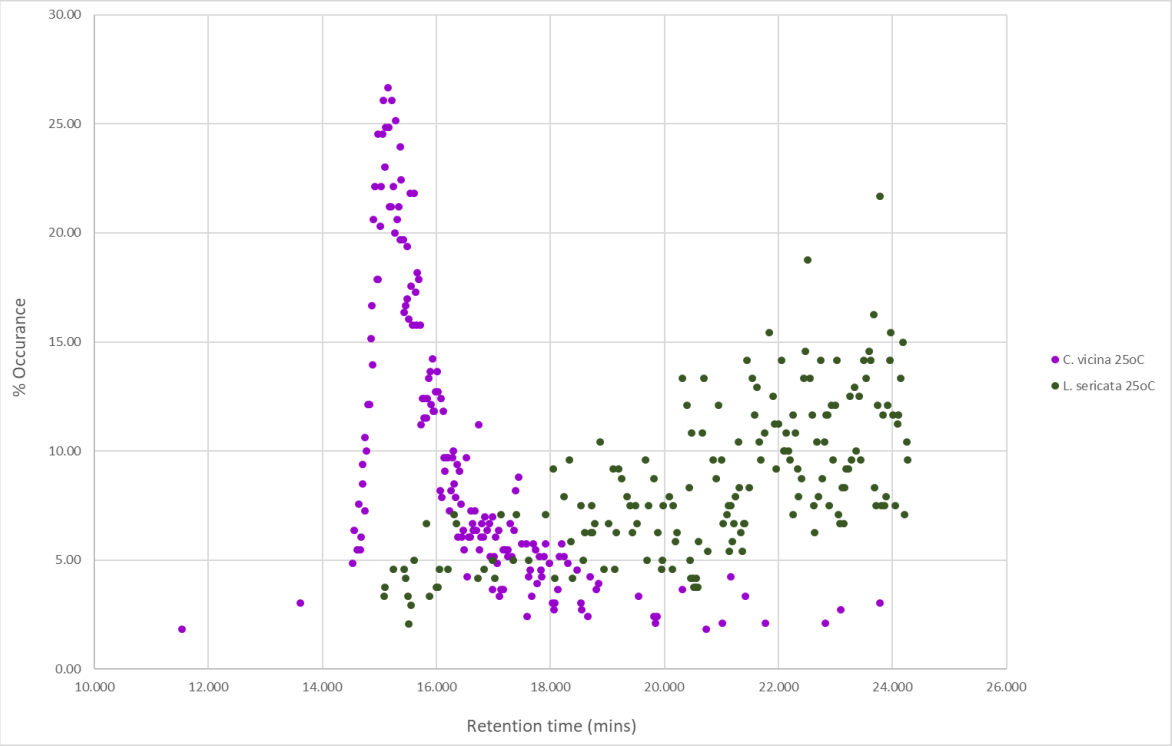


Figure 4.5 The retention times for *C. vicina* and *L. sericata* comparison at 25°C

Figure 4.5 shows clear differences for the retention time peak occurrences for 25°C to the 10°C and 20°C studies. This is shown by the peak spiking between 14.000 mins and 16.000 mins, which is still prominent for *C. vicina* and consistent with the 10°C and 20°C studies, but the *L. sericata* occurrences within the key peak area of 14.000 mins to 16.000 mins has severely reduced. The resulting percentage peaks for the remainder of the retention

times up to the end of the GC-MS temperature programme at 24.330 mins appear to increase in an ascending wide curve, this would indicate that the higher temperature has an impact on the component found at these retention times.

Although there was a clear difference to the overall retention time peak occurrences trend, as with the 10^{o}C and 20^{o}C temperature studies it was decided to focus on the distinct apex of peak occurrences between 14.000 and 16.000 mins and to exclude the data around this specific retention time peak period for the remainder of the temperatures, this would enable an analogous comparison of the data.

To further concentrate on this key area of 14.000 mins to 16.000 mins, again the data was normalised, to visualise the key differences and to be able to compare these key retention times more accurately. Once normalised a threshold of 0.8% of the most abundant retention time peak occurrences were then plotted. This would have the overall effect of concentrating the peak occurrences percentages, therefore focussing the area of interest and the retention time peaks within this range.

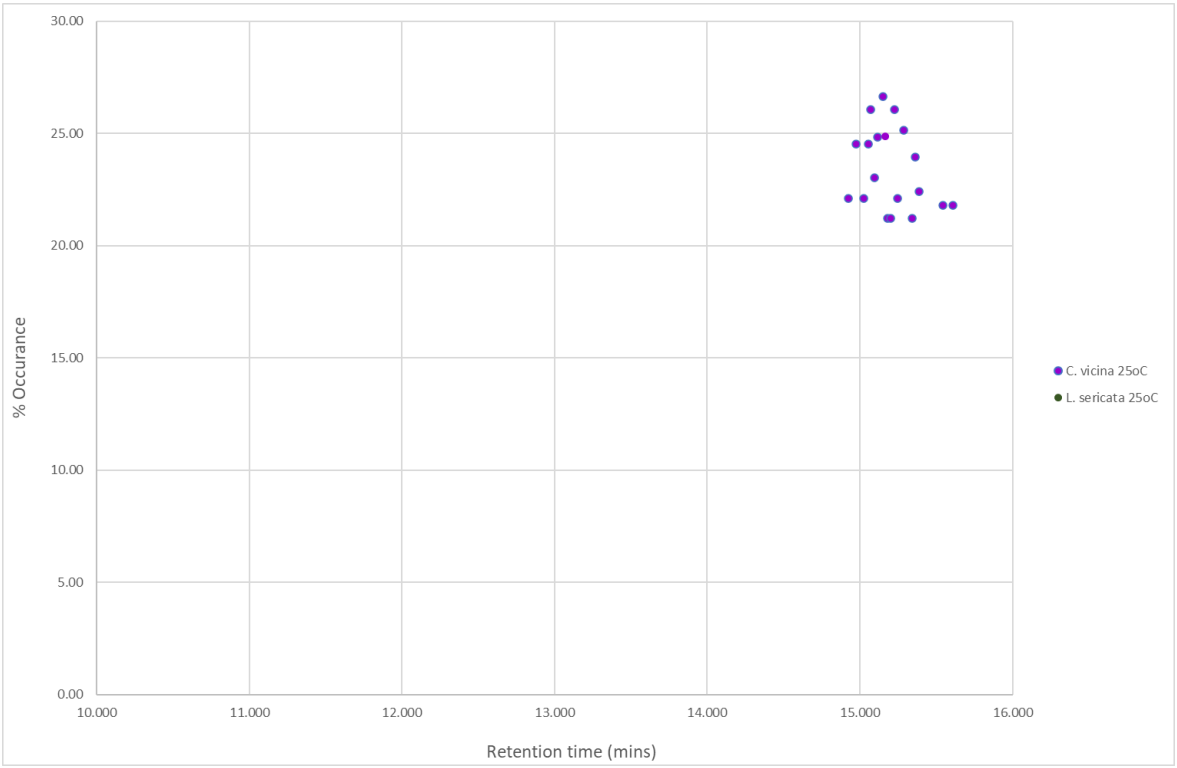


Figure 4.6 A comparison of the top % occurrences for *C. vicina* and *L. sericata* at 25°C

As seen in figure 4.6, when normalised and the threshold of 0.8% of the peaks were plotted the *L. sericata* are no longer observed, this was due to the reduction of the trending peak that was observed along with the *C. vicina* peak occurrences within the 14.000 mins to 16.000 mins range. This would indicate that the higher temperature had affected or influenced the components within the CHCs for *L. sericata,* therefore these were not detected by the GC-MS as with the 10°C and 20°C studies.

To illustrate where the 0.8% threshold occurrences of the retention time peaks and their shift within the temperature programme run of the GC-MS, the axis range was amended for the X-Y scatter plot. It can be seen from figure 4.7 that the threshold occurrence peaks are reduced in abundance and are much later in the GC-MS temperature programme, and observed to be as the temperature programme is ending.

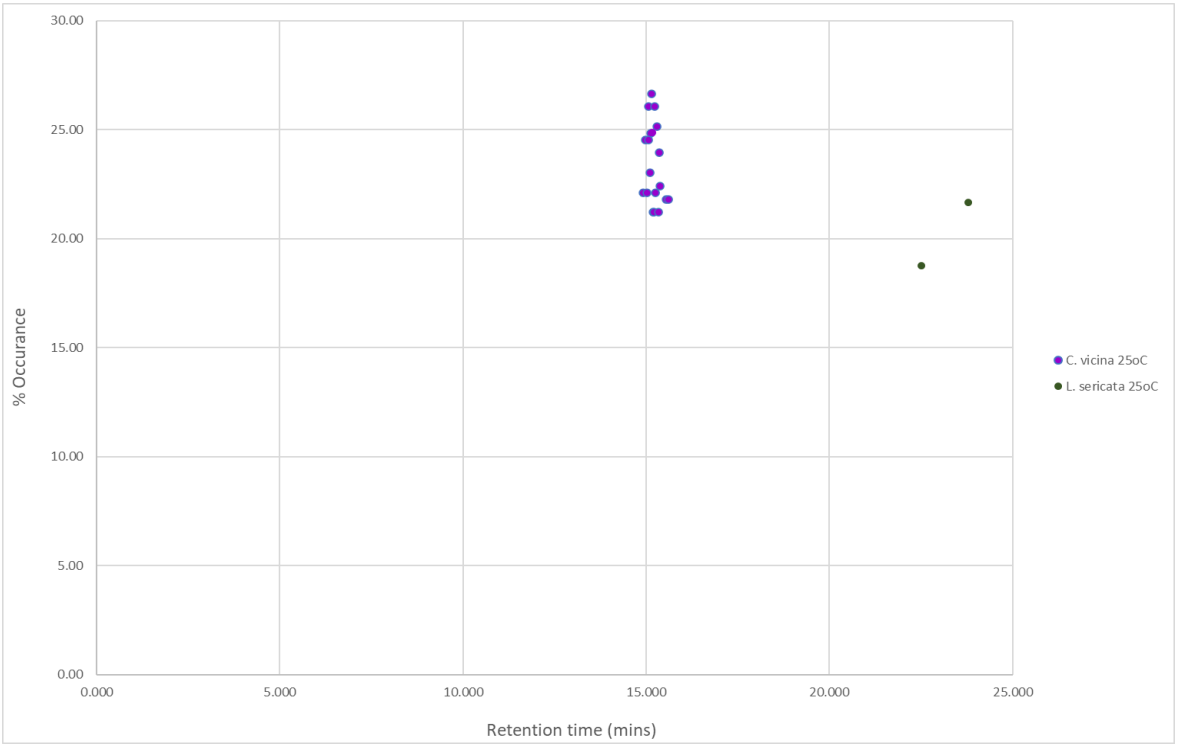


Figure 4.7 An amended axis range comparison of the top % occurrences for C. vicina and L. sericata at 25°C

When comparing the threshold key peak range for the normalised data for the key retention times for 20°C it was observed that, although key peaks for both the species with the 10°C and the 20°C studies showed trends of similarities, there were more key peak differences observed for this 25°C study, as shown in table 4.9. Peak 15.124 that was observed in both chromatograms for the key peaks of both species for the 10°C and the 20°C studies, was not detected for this temperature. This indicates that the sampling method was detecting key observable peak shifts, which would be paramount in showing differences between the species.

Table 4.9 The key retention time occurrences to 0.8% threshold for *C. vicina* and *L. sericata* at 25°C

Occurrence Ranking	*C. vicina*	*L. sericata*	Occurrence Ranking
1	15.149	23.780	1
2	15.070	22.520	2
3	15.224		
4	15.287		
5	15.116		
6	15.170		
7	14.974		
8	15.053		
9	15.362		
9	15.095		
10	15.387		
11	14.924		
12	15.024		
13	15.245		
14	15.541		
15	15.608		
16	15.183		
17	15.199		
18	15.341		

This comparison as with the previous two temperatures was expanded to include the complete data for the species peak data for 25°C, and in contrast to the 10°C and 20°C studies, there was only one observed peak at 15.462 within the threshold range for both species. The disappearance of any mutual peaks indicates that the silica rub methodology detected this and the shift in the retention times that fell within the normalised threshold. All other peaks were found to be individual to the species samples once again. This clearly indicates that distinct differences can be observed from using the silica rub methodology between *C. vicina* and *L. sericata* at 20°C.

4.4.4 All temperatures

When comparing the sample measurements for each of the instar stages from egg to pupation in figures 4.8 and 4.9, it is noticed that for both *C. vicina* and *L. sericata* that the eggs as a constant before eclosion have a mean measurement that is consistent, although the number of days spent at this stage increases the lower the temperature becomes, see tables 4.1 and 4.2. The number of samples also increases with the additional days spent at this stage as illustrated in tables 4.1, 4.2, 4.4, 4.5, 4.7, and 4.8. It is noted that the s.d. is not consistent within any of the measurement data. Although this gives the impression of varied values, it is a positive outcome for data consistency. Random sampling of any of the instar stages will increase the accuracy of the aging of the samples, this was documented by Donovan et al. (2006), who state that sampling the largest of the instar samples can only result in inaccurate results. This could impact any results by skewing the timeline of development due to these larger specimens, with the smaller specimens appearing a slower developing samples.

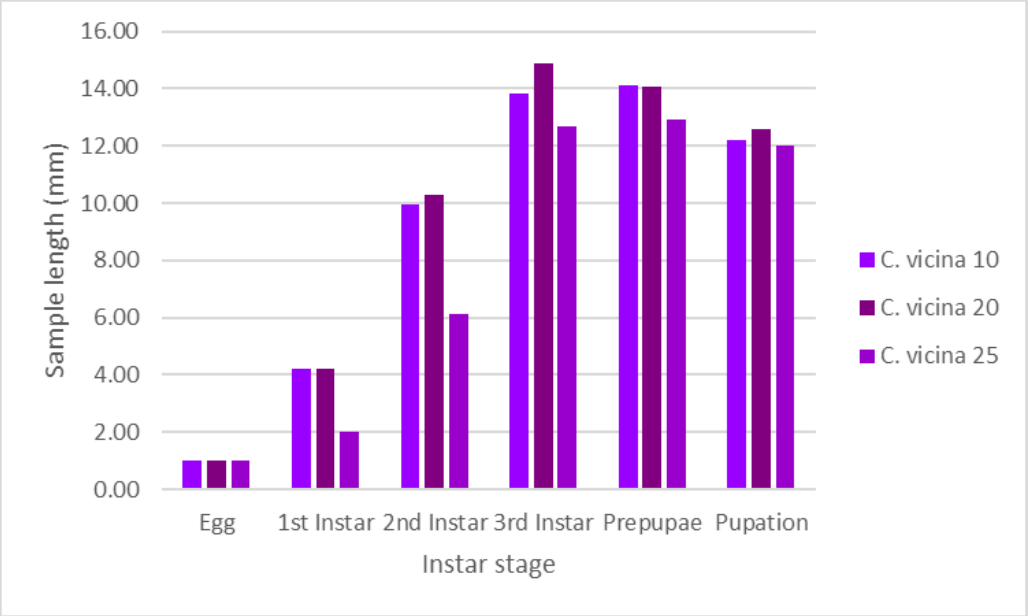


Figure 4.8 Temperature comparisons of mean sample length for *C. vicina*

It can be seen from figure 4.8 that *C. vicina* show a consistent larval growth rate through to the third instar stage. Their lengths then plateau into their prepupae stage as they are no longer feeding and then as the larvae shrinks to pupate the length decreases. It is noted that the first and second instars for the 10°C and 20°C studies show a distinct growth rate to their third instar stage, but for the higher 25°C temperature study that this is restricted through the first and second instar stages, but then there is a noticeable increase in size with the third instar stage. This would suggest that the larvae are consistently feeding and growing to keep their life cycle progressing.

When compared to *L. sericata* in figure 4.9 it is observed that, like the *C. vicina* growth rates, the growth rate for *L. sericata* is consistent again through to the third instar stage. Their growth then displays a period of reduction that correlates to the larvae no longer feeding and migrating away in their prepupae stage. The further reduction in size as with the *C. vicina* illustrates the larvae shrinking to pupate.

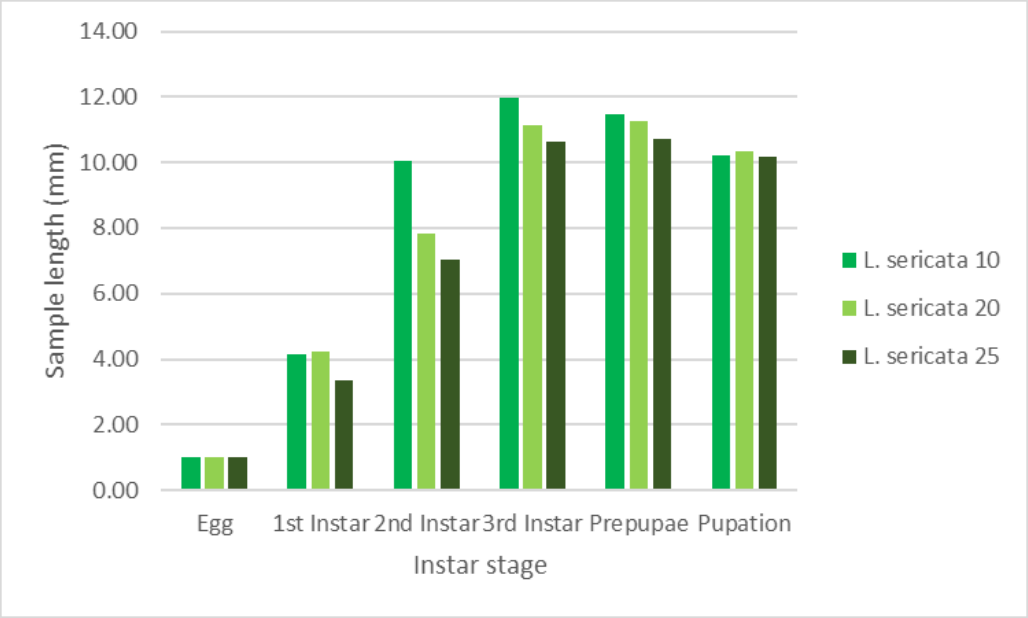


Figure 4.9 Temperature comparisons of mean sample length for *L. sericata*

Figure 4.10 illustrates the clear differences for the retention time peak occurrences for all the temperature studies. The peak occurrences spikes between 14.000 mins and 16.000 mins, which are prominent for *C. vicina* and *L. sericata* for both 10°C and 20°C are severely reduced for the *L. sericata* occurrences for 25°C. The resulting percentage peaks for the remainder of the retention times up to the end of the GC-MS temperature programme at 24.330 mins show that the peaks for *L. sericata* at 25°C increase in an ascending broad arc, this would indicate that the higher temperature has an impact on the component found at the retention times within the 14.000 mins to 16.000 mins range. While all the other retention time peak occurrences for the other temperatures and for *C. vicina* appear not to have been affected as intensely by the higher temperature.

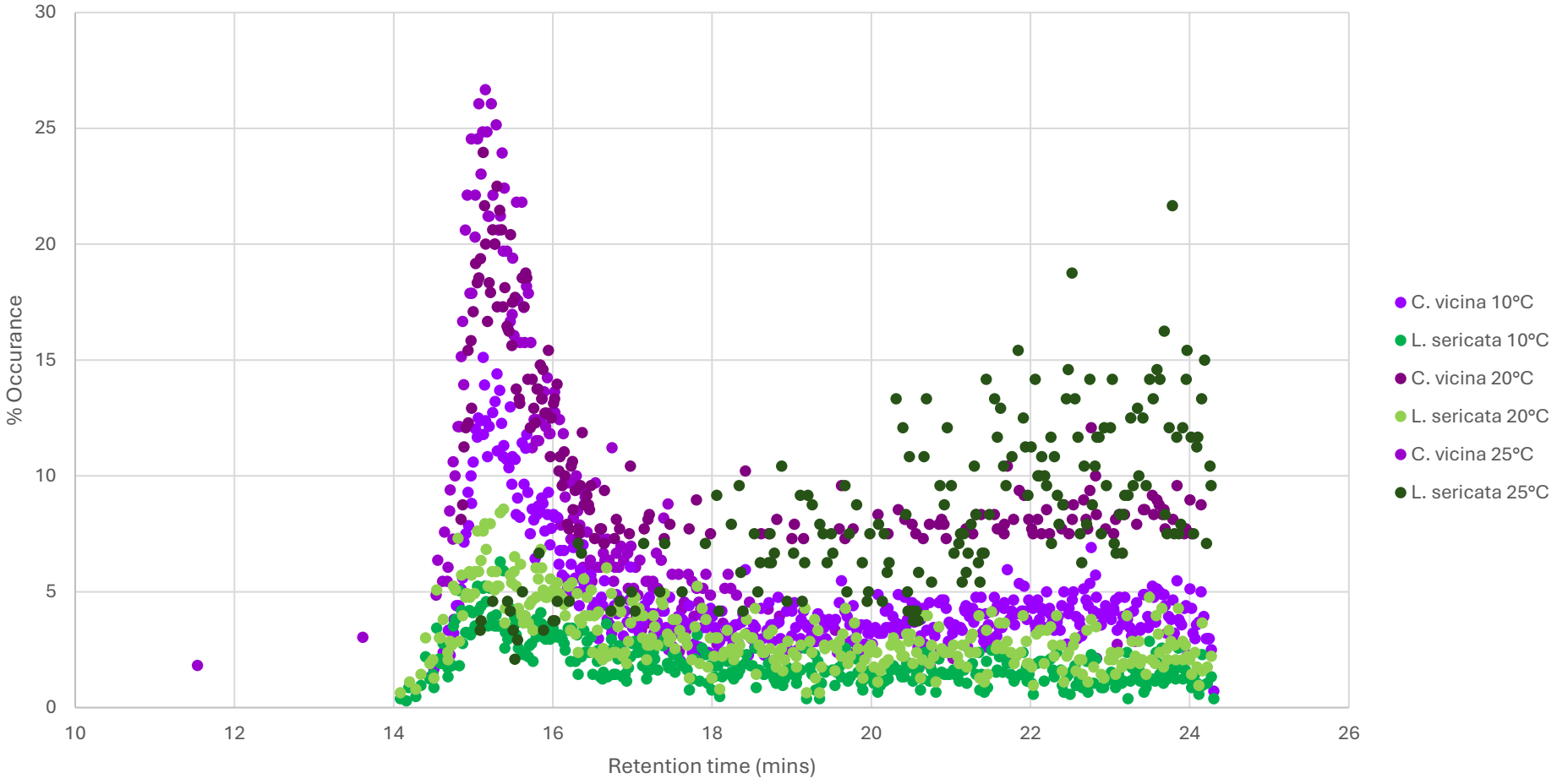


Figure 4.10 The retention times for *C. vicina* and *L. sericata* comparison for all three temperatures.

Again, the comparison for all the normalised data for the temperatures was plotted to observe the overall trends for the peaks for each species in the key area of 14.000 mins to 16.000 mins. This normalised data visualises the key differences and enables the comparison of the key retention times peak abundancies more accurately.

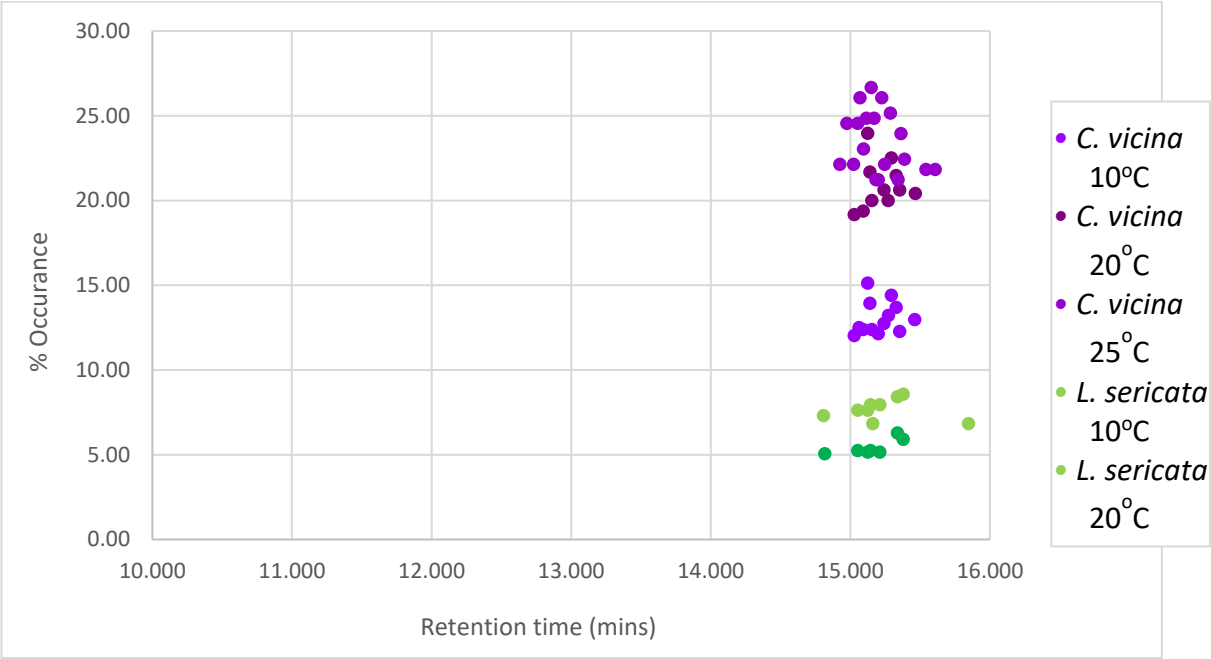


Figure 4.11 A comparison of the top % occurrences for *C. vicina* and *L. sericata* for all three temperatures. (*L. sericata* at 25°C does not appear in this data range, this is shown in figure 4.12)

It can be clearly seen in figure 4.11 that plotting the normalised data eliminates the *L. sericata* data from the selected peak area, this is due to the occurrence of the peaks in the target area of 14.000 mins to 16.000 mins disappearing. There were found to still be a small number of peaks within this time range, but the occurrence of these were individual and not as abundant as with the lower two temperatures.

To illustrate where the 0.8% threshold occurrences of the retention time peaks and their shift within the temperature programme run of the GC-MS, the axis range was amended

for the X-Y scatter plot. It is observed in figure 4.12 that the threshold occurrence peaks have reduced in numbers and there are only visible two abundant retention time peaks much later in the GC-MS temperature programme as the temperature programme is ending. Rajpurohit et al. (2021) revealed that at higher temperatures it was noticed that CHC peak profiles shifted showing that the chain lengths of these CHC had lengthened. This would support Gibbs and Pomonis (1995), who suggested that higher retention times are associated to longer carbon chain lengths. This would indicate that the chain lengths of the carbons found in the cuticles of the *L. sericata* samples at 25°C were lengthening due the temperature increase when compared to the 10°C and 20°C studies.

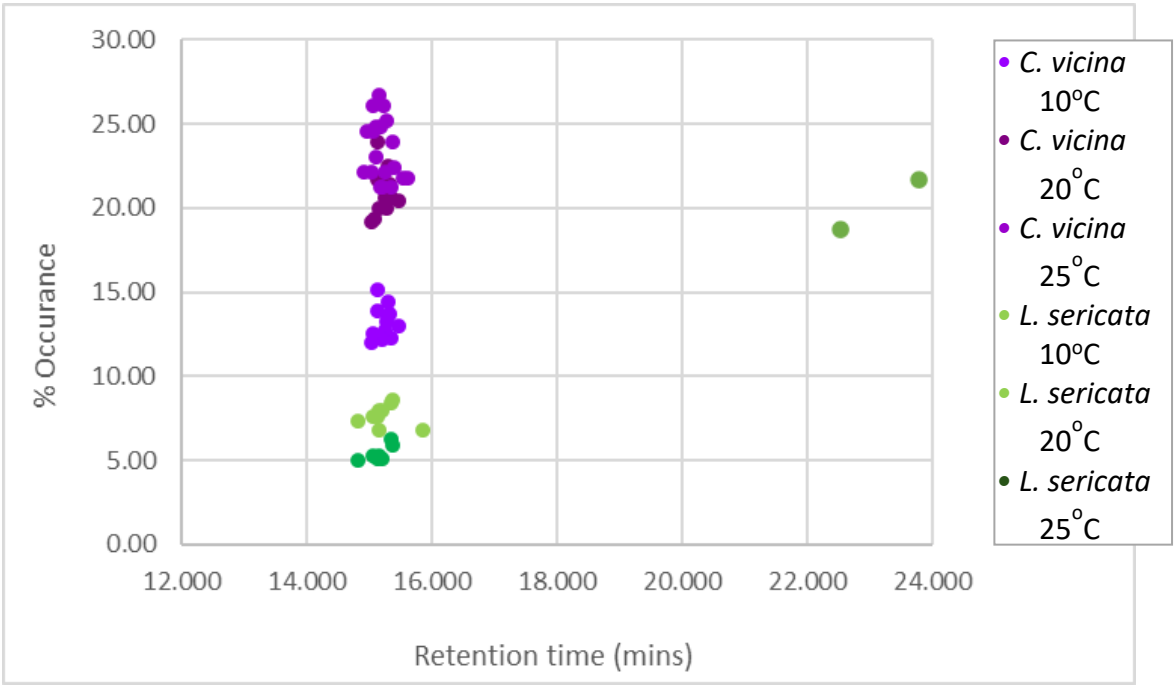


Figure 4.12 An amended axis range comparison of the top % occurrences for *C. vicina* and *L. sericata* for all three temperatures.

4.5 Discussion

When observing the retention time peak occurrences, it was found that there are clear differences between *C. vicina* and *L. sericata* retention peak occurrence profiles. This was found for all the three temperatures studied. There were several individual peak occurrences that were present for both species, but these were not always found within the threshold of the normalised data. This although focussing the attention on the most abundant or occurring peaks, does not allow for a full representation of the total peaks found for a species. This was noted when the threshold normalised peaks were plotted, and further comparisons were made of the additional peaks for the rest of the life cycles. This revealed that although there was a distinct trend with the peak occurrence pattern for both the species, there were still only several peaks that were consistently present for both.

It was observed that temperature, as discussed in chapter 1, influences the larval growth and instar stages. This was initially observed with the larval growth measurements and the varied timescale for each species at the three different temperatures. This was then echoed with the plotted normalised data at each individual temperature, in figures 4.1, 4.5 and 4.10, as the distinct peak pattern trend was only fully observed for the 10^{o}C and the 20^{o}C studies. When the temperature was increased the *C. vicina* peak was still observed while the peaks for *L. sericata* had disappeared and the initial noise of the peaks above 16.000 was observed to be increasing in an upward arc. This would infer that the components within the cuticle were changing as the samples developed within the faster timescale, therefore as the temperature increased this affected the life cycle and noticeably the stability of the CHCs within the cuticle causing the carbon bonds to

lengthen, therefore spending longer on the GC-MS column for detection. This correlates to Cini, Casacci and Nehring (2021), who when discussing social communications within insects CHCs, discussed the varying compositions of CHC profiles through ant species, and these were frequent through all the research they had investigated. This was found by Sprenger et al. (2021), who considered the variances of ant species CHCs, they determined that different body parts that were rich in some of the solid CHCs, with higher temperature melting points, when compared to other body parts where fluid CHCs, were encountered that melted at lower temperatures.

4.6 Conclusion

The main aim of this study was to determine whether the non-destructive methodology investigated in the chapter 3, could be used to determine the differences of two forensically important Dipteran species when reared at different temperatures. Having the ability to sample larvae in the field would reduce time and therefore money in criminal cases, if professional entomologists were not required or the need for rearing larvae through to adult flies was removed.

Using this quick and simple non-destructive silica rub technique the eggs, larvae and pupae of the two Dipteran species, *C. vicina* and *L. sericata* have been analysed and the results compared. These were shown to differentiate between the two species at the different temperatures, which is an important step when species is identified to aid in the determination of the $_{min}$PMI.

The limitations of this study were to focus in on one key area of retention time peaks, this would need to be expanded to encapsulate the whole CHC profile of each species at each of the temperatures. This would enable the profile to be more rigorous in the composition for each of the species which again yields more evidential value for $_{min}$PMI determination. The individual CHC components once identified would also enable the strengthening of the key peak comparisons. This would also allow this research to be compared fully to the destructive methodology currently being utilised in forensic entomology. Being able to compare the known CHC components would strengthen this methodology, making it more prevalent for utilisation for smaller sample sizes and also for police forces or environmental agencies to enable them to use their own employees for sampling and interpretation of results, reducing their costings.

Additional investigations on other carrion successors would also prove beneficial for determination of $_{min}$PMI, considering that flies once hatched can leave the crime scene leaving no evidence to be sampled if pupae casings are hidden or removed by birds and there are no observed larvae.

CHC analysis has been used prominently in entomological analysis for determination of entomological and zoological specimens, but this has always involved the specimen being killed and prepared. This non-destructive methodology could also prove valuable to these additional environments at temperatures most commonly found in the UK.

5.0 General discussion

The main aim of this research was to determine whether there was a non-destructive method that could be used at the crime scene to enhance CHC analysis of forensic entomology samples. Although research is still ongoing in CHC analysis (Kula *et al.*, 2023; Shang *et al.*, 2023; Sharif, Wunder, Kaleem Khan, *et al.*, 2023; Sharif, Wunder, Khan, *et al.*, 2023) these are all utilising the destructive methodology, so this research is still unique with the sampling methodology.

From this aim the first objective was to determine whether a non-destructive method of CHC analysis could give a clear peak pattern for quick and simple species identification. The use of destructive sampling for CHC analysis is already widely researched, but if larvae samples were of limited supply, then these destructive methods do not leave a living sample for further research or for rearing through for forensic entomologist identification.

5.1 Method development

When considering the method development for this research it was apparent from all the research that had been examined so far, that all methods apart from initial morphological examination and general optical microscopy, were of a destructive nature. Although it is stated that an expert forensic entomologist usually completes this, this is an additional expertise that requires external sourcing and can be extremely expensive. The time factor also increases the cost if any entomological sample collected from a crime scene requires them to be reared through their life cycle to adult form to enable identification.

This was when research was conducted into non-destructive methods to determine whether there was a feasible alternative to methodology currently used. All other research for current and future techniques as discussed in chapter 2 still inferred that there would still need destruction of the sample to complete the analysis. Choe, Ramírez and Tsutsui (2012) introduced the idea of a silica rub methodology that would potentially process the sample leaving it alive. This was investigating Hymenoptera: Formicidae (ants) and Hymenoptera: Apidae (bees) and whether the species could be discerned from each other. As species were distinguished from each other this gave credibility to adapt this for species comparison for these forensically important fly species.

The first component that was investigated was the silica rub method used by Choe, Ramírez and Tsutsui (2012), in comparison to the currently used method of the hexane soak method used by and Hannah E. Moore, Adam and Drijfhout (2013; Moore, 2013; Moore, Adam and Drijfhout, 2014; Xu, G. Y. Ye, *et al.*, 2014; Moore *et al.*, 2016; V. Bernhardt *et al.*, 2017). The methodologies were the conducted in relation to collection and storage parameters as mentioned in (Gennard, 2012) as discussed in chapter 3.

It was observed that the boil/freeze and freeze methods and the silica rub and soak method were comparably paired with each other. The similarities between the boil/freeze and the freeze method suggested that the larvae surface was more susceptible to the sampling methods, which could be attributed to the degradation of the surface of the specimen.

It was clear within the completion of the first investigation contained in chapter 3, that the creation of a standard set protocol, which is non-destructive, quick, and simple to complete and for non-skilled personnel could be a valuable tool for use in the field. This could also be developed for other experiments to be emulated by forensic investigators in

the field with other entomological specimens and could also become a beneficial tool for entomologists too. This protocol could then be used on varied species and temperatures globally.

This non-skilled, non-destructive sampling methodology would be easy to implement into crime scene kits. Vials containing silica gel could be pre-prepared ready for taking along with other evidence packaging. Although vortex agitation was utilised during the practical component, the shaking of a vial of silica gel would be a reasonable adjustment for the field, negating the need of the vortex in the field. Once agitated the specimen could be removed from the vial and then stored in a breathable container as previously used for identification by an entomologist. This would then leave the insect sample alive for any further analysis and the vial sent off for analytical analysis. The limitations to this would be the process including the washing process to remove any scene contaminants. Crime scenes can require timely processing, any increase in processes to the evidence collection could prove to be challenging. This could be circumnavigated by collection of the specimen at the scene and processed as soon as possible once back at a processing laboratory. Again, a limitation of this would again be time and resources. Having personnel free to process the samples for sending off for analysis could again prove problematic if smaller police forces are involved.

The practical use for the further development of this protocol could show enormous potential to be used as a complementary methodology in environmental health, for food spoilage, pest control and cases of neglect. The uses within least developed countries, where pest control is a common problem in health problems and crop growth, due to their environmental conditions.

5.2 Temperature

Once the first objective of a non-destructive method development was completed, this was then implemented to complete the second objective. This was to determine whether this non-destructive method could be used to analyse and identify differences of the two forensically important fly species, *C, vicina* and *L, sericata*, at three set temperatures. As already mentioned in previous chapters and demonstrated in figures 4.11 and 4.12 for the *L.sericata*, temperature is known to impact the growth rates and development of fly species, and it is already becoming a more abundant focus in research (Hill, Gilbert and Coetzee, 2020; Lutz and Amendt, 2020; Hu *et al.*, 2023; Park, Park and Do, 2024).

This was observably confirmed by the initial measurement and growth data that was collated from the three temperature variables that were considered for this research. The larval development was consistent throughout all the temperature variables, although the offset of the reduced timescale of the 25^{o}C trial demonstrated that the growth was concentrated jumps from the 1st instar through to the 3rd instar stages. This would correlate with fewer samples being analysed due to less days being observed. As with Kamal (1958), Greenberg and Tantawi (1993), Anderson (2000), Byrd and Allen (2001), Grassberger and Reiter (2001), Marchenko (2001), M Grassberger and Reiter (2002) Martin Grassberger and Reiter (2002)and Warren and Anderson (2013), the greater the collection of measurement and timescale data for forensically important flies that is compiled, when employed with a significant and organised approach that enables full utilisation of the data, this can only help to reinforce any forensic entomology evidence.

The results produced in chapter 4 for the non-destructive methodology, has illustrated that it can differentiate between these two species. Although keeping this investigation

as a non-destructive methodology for non-skilled individuals it is not fully comprehensive for complete CHC profiles. This is determined to be a promising step for the non-destructive method to be able to obtain full identification profiles, which in conjunction with the life cycle timelines would create an inclusive catalogue of larval behaviour. Whilst a database is currently being collated by Cranfield University (2019), this is not fully comprehensive of species yet but does show the need for this information for forensic entomology, to which this research would be a considerable contribution. Which is a crucial element for any forensic entomology evidence and would eliminate the cost and time for an expert entomologist to be appointed.

The inclusion of the temperature variances to the instar stages, would again increase the accuracy for any timescale in determining the $_{min}$PMI. As previously discussed by Ireland and Turner (2006) and Heaton, Moffatt and Simmons (2014) the ADD and ADH do not allow for temperature fluctuations. The non-destructive method demonstrated in chapter 4, show that changes to the CHC profile were evident due to the abundance of the identifiable retention time peaks. These again over the life cycle of the larvae, would give a clear profile which would be an additional reinforcement tool for a database of insect information to be able to relate to for any criminal case.

5.3 Further work

Additional work should be investigated on the temperature programme for the GC-MS to investigate whether, with increasing the temperature ramp and hold, would give any additional corroborating peaks for the whole CHC profile. Therefore, increasing the percentage of occurrence to strengthen the pattern determined for each species.

As sampling of all the instar stages has proved comparable for survivability increasing the CHC profile observations would be able to be described at each instar, this would be an additional instrument for the determination of age. If the age profiles could also be determined through the comprehensive investigation of the whole CHC compositions, this would again strengthen the evidential value for the determination of $_{min}$PMI.

The next stage for this research would be to validate the methodology with specimens collected from the field. Although this research has been completed with flies from differently reared batches meaning they were interrupted cultures, including wild and laboratory cultured batches. Analysing specimens directly from the field would give credence to the work and show the adaptability and authenticity to the specimens analysed and therefore an increased validity to the results obtained.

Once the laboratory methodology has been validated with field specimens the next phase would be to validate the collection methodology within the field. This would test the reliance, robustness, and rigour for use in the field, therefore increasing the validity for the implementation at crime scenes. Once the entire process has been validated then this would need additional trials and validation with SCEs for implementation at the crime scenes.

Once the methodology is confirmed as consistent and strong for species from the field, the temperature variables necessitate further work. Further research into fluctuation in temperatures would be extremely beneficial. The more data that is collected and collated relating to the irregularities that temperature has on insect development would give confidence to and aid in strengthening any forensic entomology evidence. Although this research currently demonstrates a strong protocol to be implemented at crime scenes, the additional information gained from the fluctuating temperatures will only strengthen

this species information and make data from crime scenes more robust for legal proceedings.

The benefit of this technique being non-destructive also has importance within the agricultural field for pest control and farming. Most insects are known for their benefits they bring to not only their natural pest control but also their pollination properties for crops. Having a method that could potentially reduce the need for pesticides would save money and the risk of crop contamination.

This research has found that investigations for further analysis methods is important and imperative to the further progress of sampling methodology. It is proved through time that methodology for any process is constantly evolving, and this can only strengthen these processes. Anything involving crime scene evidence is constantly being scrutinised in order to improve the validity of that evidence, implementing protocols for this evidence can again only improve the strength and rigour of the legitimacy of the evidence and therefore any convictions. It is believed that this non-destructive entomology collection protocol would prove invaluable at both crime scenes any environmental agency sample collection due to it being able to be utilised by non-skilled personnel.

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