CRANFIELD UNIVERSITY

CANAN KULA

THE EFFECTS OF PRESERVATIVE METHODS ON THE CUTICULAR HYDROCARBONS OF FORENSICALLY IMPORTANT INSECT SPECIES: *CALLIPHORA VOMITORIA*

CRANFIELD FORENSIC INSTITUTE
MSc Forensic Investigation

FORENSIC MODULAR MASTERS THESIS
Academic Year: 2016 - 2017

Supervisor(s): Dr Hannah Moore and Dr Karl Harrison
July 2017

CRANFIELD UNIVERSITY

CRANFIELD FORENSIC INSTITUTE
MSc Forensic Investigation

FORENSIC MODULAR MASTERS THESIS

Academic Year 2016 - 2017

CANAN KULA

The Effects of Preservative Methods on the Cuticular Hydrocarbons of Forensically Important Insect Species: *Calliphora vomitoria*

Supervisor(s): Dr Hannah Moore and Dr Karl Harrison
July 2017

This thesis is submitted in partial fulfilment of the requirements for the degree of Master of Science
(NB. This section can be removed if the award of the degree is based solely on examination of the thesis)

ABSTRACT

Estimation of post-mortem interval (PMI) is an important issue in crime investigations. In the field of forensic entomology, cuticular hydrocarbons analysis has been used for PMI determination, as in the case of cuticular hydrocarbons in *Calliphora vomitoria* to estimate the age of some species of fly larvae. Larvae generally abound in crime scenes and can provide the most useful information in predicting the post-mortem interval. With morphological techniques it is possible to identify 3rd instar blowfly larvae, although it may be more difficult to identify immature larvae. It is usually necessary to identify species by rearing larvae in adult flies.

Cuticular hydrocarbons have the potential to predict the age of adult blowfly, especially when there is significance in forensic applications. Hydrocarbon analysis is considered to have an advantage over morphological identification techniques because it does not require that the larvae be 3rd instar. As a result, this method, which can provide species identification in immature larval stages, would be advantageous by greatly cutting the time required for PMI estimates at a critical stage of the criminal investigation.

The aim of research project is to examine the effect of a preservative method (using 40%, 60%, 70% and 80% ethyl alcohol solutions and killing in near-boiling water prior to storage) on the cuticle hydrocarbon profiles of the forensically important blowfly *C. vomitoria* using gas chromatography–mass spectrometry (GC–MS), with the main focus being on the nonpolar hydrocarbon compounds. With the aid of this analysis, the chromatograms have been closely examined to see if there are any chemical changes of the hydrocarbon profile over time. In this way, the appropriate preservation conditions can be determined without deterioration of the hydrocarbon structure.

Keywords:

Forensic entomology, hydrocarbon analysis, preservation

DISCLAIMER

This report was written by a student on the Forensic Modular Master’s Programme at Cranfield Defence and Security, Cranfield University, Shrivenham (UK). It has not been altered or corrected as a result of assessment and it may contain errors and omissions. The views expressed in it, together with any recommendations, are those of the author, and not of the Defence Academy College of Management & Technology, Cranfield Defence and Security, or any individual member of staff thereof. This document is printed on behalf of the author by the College, but it has no official standing as an MOD or College document.

ACKNOWLEDGEMENTS

I would like to thank Dr Hannah Moore, my supervisor, for her endless patience, interest and relevance, for supporting and leading me through her knowledge and experience in the process from her idea to the conclusion of my thesis studies.

I would like to thank Dr Karl Harrison, my second supervisor, for taking advantage of his knowledge. He is guided and supported me with his valuable thoughts through my graduate studies.

I would like to thank all of the valuable staff members of the Cranfield Forensic Institute.

I would like to thank the Ministry of Education of the Republic of Turkey, which provided me with this training opportunity and support in all circumstances.

I would like to convey a huge thanks to Najiba Anwahi, who has been spiritually supportive of me throughout my university life, and I am glad to know her.

I would like to a huge thank Emine and Zeynel Bulbul, who never leave me alone and who are a kind family to me with their endless patience and understanding.

Last but not least, my special thanks go to my mother and father, Atiye and Fikret Kula, who have always supported me with their unconditional love from the beginning of my educational life. I am proud to be your daughter.

TABLE OF CONTENTS

ABSTRACT i

ACKNOWLEDGEMENTS iii

LIST OF FIGURES vii

LIST OF TABLES viii

LIST OF ABBREVIATIONS ix

1 INTRODUCTION 11

- 1.1 General 11
- 1.2 Targets of the Research Project 12

2 BACKGROUND AND LITERATURE REVIEW 13

- 2.1 Identifying Entomological Evidence 16
- 2.2 Isomorphen and Isomegalen Diagrams 18
- 2.3 The Life Cycle of the Blowfly 22
- 2.4 Modern Techniques in Forensic Entomology 24
- 2.5 Cuticular Hydrocarbons (CHC) 25
  - 2.5.1 Cuticular hydrocarbons in forensic entomology 25
  - 2.5.2 Chemical analysis of cuticular hydrocarbons 27
- 2.6 Gas Chromatography – Mass Spectrometry 28
  - 2.6.1 Gas chromatography 28
  - 2.6.2 Mass spectrometry 29
- 2.7 Current Preservative Techniques 30

3 MATERIALS AND METHODS 35

- 3.1 Material Studied 35
- 3.2 Methods 35
  - 3.2.1 Killing larvae in near-boiling hot water 35
  - 3.2.2 Preparation of control groups 35
  - 3.2.3 Preservation in ethyl alcohol (ethanol) solutions 36
  - 3.2.4 Hydrocarbon extraction 37
  - 3.2.5 Chemical analysis: Gas chromatography – mass spectrometry 39

4 RESULTS 40
  4.1 Effects of Ratios of Preservative Solutions on *C. vomitoria* Larvae 40
  4.2 Effects of Optimised Hydrocarbon Extraction 43
  4.3 Effects of 70% EtOH on *C. vomitoria* Larvae 44
5 DISCUSSION 57
  5.1 Effects of Ratios of Preservative Solutions on *C. vomitoria* Larvae 57
  5.2 Effects of Optimised Hydrocarbon Extraction 61
  5.3 Effects of 70% EtOH on *C. vomitoria* Larvae 61
6 CONCLUSION 64
REFERENCES 65

LIST OF FIGURES

Figure 2-1 Isomorphen diagram is showing all stages from oviposition to eclosion 20

Figure 2-2: Isomegalen diagram of hypothetical development data 21

Figure 2-3 The life cycle of *Calliphora vomitoria* 23

Figure 2-4 Linear long chain cuticular hydrocarbons 25

Figure 3-1 Preparation of preliminary CHC extractions 37

Figure 3-2 Preparation of optimised CHC extractions 38

Figure 4-1 The appearance of the *C. vomitoria* larvae in the preservation solutions 41

Figure 4-2 GC chromatograms for 40% EtOH, 60% EtOH and 80% EtOH solutions during 4 hours 42

Figure 4-3 Comparison of concentrations of control group extracts (preliminary and optimised hydrocarbon extraction) 43

Figure 4-4 Gas chromatographic profile of cuticular hydrocarbons extracted from larvae of *C. vomitoria* in 70% EtOH preservative solution 45

Figure 4-5 Retention times and determinations of some hydrocarbon structures on the chromatogram 49

Figure 4-6 The retention times and determinations of octadecane (min: 9.412) and octadecene (min: 9.351) (C18:H) on the GC chromatogram 51

Figure 4-7 Mass spectrum for octadecane h_1; m/z for M=254 51

Figure 4-8 Mass spectrum for octadecene h_2; m/z for $(M - H_2)$ =252 52

Figure 4-9 The retention times and determinations of tricosane (min: 14.888) and tricosene (min: 14.418) (C23:H) on the GC chromatogram 53

Figure 4-10 Mass spectrum for tricosane h_3; m/z for M=324 53

Figure 4-11 Mass spectrum for tricosene h_4; m/z for $(M - H_2)$ =322 54

Figure 5-1 Comparison of the length of post-feeding instar larva of *C. vomitoria* a) alive and b) killed in near-boiling hot water 60

LIST OF TABLES

Table 2-1 Selection of insects of forensic importance 18

Table 3-1 Sample preservation timeline for preliminary CHC extraction set 36

Table 3-2 Sample preservation timeline for optimised CHC extraction set....... 36

Table 4-1 Physical effects of preservative solutions at various ratios on larvae of *C. vomitoria* 41

Table 4-2 Hydrocarbon composition showing the number and percentage of n-alkanes, alkenes and methyl branched alkanes of *C. vomitoria* larvae 44

Table 4-3 Retention times (RT) (min) and peak areas in which hydrocarbon structures are detected 47

Table 4-4 Number of carbon atoms and molecular ion m/z of hydrocarbon profiles (z=1) 55

LIST OF ABBREVIATIONS

PMI	Post mortem interval
DNA	Deoxyribonucleic acid
MAb-ELISA	Enzyme-linked immunosorbent assay
CHC	Cuticular hydrocarbon
HC	Hydrocarbon
GC	Gas chromatography
MS	Mass spectrometry
LC	Liquid chromatography
GSC	Gas-solid chromatography
GLC	Gas-liquid chromatography
RSD	Relative standard deviation
FID	Flame ionization detector
KAA	A kind of preservative solution
AAG	A kind of preservative solution
KAAD	A kind of preservative solution
XAAD	A kind of preservative solution
XA	A kind of preservative solution
EtOH	Ethyl alcohol/ethanol solution
RT	Retention time
m/z	Molecular weight/ion

1 INTRODUCTION

1.1 General

Determining the time of the death of a corpse at a crime scene is a major significance in criminal investigations. Usually, medical techniques are used to determine the time of death; however, if a corpse is found 72 hours or more after death, the working principles of medical techniques experience various difficulties in determining the time of death with any precision. At this point, the field of forensic entomology comes into play; entomological investigations of organism (i.e. insect) activity over and around the corpse the most practical and accurate evidence used to determine the time of death of a corpse. In most environments inhabited by humans, insects arrive in the corpse immediately after death begin life cycles that will be completed in six steps, from leaving their eggs in apertures on the body. Forensic entomologists can estimate the time of the death by examining this life cycle.

In order to make this estimation, it is necessary to define the stage of life cycle of the particular fly larvae involved, determine by larval growth length; larval processes vary according to species. Given the enormous variety of entomological species that may be present in a corpse, forensic entomology is concerned to study larval stages in different species extensively; identifying the time of death entails identification of the particular species of concern in a crime scene, thus the forensic goal is contingent on waiting for completion of the larval life cycle, which can take weeks or even months; conversely, time is critically important in forensic and criminal investigations. Due to the unacceptable time required by traditional larval analysis, the hydrocarbon analysis technique was developed over recent years to obtain results more quickly and easily.

In the hydrocarbon analysis technique, both the species identification and the age determination can be performed by analysing the cuticular hydrocarbon structures in the cuticle layer of insects. Moreover, this method has a wider application area than other methods because it can be applied to both larval and pupal stages. It still faces the general issues of collecting forensic entomological evidence, such as killing with the appropriate method and preservation until analysis begins in a suitable solution.

In previous years, many studies have been carried out on the analysis of the cuticular hydrocarbon structures of insect species; however, no study investigating the effect of preservative methods on hydrocarbon structure has been encountered. It is expected that our work will form a basis for future work by eliminating the shortcomings in this direction.

1.2 Targets of the Research Project

This research aims to reach the following objectives by the post-feeding stage larvae of *C. vomitoria* which are preserved in 40%, 60%, 70% and 80% ethanol solutions within a period of 14 days:

- Determination of the effect of using ethyl alcohol as a preservative solution on hydrocarbon structure of insect larvae
- Determining the effects of using ethanol solutions with different ratios on the hydrocarbon structure of the larvae
- Determining the maximum preserved time that larvae can be used for hydrocarbon analysis in the preservative solution

If we reach our targets, the maximum time in which the larvae can stay without any deformation on the hydrocarbon structures within the preservative solution will be determined.

The most appropriate concentration of ethanol used as a preservative solution will be determined and a standard will be provided for preservation methods.

2 BACKGROUND AND LITERATURE REVIEW

Forensic entomology is the forensic and legal analysis of insect evidence (Amendt *et al.*, 2007). Forensic entomologists are often asked to examine insect evidence collected from human remains at a crime scene to determine how long the insects have been there. This time between death and the discovery of a corpse is usually interpreted as the Post Mortem Interval (PMI) or elapsed time since death (Catts & Goff, 1992). There are various natural processes, such as rigor mortis or livor mortis, related to fragmentation, which can be used to calculate the PMI; however, most of them are mutual functions and are very quickly mistaken in practice. Moreover, their precision is limited to the first 72 hours after death (Campobasso *et al.*, 2001; Bourel *et al.*, 2003).

Despite the use of various criteria and methods for the determination of the time of death in forensic medicine applications, none of these methods and criteria are fully reliable (Slone *et al.*, 2005). In contrast, insects can be a very effective evidence for estimating the minimum time since death. Recent advances have shown that entomological evidence is more accurate and reliable than medical examinations and findings (Duke, 2003). Pioneering techniques (Campobasso & Introna, 2001) have allowed experts to gather strong entomological data for post-mortem estimations, especially with regard to the storage and removal of cadavers, including post-mortem body artefacts, details on specific trauma sites, time of decapitation/dismemberment, submersion interval and drug use (entomotoxicology), which can particularly assist in the identification of sexual molestation in the context of a suspected crime scene that may otherwise be considered a simple homicide. This field was significantly advanced by the seminal work of Amendt, Richards, Campobasso, Zehner and Hall (Amendt *et al.*, 2011), which is discussed at length later in this paper.

The affiliation between insects and corpses and the use of insects in medico-criminal examinations is the essence of forensic entomology, which can be examined in three main forms (Catts & Goff, 1992; Aggarwal, 2005):

- Urban forensic entomology: Examines a number of problems caused by termites, bathrobes and other insect species that arise in environments where people live.

- Stored product pest forensic entomology: Examines the problems that arise from insects and insects found in food and other products.

- Medicolegal forensic entomology: Focuses on the use of insects for the resolution of crimes, particularly violent crime. The following considerations are particularly important in forensic investigations of crimes (Marchenko, 2001):
  - ⇨ Post mortem interval (PMI)
  - ⇨ The body's displacement after death
  - ⇨ Trauma evaluation
  - ⇨ Maltreatment and neglect
  - ⇨ Unexpected deaths
  - ⇨ Unexplained traffic accidents
  - ⇨ Source of human DNA
  - ⇨ Entomotoxicology

The first known document of forensic entomology concerning murder investigations is the Chinese work *The Washing Away of Wrongs* (1235) by Sung Tzu, translated into English by McKnight in 1981. It describes a true event of medico-criminal entomology relating to the slaughter of a farmer in a Chinese village investigated by Sung Tzu. After preliminary interrogations, Sung Tzu had made all the villagers bring their sickles in one place and stacked them in front of the crowd. No obvious visual evidence was seen on the sickles, but a large number of blowflies congregated on the invisible blood traces on a particular sickle, whereupon the owner of that sickle confessed to the murder in direct response to the entomological evidence (Benecke, 2001).

In the Middle Ages, the correlation between the larvae (i.e. maggots) on a cadaver and the oviposition of adult flies was not recognized, but by the seventeenth century the metamorphosis of insects was more widely understood (Blankaart, 1690). The first modern use of entomology in a criminal investigation was by Bergeret (1885), a researcher in Paris. In this case, a child's body was found behind the plaster surface in the wall of the house and an investigation was initiated. Bergeret pointed out that the accumulation of insects around the corpse belonged to a decay phase that took place several years ago; for this reason, the murder was not charged to the current landlord, thus the former landlord was the starting point of further investigations (Benecke, 2001). At the same time, great theoretical advances were made by J. P. Meginin who published (in France) a series of articles on medico-criminal entomology between 1883 and 1898, the most famous of which, *La Faune des Cadavres*, popularised the concept that the medical and legal professions could find entomological data useful to a wide range of forensic investigations (Benecke, 2001).

Necrophagous insects immediately colonize a fresh corpse depending on the level of accessibility and environmental conditions. The first taxa that usually arrive on the body are flies (Diptera), in particular blowflies (*Calliphoridae*). They can determine the location of an odour source with great spatial precision, and very soon after death, they can lay their eggs on a corpse. Larvae hatch from the egg and feed on underlying tissues. As they grow they go through three instar stages (with two moults in-between). A new larval instar occurs after each ecdysis (moult). When the third instar larvae finish feeding, they enter the post-feeding phase and migrate away from the corpse to find shelter in dark and sheltered areas. They then form a pupa in a protective outer sheath; these are the puparium (the hardened cuticle of the third instar larva) from which adult flies emerge upon the completion of metamorphosis (Amendt *et al.*, 2011).

The decomposition is a measurable continuous process and allows accurate minimum PMI estimates to be made, whether that be hours after death or within a few months after death, depending on the circumstances (Campobasso *et al.*, 2001). The assumption behind these estimates is that it is possible to calculate the time since death by calculating the age of the insect development on a corpse, resulting in a minimum PMI: the time when insects are colonized on the corpse rather than the actual death time. Since blowflies are usually the first group to colonize a body, the PMI estimations often focus on this family of Diptera when entomological evidence is used (Catts, 1992; Amendt *et al.*, 2007).

The insect development rate is primarily governed by temperature and may vary between closely related species (Richards *et al.*, 2009). For this reason, a three-step process is absolutely necessary for the forensic entomologist to calculate the age of a sampled insects (Amendt *et al.*, 2011):

1. Accurate identification of species found on a corpse
2. Recreation of scene temperatures
3. Modelling the development rates of immature insects on a corpse

2.1 Identifying Entomological Evidence

During the decomposition process, various insect species and other insects are observed on or around the corpse. Nevertheless, their presence on the corpse does not precisely show that they have oviposited on the corpse. Depending on the ecological and biological tendencies of the insects, they are attracted by certain olfactory receptors and colonize on the corpse at different stages of decomposition. Some species never feed on dead tissues, but they are fed by hunting other colonizing groups on the corpse.

According to Smith, four different ecological categories of insects can be can be described on a corpse:

1. Necrophagous species—which feed on the dead tissue of a corpse
2. Predators and parasites of necrophagous species—which feed on other insects. This group also includes schizophagous species, which initially feed on the carrion at first, but may predate in later larval stages.

3. Omnivorous species such as wasps, ants and some insects feeding on corpses and colony regulators.
4. Adventive species such as spiders, springtails and caterpillars that use the body as an extension of the periphery.

As a result of the investigations carried out, it has been seen that many insect species are interested in corpses at varying stages of decomposition. As the attractiveness of a decaying corpse varies between insects, changes over time and the colonization of the corpse occur in a predictable sequence. The manner of deposition or storing of a corpse will also affect the composition of the species. Two groups are very important among these species for forensic entomology: Diptera (flies) and Calliphora (insects) species (Table 2-1) (Amendt *et al.*, 2004; Amendt *et al.*, 2011).

Table 2-1 Selection of insects of forensic importance

COLEOPTERA/BEETLES	
Order/Family	**Important Genera**
Cleridae (Checkered beetles)	*Necrobia*
Dermestidae (Larder beetles)	*Attagenus, Dermestes*
Geotrupidae (Dung beetles)	*Geotrupes*
Histeridae (Clown beetles)	*Hister, Saprinus*
Silphidae (Carrion beetles)	*Necrodes, Nicrophorus, Silpha*
Staphylinidae (Rove beetles)	*Aleochara, Creophilus*
DIPTERA/FLIES	
Order/Family	**Important Genera**
Calliphoridae (Blowflies)	*Calliphora, Chrysomya, Cochliomyia, Lucilia, Phormia*
Drosophilidae (Fruit flies)	*Drosophila*
Ephydridae (Shore flies)	*Discomyza*
Fanniidae (Latrine flies)	*Fannia*
Heleomyzidae (Sun flies)	*Heleomyza, Neoleria*
Muscidae (House flies)	*Hydrotaea, Musca, Muscina, Ophyra*
Phoridae (Scuttle flies)	*Conicera, Megaselia*
Piophilidae (Skipper flies)	*Piophila, Stearibia*
Sarcophagidae (Flesh flies)	*Liopygia, Sarcophaga*
Sepsidae (Black scavenger flies)	*Nemopoda, Themira*
Sphaeroceridae (Small dung flies)	*Leptocera*
Stratiomyidae (Soldier flies)	*Hermetia, Sargus*
Trichoceridae (Winter gnats)	
LEPIDOPTERA/BUTTERFLIES	
Tineidae (Clothes moths)	*Tineola*
HYMENOPTERA/WASPS	
Order/Family	**Important Genera**
Ichneumonidae (Ichneumon wasps)	*Alysia*
Pteromalidae (Fly wasps)	*Nasonia, Muscidifurax*

(Amendt *et al.*, 2004)

2.2 Isomorphen and Isomegalen Diagrams

Isomorphen and isomegalen diagrams are measures for the estimation of post-mortem interval. Larval developmental data relate to the developmental process of immature stages (often fixed but sometimes fluctuating) recorded at different temperatures. Current techniques used to estimate the age of immature insects are size (e.g. length/weight) and developmental stage (e.g. 1st instar or pupariation). Also, developmental models use one of these two measures to estimate PMI (Amendt *et al*., 2011).

It is possible to estimate the correct PMI using the min/max error bars of these models and to establish the confidence interval for PMI by knowing the developmental stage of the oldest immature insects in the corpse and the average ambient temperature of the scene at the crime scene. An advantage of these models is that it is conducive to the simple representation of concepts in court; however, this simplicity is at the expense of higher accuracy, i.e. pertaining to fluctuations in ambient temperatures, such as outside scenarios, and the average ambient temperature of the scene used to calculate PMI. Because the isomorphen and isomegalen diagrams do not show any grading between developmental events and model events only, it is true if the prediction is derived from a living entomological evidence, but only to the next development event at a known, constant temperature.

In the isomegalen diagram, the time elapsed from egg hatching (x axis) is plotted against the temperature (y axis). Larval size is a measure of age with a higher resolution. This higher resolution input provides a more accurate estimate for PMI. For this, the largest larvae in the corpse and in the crime scene are collected, identified and measured. After then, the data is summarized and modelled with the relevant environmental temperatures to estimate PMI. In the isomorphen diagram, all morphological stages from ovulation to eclosion are considered and the area between the lines in the graph shows the morphological stages of the insect (Amendt *et al*., 2011).

On the other hand, entomological evidence does not always accumulate vividly, and in some cases; the development times from oviposition to eclosion are different in all geographical areas of the world. The life cycles of insects change according to the temperature values of the environment. If the temperature is fixed on average (corpses found indoors) the use of isomegalen and isomorphen diagrams (as shown in Figure 2-1 and Figure 2-2, respectively) are able to provide a good post mortem interval estimation (Sharma *et al.*, 2015).

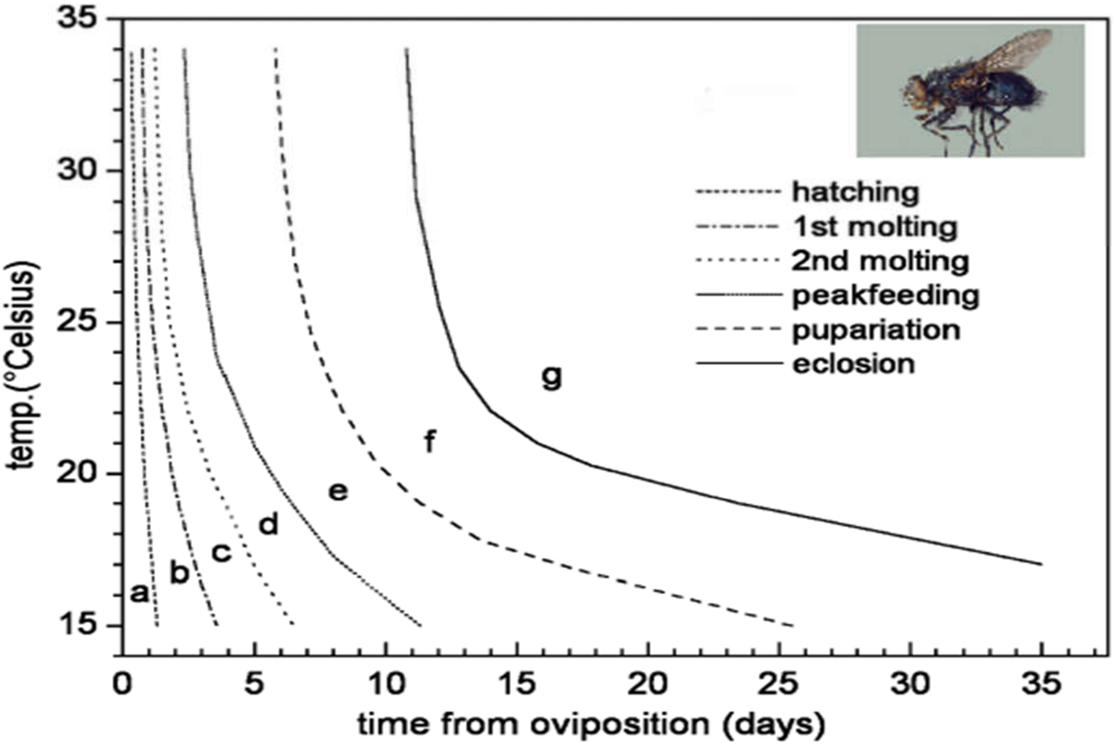


Figure 2-1 Isomorphen diagram is showing all stages from oviposition to eclosion

Source: Grassberger & Reiter (2001). Areas between lines represent identical morphological stages at various temperature. A = egg, b = 1st *instar, c = 2*nd *instar, d = 3*rd *instar, e = post-feeding larva, f = pupa, g = adult fly*

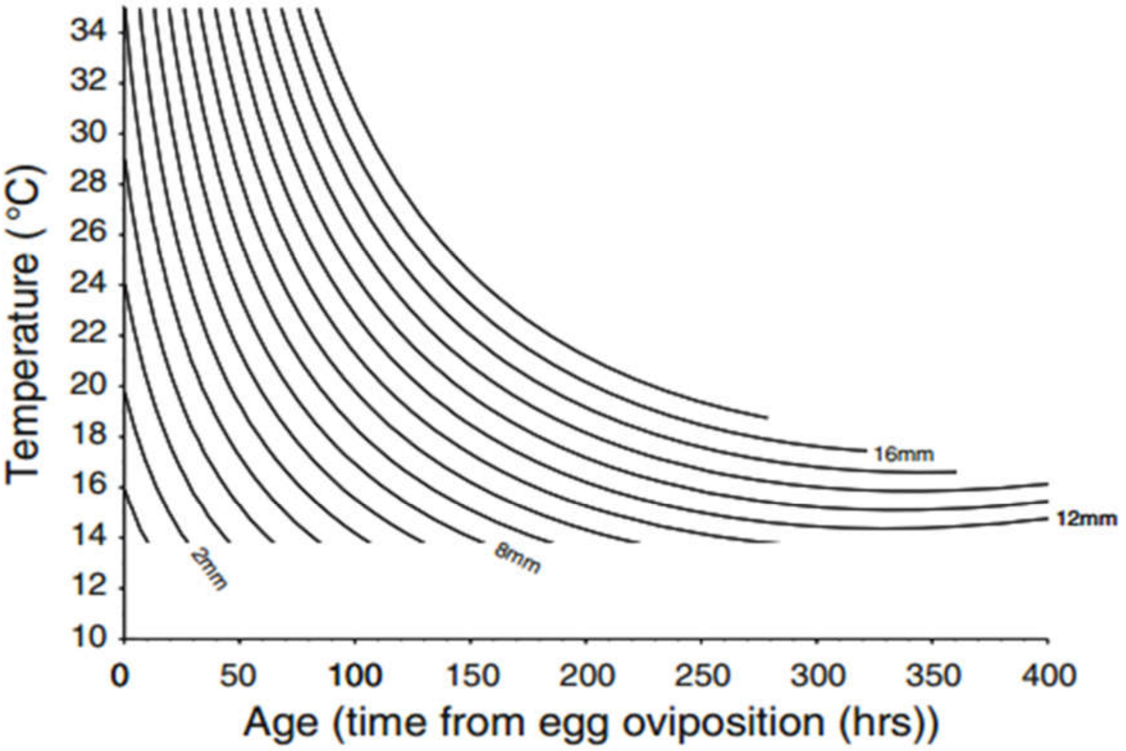


Figure 2-2: **Isomegalen diagram of hypothetical development data**

Source: Amendt et al. (2011). The diagram details the entire range of larval sizes from the beginning of the egg hatching, where each contour on the graph represents a particular larval dimension.

2.3 The Life Cycle of the Blowfly

Taxonomic status and species characteristics of *Calliphora vomitoria*

- ➔ Domain: Eukarya
  - ➔ Kingdom: Animalia (Animals)
    - ➔ Phylum: Arthropoda (Arthropods)
      - ➔ Class: Hexapoda (Insects)
        - ➔ Order: Diptera (Flies)
          - ➔ Family: Calliphoridae (Blow Flies)
            - ➔ Genus: *Calliphora*
              - ➔ Species: *Vomitoria*

It is known that blowflies (Diptera: *Calliphoridae*) are usually the first insect colonizers of a corpse. Insects' larvae are one of the best ways to estimate the time the body is exposed to insects (Boehme *et al.*, 2013). Some of the first insects that invade corpses in the UK are *Calliphoridae* (Diptera), *Calliphora*, *Protophormia* and *Lucilia* (Lane, 1975). In many temperate regions, Calliphoridae are the most important family and are widely distributed in most areas of the world (Ames & Turner, 2003). *C. vomitoria*, *C. vicina* and *Lucilia sericata* are the three most common species, are among the first wave of necrophagous insects (Moore, 2012). These species have geographical distribution in each region.

Under favourable conditions, the female adult flies lay their eggs around natural orifices or wounds of the fresh corpse within a short time after death. Depending on the season and geographical region, in several weeks, adult insects emerge and the life cycle begins again (Davies & Ratcliffe, 1994). The life cycle is depicted in Figure 2-3.

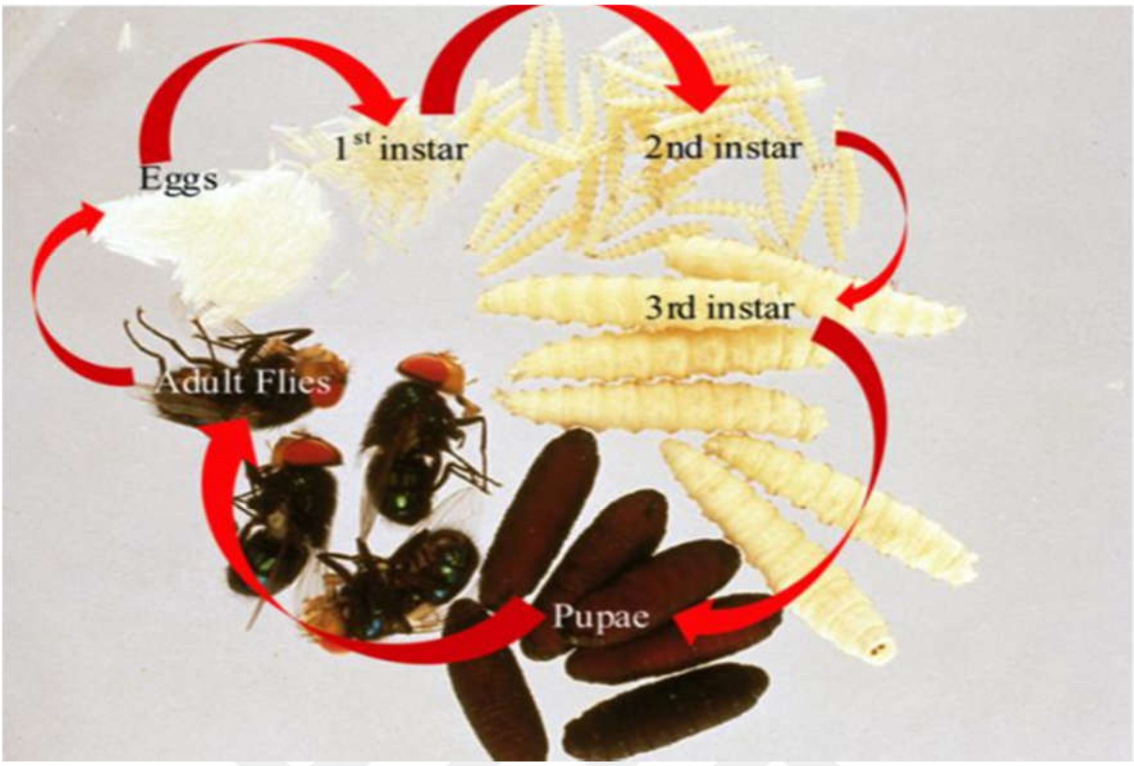


Figure 2-3 The life cycle of *Calliphora vomitoria*

L1: 1st instar larvae, L2: 2nd instar larvae, L3: 3rd/post-feeding instar larvae (Moore, 2012).

The *C. vomitoria* species in corpse indicate an ecological importance in the decomposition of human and animal remains. Additionally, they are useful in forensic cases for the detection of morphine accumulation and metabolism. The *C. vomitoria* larvae also used in the treatment of gangrene and wounds (maggot therapy), with *L. sericata* larvae or closely related *C. vicina* larvae. There is an abundance of data on the passive transfer of animal and plant pathogens with *C. vomitoria* (Gołębiowski *et al.*, 2013).

Calliphora can be collected from shallow graves. Colonization of the body without being buried cannot be excluded. In this case, the estimate is more accurate (Amendt *et al.*, 2010). Turner and Wilshire argued that cold and anoxic conditions in heavy clay soils protected corpses and significantly delayed the development of *C. vomitoria* (Turner & Wiltshire, 1999). Using this method would result in incorrect PMI estimation. Wyss and Chérix observed the spawning of *C. vicina* and *C. vomitoria* 12 days after a 10 cm deep feed, relating to a hill-walker found in in the depths of an abyss in the dark (Wyss & Cherix, 2013). The average temperature was 5°C. In this case, the location of the substrate delayed the attraction of the insects, but did not stop it. They concluded that the death of the hill-walker occurred just after disappearance. The absence of daylight is not sufficient for ovulation to be considered as a night phenomenon. The authors could not observe the time of ovulation. The main result in this case was affected by the lack of developmental data of some species in these families (Amendt *et al.*, 2010).

2.4 Modern Techniques in Forensic Entomology

In recent years, forensic entomology is evolving using new technologies, like many other fields in forensic sciences. For example, the first studies on species identification were carried out by collecting and maturating live female flies and their eggs or larvae, and then identifying adult individuals. In more recent studies, scanning electron microscopy and potassium permanganate staining techniques were used for species identification (Sukontason *et al.*, 2004; Mendonça *et al.*, 2008; Sukontason *et al.*, 2008). With advances in molecular biology, DNA technology has become a tool for forensic entomologists. It can be difficult to distinguish species using their morphological characteristics. Thanks to DNA, it is possible to get faster and error-free results in the identification of species of insects (Gennard, 2007). In addition to molecular techniques, immunological methods have been utilised in forensic entomology. Two highly species-specific monoclonal antibodies were used in an enzyme-linked immunosorbent assay (MAb-ELISA) to determine the species of eggs, larvae, pupae and adult flies (Figarola *et al.*, 2001). Another study achieved isolation of species-specific antigenic proteins and their subsequent immunological detection, which led to species determination (McDonagh *et al.*, 2009).

Nowadays, this application is used in 48 countries covering Asia, Europe, North America, South America and Australia. Today, this practice is being conducted in 48 countries covering Asia, Europe, North America, South America and Australia (Tomberlin & Benbow, 2015).

2.5 Cuticular Hydrocarbons (CHC)

Newly developed forensic entomology techniques have beneficial to determine the PMI faster and more securely. One of the methods involves identifying hydrocarbons present on the cuticle of the insects (Frederickx *et al.*, 2012).

Hydrocarbons are long linear molecules that exist in saturated and unsaturated forms on insect cuticles and can have one or more methyl groups depending on the chain length (Figure 2-4). Saturated forms, such as n-alkanes or paraffin, contain all carbons joined together in single bonds, but may contain one or more methyl groups. In unsaturated forms, like olefins, there can be one (alkene), two (alkadiene) or three (alkatriene) double bonds along the chain length. The olefins may be in the form of two isomeric structures termed cis-alkenes (Z-alkenes) or trans-alkenes (E-alkenes) (Moore, 2012). Insect hydrocarbons usually consist of n-alkanes, Z-alkenes and methyl-branched alkanes.

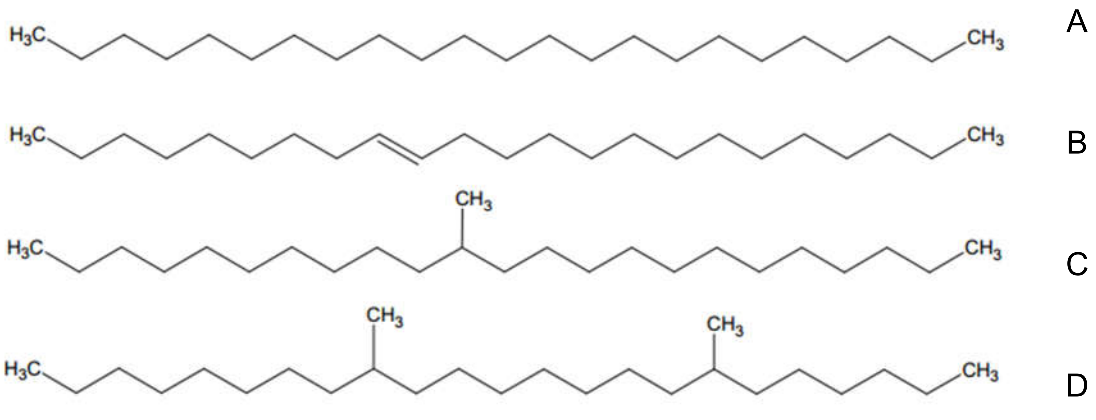


Figure 2-4 Linear long chain cuticular hydrocarbons

A) Linear alkane: Tricosane, B) Z-Alkene: (Z9) - tricosene, C) Mono methyl branched alkane: 11-methyltricosane, D) Dimethyl branched alkane: 9,17-dimethyltricosane (Amendt et al., 2010; Moore, 2012).

2.5.1 Cuticular hydrocarbons in forensic entomology

Cuticular hydrocarbons have the potential to predict the age and identification of blowfly, especially when there are no larval specimens at the scene (Zhu *et al.*, 2006; Drijfhout, 2009). Although there are various methods used to estimate the age of the adult blowfly (e.g. evaluating pteridine levels in the eye, counting cuticular bands, and evaluating female ovarian status), hydrocarbons offer reliable results for insect age. The cuticle of the insect is covered with a wax layer consisting of cuticular hydrocarbons (CHC), such as n-alkanes, alkenes, monomethyl alkanes and polymethyl alkanes, together with other compounds such as fatty acids, sterols, waxes, acylglycerides, phospholipids, glycolipids and alcohols (Moore *et al.*, 2013). The function of these hydrocarbons is to act as pheromones or kairomones, which are important for preventing toxins such as insecticides, bacterial and fungal pathogens, or for reducing toxin effects and regulating adult insect behaviour, for example mate selection and ovulation (Ye *et al.*, 2007). In addition, they play an important role in preventing water evaporation through the cuticle to limit water loss and at the same time serving as communication signals. In the area of forensic entomology, cuticular hydrocarbons have been utilized to estimate the identification of some species of blowfly larvae, and further aid the determination of the PMI (Xu *et al.*, 2014).

Previous studies describing hydrocarbon compounds in Calliphora have led to the discovery of specific hydrocarbon profiles for individual taxa with specific profiles resulting from gender and developmental stage differences within each taxa. For example, hydrocarbon profiles have been used to distinguish six necrophagous fly pupas; this is important because the pupa taxonomical keys are not complete from other life stages, like adults and third instar larvae, and pupae or empty puparial cases are usually collected as entomological evidence in crime scene investigations (Ye *et al.*, 2007). Hydrocarbon profiles have also been identified at different stages of life cycle in Calliphoridae (Pechal *et al.*, 2014). Cuticular hydrocarbons generally vary in composition depending on gender, sexual maturity, and age, consisting of more than 100 compounds (Xu *et al.*, 2014).

Different hydrocarbons between males and females have been reported, and there are differences in adult hydrocarbon composition. For example, the alkalinity in the female is richer than in the male (Brown *et al.*, 1992; Trabalon *et al.*, 1992). In addition, cuticular hydrocarbons have shown promise in determining geographic regions of origin based on adult profiles (Brown *et al.*, 1998). Many of these studies have direct implications for the use of a forensic scientist to estimate the age of an insect specimen, as several insect species provide extensive identification of available hydrocarbon profiles during development according to taxonomy (Pechal *et al.*, 2014). In the field of forensic entomology, cuticular hydrocarbons have been used for PMI determination, including *C. rufifacies* (Macquart), *C. vomitoria* (Linnaeus), *C. vicina*, and *P. terraenovae*, as in the case of cuticular hydrocarbons in *L. sericata* (Meigen) to estimate the age of some species of fly larvae (Xu *et al.*, 2014).

2.5.2 Chemical analysis of cuticular hydrocarbons

The technique of using cuticular hydrocarbons for identification is described in many studies. Goodrich was the first to investigate the cuticular lipids of *Lucilia cuprina*, taken from adults and pupa (Goodrich, 1970). Roux *et al*. successfully presented cuticular hydrocarbon separations between *C. vicina*, *C. vomitoria* and *P. terraenovae* and demonstrated the age-related difference between developmental life stages (Roux *et al.*, 2008). Ye *et al.*, who were using cuticular hydrocarbons extracted from the pupa, which could distinguish between six forensically important blowfly species (Ye *et al.*, 2007).

Hydrocarbon analysis is considered to have an advantage over morphological identification techniques, since it does not require that the larvae to be 3rd instar. Due to the size of the first instar larvae it is particularly difficult to identify morphologically, and there are very few taxonomists who can determine the species level at this age. However, CHC analysis can also be applied to eggs, 1st and 2nd instar larval to determine the identification (Moore *et al.*, 2014).

Cuticular hydrocarbon analysis is used as an additional taxonomic tool to characterize various Diptera disease vectors, such as Anopheles, Simulium, sandfly and Glossina sp (Roux *et al.*, 2006).

2.6 Gas Chromatography – Mass Spectrometry

2.6.1 Gas chromatography

Chromatography is a separation method in which fractions of a sample are separated between two phases: a stationary phase with a large surface area and a gas phase (mobile phase) flowing through the stationary phase. The sample is evaporated and carried along the column by the mobile gas phase (carrier gas). The samples are separated by the stationary liquid phase based on their solubility at a given temperature. The components of the sample are separated from each other on the basis of affinity for the vapour pressure and the stationary phase relative to each other and are called solutes or analytes. This chromatographic processing is referred to as elution (McNair & Miller, 2011).

Chromatographic processes are named according to the physical state of the mobile phase. In gas chromatography (GC), the mobile phase is a gas whereas in liquid chromatography (LC) the mobile phase is liquid. A sub-classification is made according to the physical state of the stationary phase; if the stationary phase is solid, the gas chromatographic technique is called gas-solid chromatography (GSC), while if it is liquid it is called gas-liquid chromatography (GLC). Advantages of gas chromatography include (McNair & Miller, 2011):

- Faster analysis; results are usually obtained within minutes.
- More efficient; it provides higher resolution.
- More sensitive; it is easy to get results in ppm and ppb.
- Non-destructive; on-line coupling is possible.
- Extremely accurate quantitative analysis is obtained, generally RSDs of 1-5%.
- Samples in small quantities are sufficient for analysis.
- Reliable and relatively simple results are achieved.
- Low cost.

GC can analyse both organic and inorganic materials, with molecular weights ranging from 2 to 1,000 Daltons. Therefore, over the years, gas chromatography has become one of the most important technique for the separation and analysis of volatile compounds. Gas chromatographs are one of the most widely used analytical instruments in the world (McNair & Miller, 2011).

2.6.2 Mass spectrometry

Mass spectrometry is one of the most common informative detectors. It is easily connected to a GC system. It only required microgram quantities in order to sufficiently detect a compound. It provides both quantitative and qualitative identification of unknown compounds, such as structure, elemental composition and molecular weight (McNair & Miller, 2011).

Over the last 20 years the number of studies on cuticular chemistry has increased dramatically, especially concerning the hydrocarbons of social insects (H. E. Moore, 2012). Due to the prevalence and popularity of Gas Chromatography-Mass Spectrometry (GC-MS), this technique has been preferred for CHCs studies because of the advantages, such as separation, identification and user-friendliness (Martin & Drijfhout, 2009).

Insect cuticular hydrocarbons, especially n-alkanes, have been investigated mostly by GC and GC-MS techniques for forensic applications (Amendt *et al.*, 2010). For example, different types of dipteran can exhibit different hydrocarbon contents at significant levels, thus offering possible chemotaxonomic applications (Ye *et al.*, 2007). The distribution patterns of hydrocarbon chain lengths rather than certain molecules are often described. For n-alkanes, the distribution patterns were observed to change in relation with temperature and humidity values (Roux *et al.*, 2008). Statistical analysis of the content of hydrocarbons extracted from the same species of blowfly can accurately predict larval age for nine days (sometimes with a one-day accuracy) (Moore *et al.*, 2013). Significant differences between the cuticular composition combinations of different species have led to the hypothesis that there is a specific cuticular signature that can be used for phylogenetic studies of each fly species (Roux *et al.*, 2006).

Zhu *et al*. demonstrated the use of hydrocarbons for forensic entomology applications by analysing for the first time *C. rufifacies* larvae using GC-FID and GC-MS (Zhu *et al.*, 2006). The study of larval *C. rufifacies* gave results that allow aging by adopting a simple statistical analysis of reaching eight other selected peaks by taking the nonacosane peak area (C29:H) and dividing it into the selected eight top peak areas, compared to the ratio C29:H. This ratio increases markedly with age, and the results are modelled using exponential or power functions. Nevertheless, the peak ratios can vary significantly between individuals and the analytical method of selecting the other peaks is a highly subjective analysis tool. The GC-MS technique has been used many times to analyse hydrocarbons, and significant information has been obtained about the changes of the abundances of the compounds relative to the time of disintegration. The abundance of even numbered low molecular weight n-alkanes (C22: H, C24: H, C26: H) has increased over time, allowing an age of approximately 90 days to be determined (Moore, 2012).

2.7 Current Preservative Techniques

The larvae stages of the blowflies (Diptera: Calliphoridae), such as feeding the first, second and third instar and post-feeding the third instar larvae are usually found a crime scene. They constitute the majority of the entomological evidence. For this reason, recognizing and using the correct procedures for the collection, conservation and processing of entomological evidence is very important in estimating a PMI (Richards *et al.*, 2013).

Larval length is always measured from the dead insects, because in live blowfly the larva is constantly moving and shrinks if disturbed, changing the length of its body to a significant degree. A guide is provided in the current literature on a range of methods for killing and preserving the larvae. Diverse killing solutions have been suggested to correct the samples. The fixation is at the initial stage of preservation, wherein the freshly killed material is stabilized by protein coagulation or fixative chemically coupled, which prevents cellular lysis that would otherwise occur through the action of the bacterial fauna and the enzymes of the larval gut (Adams & Hall, 2003).

The crime scene environment is very different compared to the laboratory, and when an entomologist cannot enter a crime scene, the crime scene investigators collect entomological evidence instead of an entomologist. However, such personnel may not preserve the larvae as recommended in the literature, since the necessary chemicals or diluents are not available or the techniques recommended for killing the larvae may not be practical or even possible at a crime scene (Day & Wallman, 2008).

The length of the insect body are used to estimate the age of dead specimens. However, it is influenced by the way in which the insects are killed and preserved as evidence. The length can vary during storage, especially as the lipids can be removed by the preservation solution and the water content in the samples can vary. These two processes may affect the length of the sample and cause erroneous data. Some killing methods are preferred because they cause less variation than others in a longer time period. To overcome this problem, it is suggested that insect specimens gathered at the scene should be killed by the same method used to construct the model of the age of insect larvae. If different killing methods have different but predictable effects on the visible age of the larvae, then corrections for the effects can be developed and then any method of assessment can be applied independently of the killing method used in a particular investigation (Midgley & Villet, 2009).

Ethanol appears to be the preferred preservative, but the concentrations used by individuals vary, and it is understood that hot water is not always used as a killing agent, although it is highly recommended. In most cases, the ethanol concentration is not specified and some investigators say that preservation has occurred, but they cannot identify the concentration of the preserving solution. In experimentation, experts have killed larvae in hot-water and preserved them in ethanol solution (Day & Wallman, 2008).

Lord and Burger were the first researchers to mention the killing methods and the negative effects of preservative solutions on immature and soft-bodied insects (Lord & Burger, 1983). Wells and Kurahashi removed all the larvae and killed them by boiling in 70% ethanol solution (Wells & Kurahashi, 1994). Adams and Hall explored in more detail the effects of ‘hot water killed’ larvae before applying preservative solutions (Adams & Hall, 2003). They tested the change in length of post-feeding instar *L. sericata* and *C. vomitoria* larvae killed in boiled water and stored in 80% ethanol, 90% ethanol and 10% formaldehyde solutions for different periods of time (Day & Wallman, 2008). In addition, Adams and Hall have examined the effects of placing live larvae in these solutions and the effects of water temperature on killing (Adams & Hall, 2003).

For the most reliable results larvae are killed by immersing them in near-boiling water for 30 seconds, immediately measured and then transferred to 80% ethanol for preservation. This recommendation has been accepted as the standard practice for collecting and preserving entomological evidence (Amendt *et al.*, 2007; Richards *et al.*, 2013). Other related handbooks suggest proper fixation and preservation in ethyl alcohol, and treating them with near-boiling water before they are transferred to 70–80% alcohol or Pampel's fluid (6 parts 35% formalin, 15 parts 95% ethyl alcohol, 2 parts glacial acetic acid and 30 parts distilled water), which is particularly suitable for dipteran larvae requiring dissection (Upton & Norris, 1980; Day & Wallman, 2008). However, some technicians immediately kill eggs and larvae and mount them on glass slides.

Rodriguez and Bass immediately killed the insects and kept them in a solution containing 85 cm^3 of 90% ethanol, 10 cm^3 of 40% formalin and 5 cm^3 of glycerine (Rodriguez & Bass, 1983). Similarly, Wolff *et al*. preserved their samples in 70% ethanol solution (Wolff *et al.*, 2001). Rodriguez and Bass were not the only scientists who used comparatively complex preservation solutions (Day & Wallman, 2008). Shean *et al*., after placing the first fixed samples in the KAA solution (85 ml 95% ethanol, 10 ml kerosene, 20 ml glacial acetic acid and 10 ml dioxane), transferred them to 75% ethanol and 3% glycerine as soon as possible (Shean *et al.*, 1993). Wells and LaMotte preserved larvae in Kahle's solution (30 ml 95% ethanol, 12 ml formaldehyde, 4 ml glacial acetic acid and 60 ml water) (J. Wells & LaMotte, 1995). The use of preservative materials has discouraged some researchers because it can affect dry weight (Williams, 1984).

A recent articles for forensic entomology students suggests fixing larvae by killing them in Kahle's solution or KAA, or by boiling larvae in hot water. The samples should then be taken from the fixative and placed in 80% ethanol. Fixing in hot water is amenable to the limited resources of law enforcement agencies and avoids samples becoming brittle from being in chemical fixation for long periods (i.e. over 12 hours) (Byrd & Castner, 2009).

Tantawi and Greenberg first investigated how larval killing method and preservative selection affected the length of larvae and larvae (Smith, 1986). They tested the effects of 15 common preservatives on the length of third instar and post-feeding larvae of *P. terraenovae* and *C. vicina*, also using the disciplines of entomology and pathology. The killing and preservative solutions were:

- 70% ethanol
- 80% ethanol
- 90% ethanol
- Benzene
- Kerosene
- Formalin
- 99.7% isopropanol
- 70% EtOH/AAG (3 parts 70% ethanol: I part glacial acetic acid)
- 90% EtOH/AAG (3 parts 90% ethanol: 1 part glacial acetic acid)
- KAAD (80 mL 95% ethanol, 20 mL glacial acetic acid, 10 mL kerosene, and 10 mL dioxane)
- KAA (1 part kerosene: 1 part glacial acetic acid: 30 parts 95% ethanol)
- XAAD (40 mL xylene, 60 mL isopropanol, 50 mL glacial acetic acid, and 40 mL dioxane)
- XA (50 mL xylene and 50 mL 95% ethanol)
- Pampel’s fluid (6 parts formalin: 15 parts 95% ethanol: 2 parts glacial acetic acid : 30 parts distilled water)
- San Veino (Embalmer’s Supply Co. Stratford, CT)

According to studies of Tantawi and Greenberg, the type of solution used to kill or preserve larvae has been found to have a significant influence on the length and estimated age, thus may lead to miscalculations of the post-mortem interval estimation. Larvae preserved in solutions such as 70% ethanol maintain the flexibility and extensibility of the cuticle, which can cause more errors when measuring the length (Tantawi & Greenberg, 1993). Although the size of the larvae still shrinks, it has been found that 70% ethanol has the lowest impact on larval length while achieving the required preservation; however, several authors have observed that the use of ethanol for larvae preservation in general causes more length change than other methods, such as using protective solutions after immersion in boiling water to prevent shrinking (Richards *et al.*, 2013). Furthermore, such studies refer to fly larvae (Midgley & Villet, 2009).

3 MATERIALS AND METHODS

3.1 Material Studied

It was intended to keep the preservation methodology simply, such that a non-specialist could carry out the hydrocarbon extractions from blowfly larvae with ease. This sampling method was developed at the start of the project and applied to the post-feeding larvae of *C. vomitoria* used in this work. To compensate for any variation within the extractions, 10 replicates were always taken from each extraction period. Post-feeding instar larvae of blowfly *C. vomitoria* were purchased from an angling shop and stored.

3.2 Methods

3.2.1 Killing larvae in near-boiling hot water

A number of time-dependent preservation experiments have been conducted to determine how different solutions affect the hydrocarbon profile of *C. vomitoria* larvae. Post-feeding growth stages of *C. vomitoria* larvae were put in near-boiling hot water during 30 seconds to kill them.

3.2.2 Preparation of control groups

In order to be able to compare the CHC profiles between preserved larvae and immediately killed larvae, untreated controls were prepared. The control larvae were killed directly in hexane and the hydrocarbon profiles were extracted in the same way.

In addition, some of the post-feeding larvae were kept alive without being killed in order to be able to identify the type of insect in the adult stage (Day & Wallman, 2008). It took about three weeks in standard room temperature (laboratory temperature was 25°C) for the insect larvae to complete the life cycle and become adult flies, microscopic examination of which revealed that they were *C. vomitoria*.

3.2.3 Preservation in ethyl alcohol (ethanol) solutions

Larvae of *C. vomitoria* were divided into groups. The larvae samples were killed by near-boiling hot water, then preserved in jars of the following concentrations: 40% EtOH, 60% EtOH, 70% EtOH and 80% EtOH.

The samples were placed live into the preservatives and extracted with hexane after: 4 hours, 8 hours, 1 day (24 hours), 2 days (48 hours), 3 days (72 hours), 4 days (96 hours), 7 days and 14 days (Table 3-1). A period of 14 days was chosen to see how long larvae can stay in preservative solution for without the CHC profiles being affected. In addition, a period of 14 days preservation period is the average amount of time required by the researcher for sample collection, transportation, transport, autopsy and time management (Wells & Kurahashi, 1994).

Table 3-1 Sample preservation timeline for preliminary CHC extraction set

16.05.17	17.05.17	18.05.17	19.05.17	23.05.17	26.05.17	02.06.17
Control (Day 0) 4th hours 8th hours	Day 1	Day 2	Day 3	Day 4	Day 7	Day 14

In order to obtain optimised result, *C. vomitoria* larvae were killed in near-boiling water and then preserved in 70% EtOH solution. The samples were placed live into the preservatives and extracted with hexane after: 4 hours, 8 hours, 1 day (24 hours) and 4 days (96 hours) (Table 3-2).

Table 3-2 Sample preservation timeline for optimised CHC extraction set

22.06.17	23.06.17	26.06.17
Control (Day 0) 4th hours 8th hours	Day 1	Day 4

3.2.4 Hydrocarbon extraction

For the extraction of hydrocarbons, a non-polar solvent such as hexane is preferable, as the hydrocarbons themselves are non-polar. Using polar solvents, such as methanol or ethanol, would only extract more polar compounds (or even internal compounds).

3.2.4.1 Preliminary CHC extractions

One post-feeding instar larvae was placed into a 2 mL GC vial with 300 µL hexane, ensuring that the insects were fully submerged, and left for 10 to 15 minutes. The extract was then transferred in a clean vial and the hexane was left to evaporate and left to dry down completely. All samples were stored dry in the refrigerator at 4°C until required for analysis. The dried extract was then reconstituted in 30 µL hexane before GC-MS analysis. Each sample was washed in hexane to obtain only the hydrocarbon compounds (Figure 3-1).

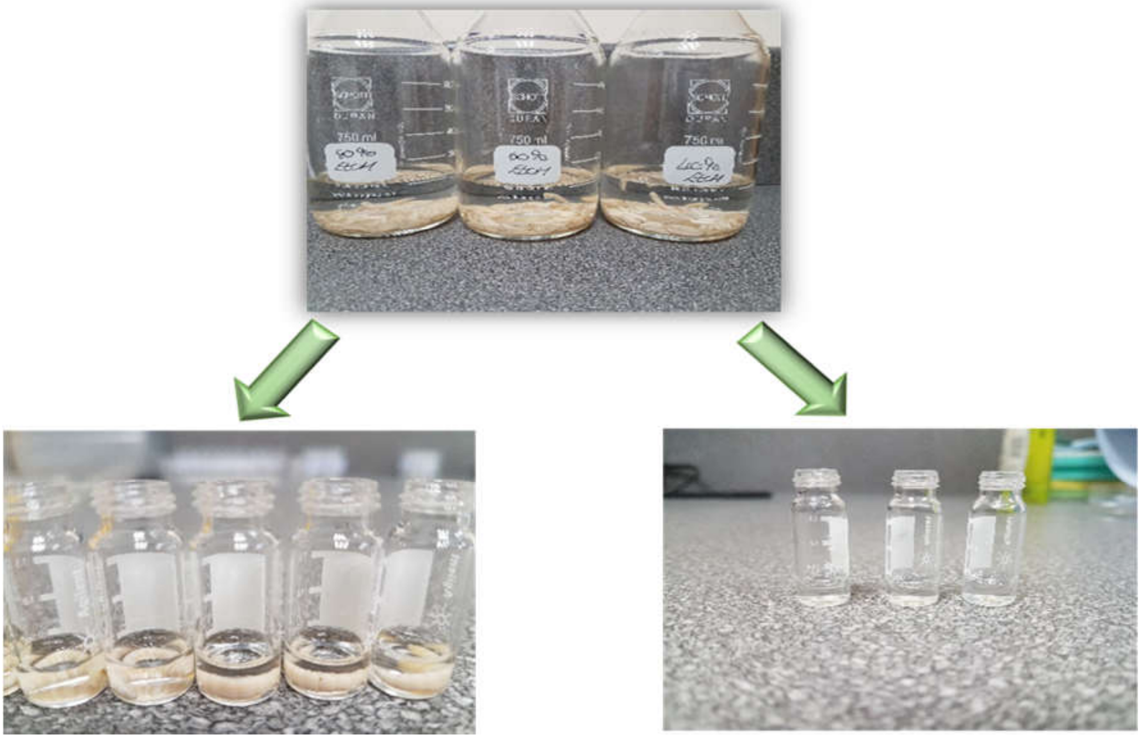


Figure 3-1 Preparation of preliminary CHC extractions

3.2.4.2 Optimised CHC extractions

Three post-feeding instars larvae were placed into a 2 mL GC vial with 500 µL hexane, ensuring that the insects were fully submerged, and left for 10 to 15 minutes. The hexane extract was then transferred to a silica gel column. The larvae were the only life stage needed to have column chromatography applied due to contaminants the larvae encounter when feeding on meat. The eluted column extract was collected in a clean vial. All samples were stored dry in the refrigerator at 4˚C until required for analysis (Figure 3-2).

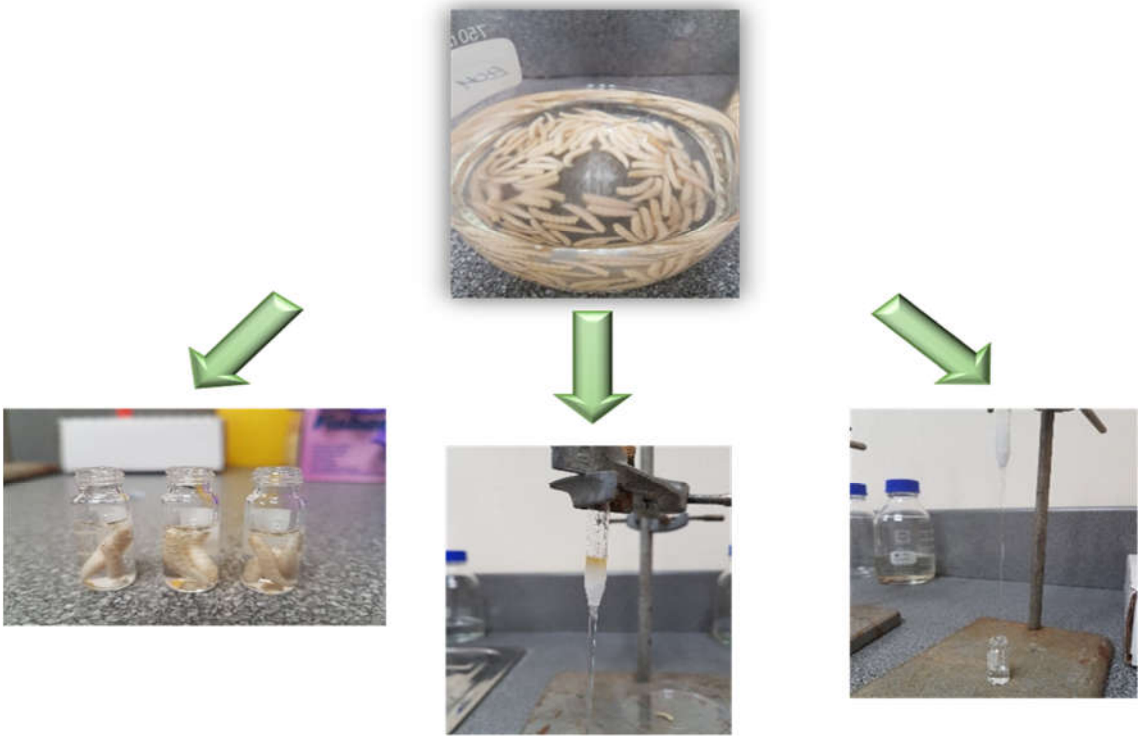


Figure 3-2 Preparation of optimised CHC extractions

3.2.4.2.1 Column chromatography

In order to separate out the polar compounds from the non-polar hydrocarbons, column chromatography was used on larval extracts, as this stage was found to contain the highest number of polar contaminants. The column was prepared by using a Pasteur pipette, plugged with glass wool and then layered with approximately 1 cm of silica gel. The column was first wet with 100 µL of hexane before the extract containing the insect specimen was transferred onto it, and an additional 500 µL of hexane was then added. The eluted hexane was collected into a clean 2 mL GC vial and the extract was left to evaporate to dryness.

3.2.5 Chemical analysis: Gas chromatography – mass spectrometry

The dried extract was then reconstituted in 30 µL L of hexane for GC-MS analysis (hexane could have evaporated slightly before the sample was analysed, thus the autosampler could not be relied upon to draw up the extract from the insert); the samples were introduced to the GC-MS via the autosampler.

Chemical analysis of all extracts was carried out on an Agilent Technologies 6890N Network GC system with a split/splitless injector at 250˚C, a Restek Rxi–1MS capillary column containing an SP of 100% Polydimethyl siloxane (30 m x 0.25 mmID, 0.25 µm film thickness) coupled to an Agilent 5973 Network Mass Selective Detector. The GC was connected to a computer and the data was processed with Agilent Chemstation software. Elution was carried out with helium at 1 mL/min. The oven temperature was programmed to be held at 50˚C for 2 minutes then ramped to 200˚C at 25˚C/min, then from 200˚C to 260˚C at 3˚C/min and finally from 260˚C to 320˚C at 20˚C/min where it was held for 2 minutes. The mass spectrometer was operated in Electron Ionisation at 70 eV, scanning from 40 – 500 amu at 1.5 scans s^{-1}. Hydrocarbons were identified using a library search (NIST08), the diagnostic fragmented ions and the Kovats Index.

An n-alkane standard solution, ranging from henicosane (C14:H) to tetracosane (C40:H), was analysed on the GC-MS under the parameters stated above.

4 RESULTS

4.1 Effects of Ratios of Preservative Solutions on *C. vomitoria* Larvae

C. vomitoria larvae were preserved in 40%, 60% and 80% EtOH solutions for varying durations of 4 hours, 8 hours, 1 day (24 hours), 2 days (48 hours), 3 days (72 hours), 4 days (96 hours), 7 days and 14 days. GC-MS analyses were performed by performing hydrocarbon extractions. Figure 4-1 shows the GC chromatograms of larvae samples preserved in 40%, 60% and 80% EtOH solutions at the end of 4th hours. It can clearly be seen that the concentrations of larvae extraction were too low, due to which the hydrocarbon peaks cannot be determined. To overcome this problem, the number of larvae used for extraction was increased and a new optimisation section was prepared.

The physical effects of 40% EtOH, 60% EtOH and 80% EtOH solutions on *C. vomitoria* larvae at Day 7 and Day 14 are shown in Table 4-1. The colours of the larvae in the preservative solutions varied, with cream, brown and black. All of the larvae in the preservative solutions were cream coloured the first day, while the colour of the larvae in 40% EtOH preservative solution was still preserved on Day 7, and by Day 14 the colours of some of the larvae turned brown. For the larvae in other ethanol solutions, it was seen that on Day 7, both brown and black larvae were found in the solution, whereas on Day 14, the number of larvae in these colours increased. The browned/darkened images of larvae indicate that they are not preserved in solution, and that the degradation has begun. Physically, larvae with this status cannot be used for the analysis of hydrocarbons

Table 4-1 Physical effects of preservative solutions at various ratios on larvae of *C. vomitoria*

Day 7

Preservative type	Colours observed		
	Cream	Brown	Black
40% EtOH	Yes	No	No
60% EtOH	Yes	Yes	No
80% EtOH	Yes	Yes	Yes

Day 14

Preservative type	Colours observed		
	Cream	Brown	Black
40% EtOH	Yes	Yes	No
60% EtOH	Yes	Yes	Yes
80% EtOH	Yes	Yes	Yes

Day 0 Day 7 Day 14

Figure 4-1 The appearance of the *C. vomitoria* larvae in the preservation solutions

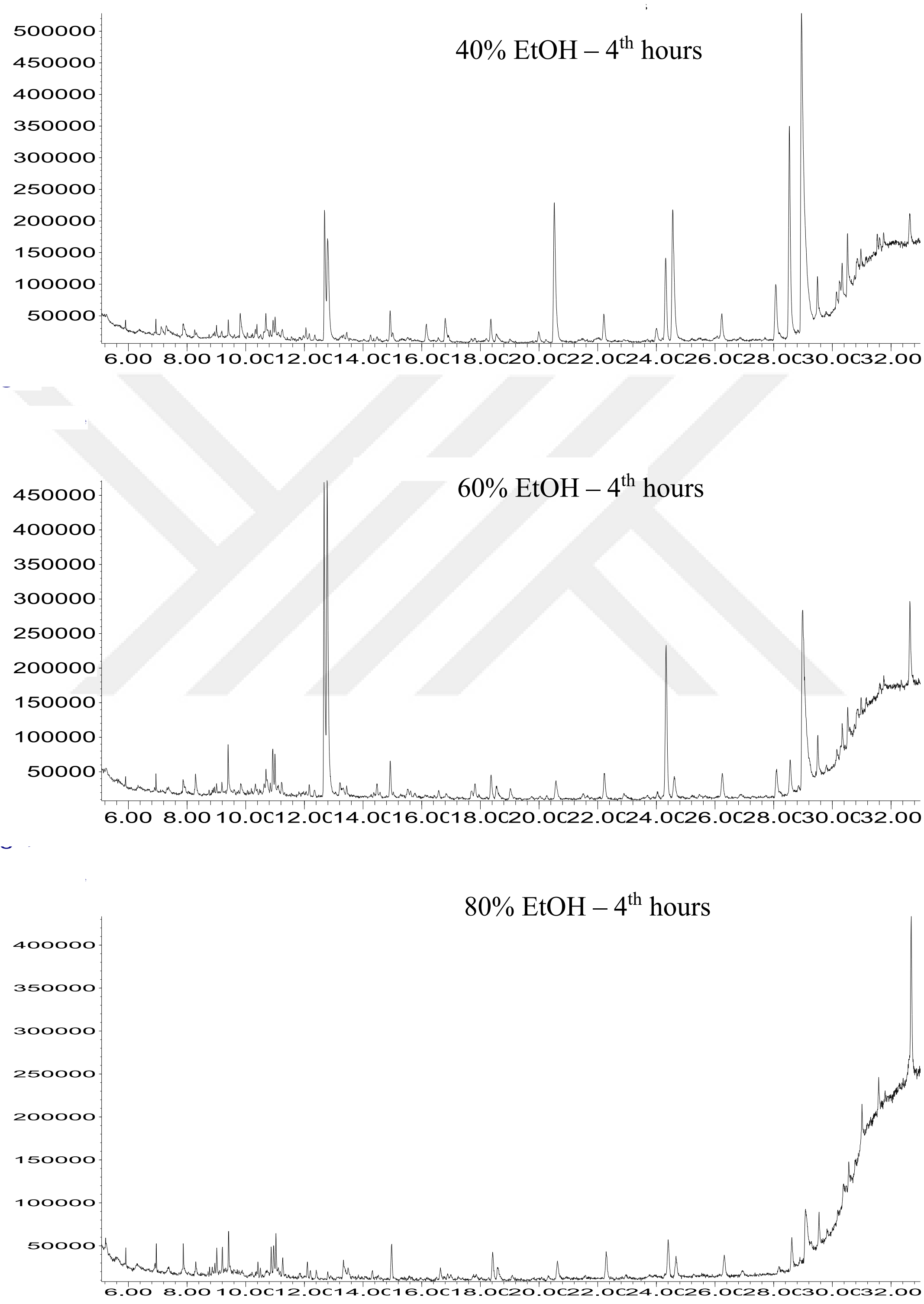


Figure 4-2 GC chromatograms for 40% EtOH, 60% EtOH and 80% EtOH solutions during 4 hours

4.2 Effects of Optimised Hydrocarbon Extraction

In order to see the effect of the hydrocarbon extraction concentration on the analysis results, more larvae were used, unlike the preliminary hydrocarbon extraction. The chromatogram in Figure 4-3 shows two control samples of larvae belonging to optimised and preliminary hydrocarbon extractions. Hydrocarbon structures cannot be analysed due to the very low concentration of preliminary hydrocarbon extraction associated with the use of a single larva, while the hydrocarbon structures can easily be obtained in the optimised hydrocarbon analysis using three larvae for extraction.

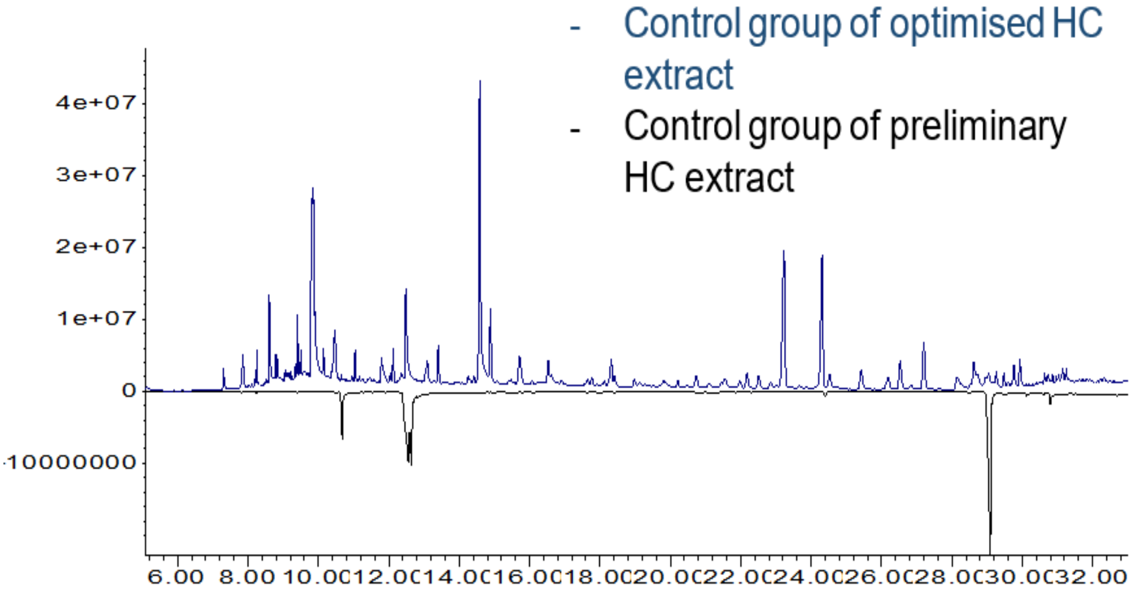


Figure 4-3 Comparison of concentrations of control group extracts (preliminary and optimised hydrocarbon extraction)

When looking at the chromatograms given in Figure 4-1 it is understood that there was a very high amount of contamination in the analysis. This problem was solved by the silica gel column added during the preparation of the hydrocarbon extracts. The silica gel wetted with hexane retained the contaminants and polar compounds and removed the impurities that prevented the analysis of the hydrocarbon structures during the analysis. It takes off the fatty acids etc. and cleans up the profile. Thus, it can just see the non-polar hydrocarbons.

4.3 Effects of 70% EtOH on *C. vomitoria* Larvae

C. vomitoria larvae were preserved in 70% EtOH solutions during 4 hours, 8 hours, 1 day (24 hours), 4 days (96 hours) and GC-MS analysis were performed by performing hydrocarbon extractions.

A typical gas chromatographic profile of CHC of larval *C. vomitoria* is shown in Figure 4-4. The hydrocarbon structures were identified by GC–MS and found mainly to be a mixture of n-alkanes, methyl-branched alkanes and alkenes, with carbon chain length of C14–C33. In Table 4-3, the analysis results of these compounds are given according to the preservation times. The summarized data of the hydrocarbons profiles of the larvae are shown in Table 4-2. The other polar compounds were excluded because the focal point is hydrocarbon profiles. The desired hydrocarbon analysis is shown to be as expected, even after 4 days.

Table 4-2 Hydrocarbon composition showing the number and percentage of n-alkanes, alkenes and methyl branched alkanes of *C. vomitoria* larvae

	***C. vomitoria* (%)**
Alkanes	17 (37)
Alkenes	14 (30)
Methyl branched HC	15 (33)
Total	**46 (100)**

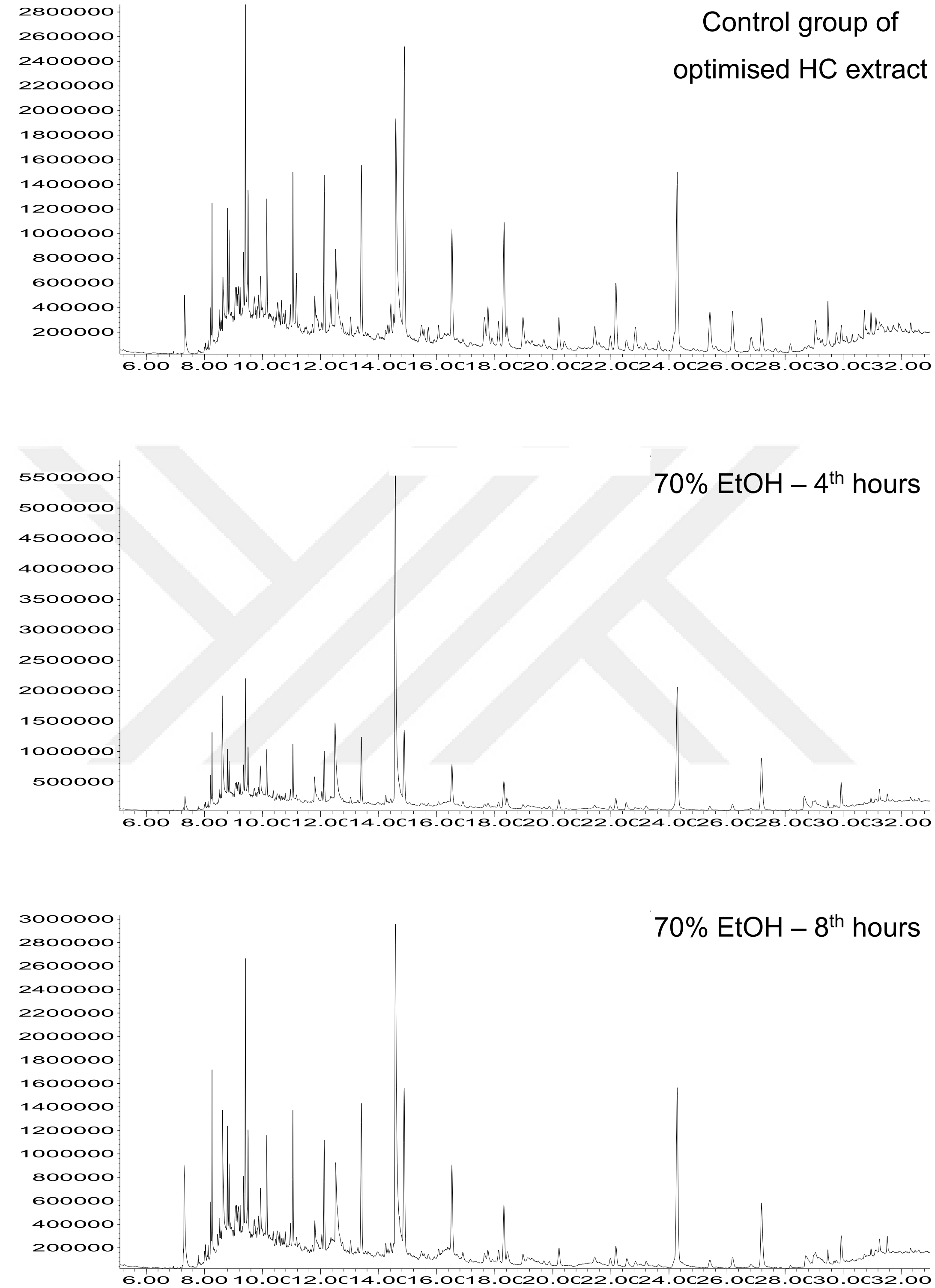
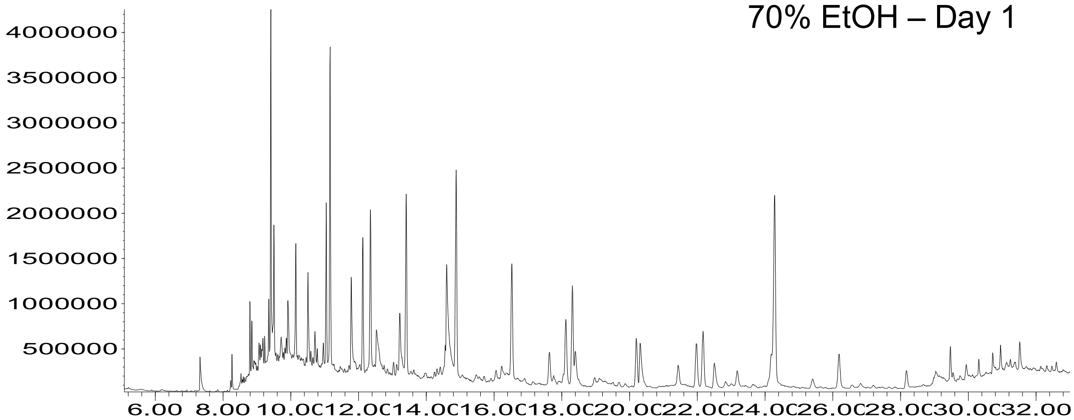


Figure 4-4 Gas chromatographic profile of cuticular hydrocarbons extracted from larvae of *C. vomitoria* in 70% EtOH preservative solution



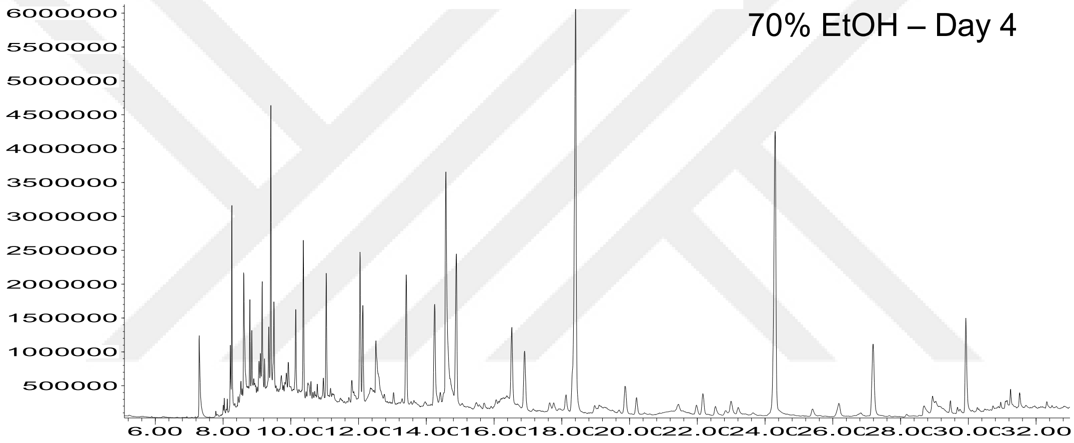


Figure 4-4 cont.

Table 4-3 Retention times (RT) (min) and peak areas in which hydrocarbon structures are detected

ID	RT	Control	4th Hours	8th Hours	Day 1	Day 4
Tetradecene	8,040	-	2192046	4872856	-	3453246
Tetradecane	8,118	-	1838909	4175094	-	3374875
Hexadecene	8,219	5906578	5880207	11812059	3132222	10919847
Hexadecane	8,264	13940913	15033895	29967153	5475176	33603794
Methyl branched HC	8,531	7278070	10552153	13599688	3871617	6703378
Methyl branched HC	8,568	2825295	34220228	4690000	-	-
Heptadecene	8,175	21793241	3634984	-	74000343	28653069
Heptadecane	8,794	13532754	14431660	21626002	13971356	20010646
Methyl branched HC	8,850	16078027	14244624	21941040	13005600	15489264
Methyl branched HC	9,065	9471196	10179477	17570207	9505976	11422247
Methyl branched HC	9,106	6446574	6366948	10321230	6378695	8368147
Methyl branched HC	9,174	12259460	13294772	20079234	8170527	-
Octadecene	9,353	17047907	18025052	20626256	17584855	24450669
Octadecane	9,413	32324088	32294732	45693643	44248507	52751114
Methyl branched HC	9,505	21905013	22772526	34012999	35337656	26347136
Methyl branched HC	9,718	8194573	19193286	17252860	12600945	14199345
Nonadecene	9,924	26212658	22496340	-	-	-
Nonadecane	10,150	22338902	20834634	27970990	26930201	30318846
Methyl branched HC	10,653	3508647	4488813	5466255	-	-
Eicosene	10,962	6747187	5987436	7960529	9249627	9172750
Eicosane	11,048	20921698	18987062	26706144	28738202	29265616
Heneicosene	11,793	29200277	-	-	-	-

Table 4 3 cont.

ID	RT	Control	4th Hours	8th Hours	Day 1	Day 4
Docosene	13,161	3920191	-	1877491	-	-
Docosane	13,408	27852379	26892469	34600185	40337844	38893061
Tricosene	14,419	10836105	4050741	3873180	13683691	11944107
Tricosane	14,885	62506222	40248249	46850836	54200224	53272700
Methyl branched HC	15,475	7792343	3917205	6214861	-	-
Methyl branched HC	15,710	4897092	2219783	6018866	-	-
Tetracosane	16,525	22465093	26689400	32868567	31483759	33524007
Methyl branched HC	17,647	9935971	5417962	9329785	11590480	11549301
Pentacosene	17,765	12549613	6685137	4458615	11590480	6771506
Pentacosane	18,320	33424982	15591327	18490731	23194374	9234702
Methyl branched HC	18,977	12061021	5109650	17789567	-	12827767
Hexacosane	20,210	8562965	5569656	7714514	12708992	7698412
Heptacosene	21,442	10088106	2876058	4468324	-	6627114
Heptocosane	21,979	16456070	7197330	8663531	14699080	10291032
Methyl branched HC	22,846	9744179	3125457	14139076	-	5459150
Nonacosene	25,542	19248393	3330488	5130116	-	5554699
Nonacosane	26,113	22877577	4902416	4894326	11917977	11172098
Methyl branched HC	27,197	34498459	14678376	90980411	82941103	71287486
Hentriacontene	29,261	21525600	-	3347616	-	
Hentriacontane	29,475	10346733	3238017	-	8516368	4509948
Dotriacontane	30,312	-	-	-	5731767	-
Tritriacontane	30,980	5316147	-	-	-	-

A detailed chromatogram of the extract obtained from larvae of *C. vomitoria* preserved for 4 days in the solution prepared using 70% ethanol is given in Figure 4-5. The results obtained are very important in that they show that the hydrocarbon analyses can still be made despite 4 days in the preservative solution. Examples of some hydrocarbon structures on the GC chromatogram are shown. From left to right, the hydrocarbon profiles mentioned in red colour (in Figure 4−5) belong to heptadecane, octadecane, nonadecane, eicosane, heneicosane, docosane, tricosane, tetracosane, pentacosane and heptacosane alkane structures; and those mentioned in purple colour (in Figure 4−5) belong to octadecene, nonadecene, eicosene, heneicosene, tricosene, pentacosene and haxacosene alkene structures. The profiles indicated by the green colour (in Figure 4−5) belong to methyl branched hydrocarbon structures.

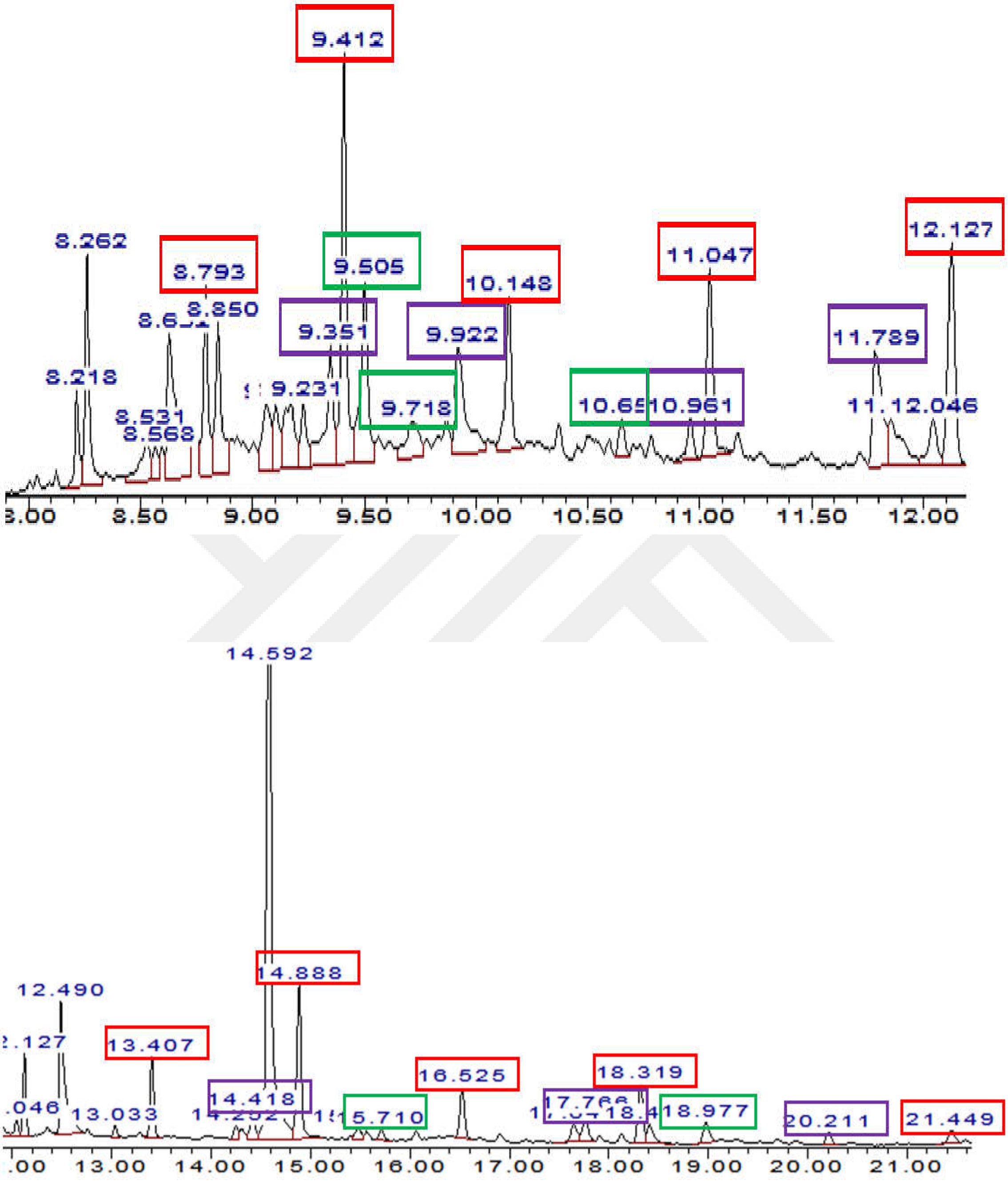


Figure 4-5 Retention times and determinations of some hydrocarbon structures on the chromatogram

GC chromatogram of Day 4 larvae – Alkanes – Alkenes – Methyl branched HC

Figure 4-6 and Figure 4-9 show the chromatograms of the larvae which preserved in the ethanol solution for 4 days. Pertaining to determination of the structures found in the chromatogram results, mass spectrometry, one of the most popular detectors used for gas chromatography analysis, was used to identify the undefined compound at retention time 9.412 (min); the spectrum from mass spectrometry is shown in Figure 4-7. Based on previous studies, the mass/ion (*m/z*) 57, 79 and 85 ratios are known as fingerprints/trace for alkane structures. In Table 4-4, the molecular weight/ion ratios for hydrocarbon structures are given (z = 1). *m/z* = 254 is the molecular weight/ion ratio for octadecane. It is determined that this structure, which is undefined by this information, belongs to the octadecane ($C_{18}H_{38}$) alkane compound.

Each individual peak in the chromatogram has its own mass spectrum, and this allows it to be identified. The retention times are also characteristic for ID purposes, as, for example, under the same conditions, a (C18:H) will always have the same retention time. Alkene structures with lower molecular weights are detected at retention times just prior to alkane constructions with higher molecular weights. This is because the presence of a double bond lowers the boiling point of the compound, making it elute before the associated alkane. Octadecane with the molecular weight (254 g/mol) is expected to come after octadecene with the molecular weight (252 g/mol). For this reason, it is expected that the retention time of 9.351 is attributed to the octadecene ($C_{18}H_{36}$) alkene structure. The mass spectrum given in Figure 4-8 belongs to the structure observed in the retention time 9.351. *m/z* = 252 is the molecular ion for octadecene. It has been determined that this structure, which is undefined by this information, belongs to the octadecene compound.

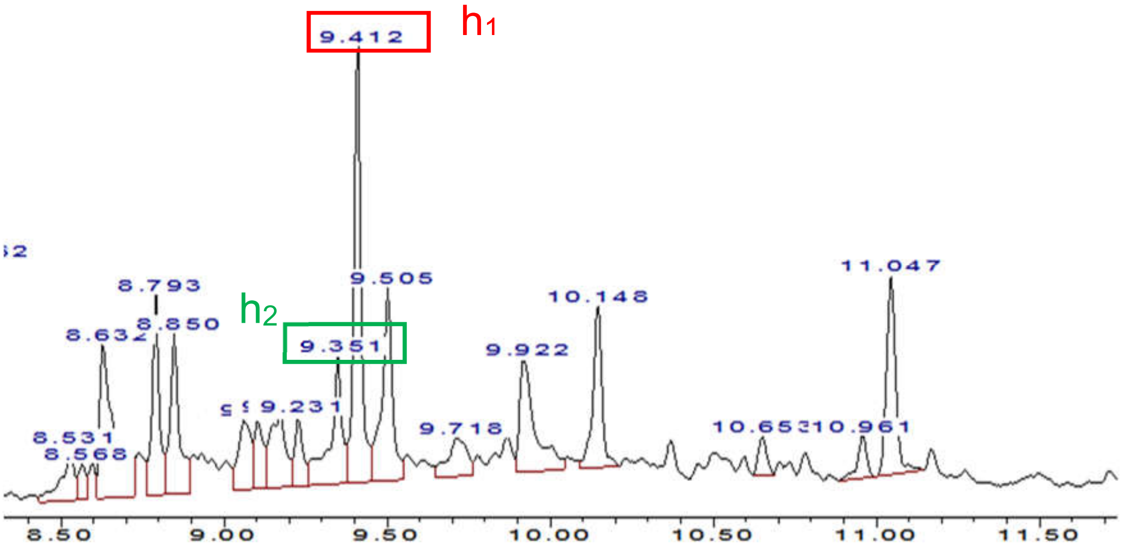


Figure 4-6 The retention times and determinations of octadecane (min: 9.412) and octadecene (min: 9.351) (C18:H) on the GC chromatogram

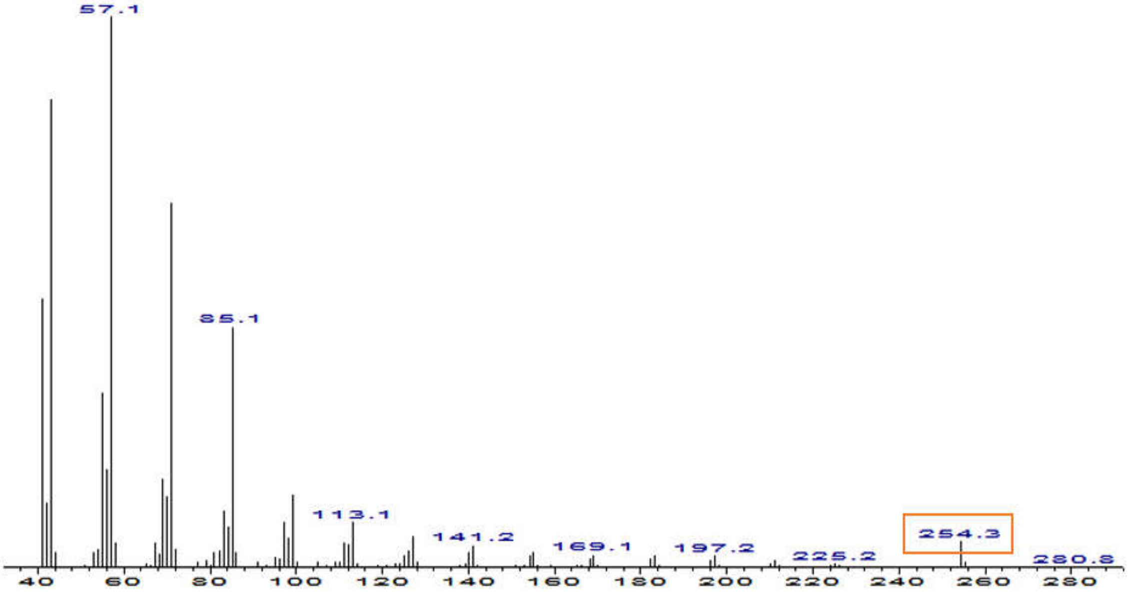


Figure 4-7 Mass spectrum for octadecane h_1; *m/z* for M=254

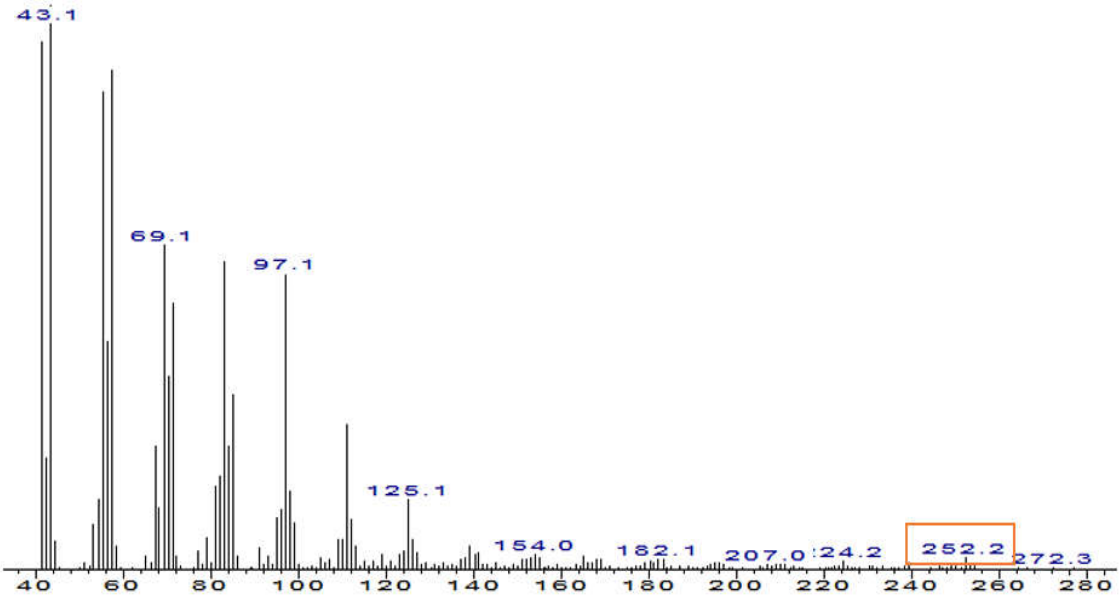


Figure 4-8 Mass spectrum for octadecene h$_2$; *m/z* for (M – H$_2$) =252

Another example of GC-MS spectrum interpretation is given in Figure 4-9. Mass spectrometry was used to identify undefined compounds with retention times at 14.418 and 14.888. Figure 4-10 shows that the *m/z* = 57, 79 and 85 alkane structures in the mass spectrum are supported. Utilizing the data in Table 4-4, *m/z* = 324 is the molecular weight/ion ratio for tricosane. Based on this information, it was determined that this undefined structure belongs to the tricosane ($C_{23}H_{48}$) alkane compound.

Tricosene, with the molecular weight 322 g/mol, was expected to precede tricosane, with the molecular weight 342 g/mol. For this reason, it is considered that the structure with a retention time 14.418 belongs to the alkene structure of tricosene ($C_{23}H_{46}$). The mass spectrum is given in Figure 4-11 belongs to the structure with the retention time 14.418. *m/z* = 322 is the molecular ion for tricosene. Due to this information, it is determined that this undefined structure belongs to tricosene compound.

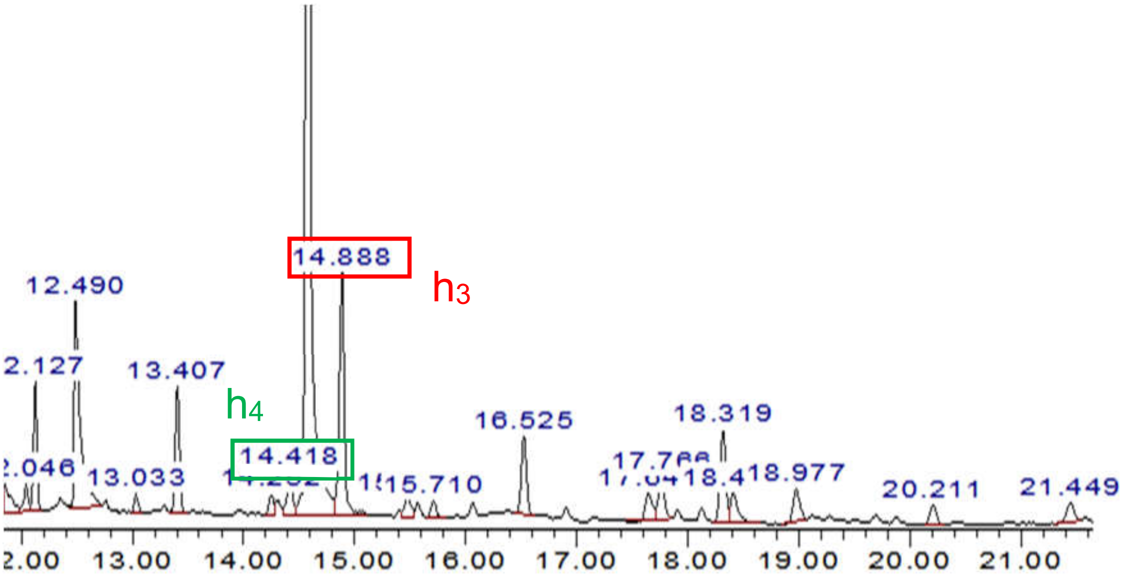


Figure 4-9 The retention times and determinations of tricosane (min: 14.888) and tricosene (min: 14.418) (C23:H) on the GC chromatogram

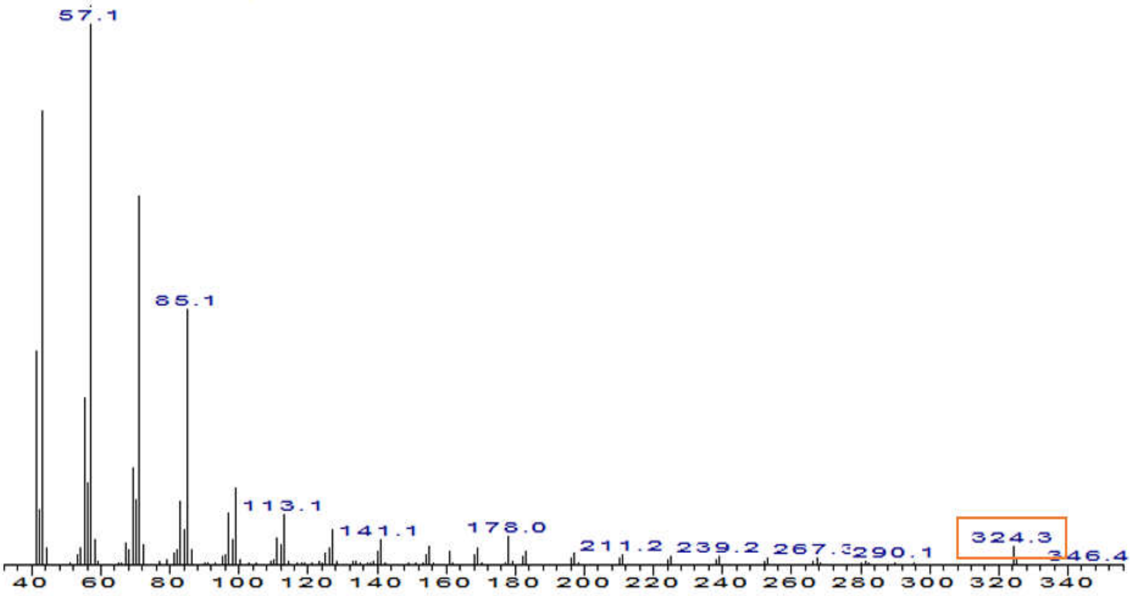


Figure 4-10 Mass spectrum for tricosane h_3; *m/z* for M=324

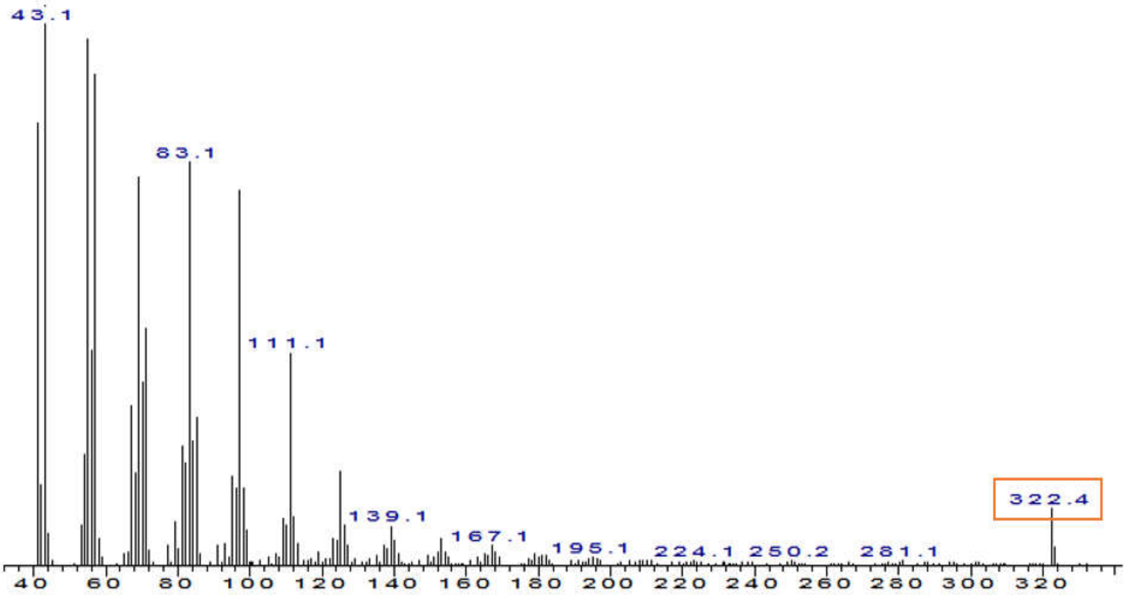


Figure 4-11 Mass spectrum for tricosene h_4; *m/z* for $(M - H_2)$ =322

Table 4-4 Number of carbon atoms and molecular ion m/z of hydrocarbon profiles (z=1)

Molecular formula	Name of straight chain	Molecular ion m/z	Molecular formula	Name of straight chain	Molecular ion m/z
CH_4	Methane	16	$C_{21}H_{44}$	n-Henicosane	296
C_2H_6	Ethane	30	$C_{22}H_{46}$	n-Docosane	310
C_3H_8	Propane	44	$C_{23}H_{48}$	n-Tricosane	324
C_4H_{10}	n-Butane	58	$C_{24}H_{50}$	n-Tetracosane	338
C_5H_{12}	n-Pentane	72	$C_{25}H_{52}$	n-Pentacosane	352
C_6H_{14}	n-Hexane	86	$C_{26}H_{54}$	n-Hexacosane	366
C_7H_{16}	n-Heptane	100	$C_{27}H_{56}$	n-Heptacosane	380
C_8H_{18}	n-Octane	114	$C_{28}H_{58}$	n-Octacosane	394
C_9H_{20}	n-Nonane	128	$C_{29}H_{60}$	n-Nonacosane	408
$C_{10}H_{22}$	n-Decane	142	$C_{30}H_{62}$	n-Triacontane	422
$C_{11}H_{24}$	n-Undecane	156	$C_{31}H_{64}$	n-Hentriacontane	436
$C_{12}H_{26}$	n-Dodecane	170	$C_{32}H_{66}$	n-Dotriacontane	450
$C_{13}H_{28}$	n-Tridecane	184	$C_{33}H_{68}$	n-Tritriacontane	464
$C_{14}H_{30}$	n-Tetradecane	198	$C_{34}H_{70}$	n-Tetratriacontane	478
$C_{15}H_{32}$	n-Pentadecane	212	$C_{35}H_{72}$	n-Pentatriacontane	492
$C_{16}H_{34}$	n-Hexadecane	226	$C_{36}H_{74}$	n-Hexatriacontane	506
$C_{17}H_{36}$	n-Heptadecane	240	$C_{37}H_{76}$	n-Heptatriacontane	520
$C_{18}H_{38}$	n-Octadecane	254	$C_{38}H_{78}$	n-Octatriacontane	534
$C_{19}H_{40}$	n-Nonadecane	268	$C_{39}H_{80}$	n-Nonatriacontane	548
$C_{20}H_{42}$	n-Icosane	282	$C_{40}H_{82}$	n-Tetracontane	562

N.B. The number of carbon atoms is indicated in the formula ($C_nH_{(2n+2)}$), e.g. CH_4 (n=1), C_2H_6 (n=2) etc.

5 DISCUSSION

5.1 Effects of Ratios of Preservative Solutions on *C. vomitoria* Larvae

This study investigated time and ethanol concentration dependent changes in the hydrocarbon structure of the post-feeding instar larvae of *C. vomitoria* preserved in ethanol solutions. This was in order to determine the maximum time that larvae can be used for the analysis of hydrocarbons and preserved in ethanol solution, and to identify the optimal ethanol concentration for this purpose. The results can be used to work with the larvae of necrophagous fly species, and future work in this area will be able to determine the concentration of ethanol and the duration of preservation that can be used for each species.

Identification of larva species is the fundamental starting point of entomological forensic post-mortem analysis, in order to reveal the time of death, extrapolated from the larval growth rate over time (which varies according to insect species). In traditional methods, the life cycle had to be completed, from eggs to adult flies, in order to identify the species; this time-consuming method could delay investigations for days or weeks, which is highly problematic for practical applications in criminal investigations. In order to determine the age of larvae, the instar stage in which they are collected from the corpse is examined; however, their latent (i.e. genetic) growth process can be affected by environmental conditions – in other words, the durations of larval stages are influenced by factors such as temperature and humidity, which directly affects age determination.

Many factors affect the life cycle of insects such as the season in which the corpse was found, summer or winter, the presence of a closed area or open area, temperature changes during the day, and the weather. The changing conditions can cause the instar stages to be shorter or longer than expected. For this reason, it is necessary to carry out a lot of studies for an accurate age determination, considering every factor affecting the age estimation. On the other hand, there is no waiting period for identification and age estimation in the hydrocarbon analysis technique.

Identification and age determination can be performed for each larval stage. Moore *et al*. (2012) demonstrated the use of hydrocarbon analysis for identification and age determination of insects by studies on *C. vomitoria*, *C. vicina* and *L. sericata* species. The hydrocarbon structure of larvae varies by larval stages and species. By creating a database, identification and larval age determination can be performed by comparing results. Closely related species, such as *C. vomitoria* and *C. vicina*, can easily be identified and differentiated from each other by hydrocarbon analysis technique (Moore *et al*., 2014).

Another important point is the collection and preservation of entomological evidence from the scene. In the vast majority of previously prepared reports, larvae collected from the crime scene were killed in hot water firstly and then preserved in 70-95% ethanol solutions by crime scene investigators; however, no precise information is given about the preservative solution, which affects both larval lengths (by remove lipids) in traditional methods and damages hydrocarbon structures in a hydrocarbon analysis technique. In both cases, this affects the species identification and the age of the larvae and the PMI estimates.

Tantawi and Greenberg (1993) examined the effect of killing and preservative solutions on larval stages of forensically important insect species. Insect larvae of known age were preserved in solutions and post-mortem length was measured 5 days after killing using traditional preservation methods with 15 different solutions including varying concentrations of ethanol and benzene; they found that ethanol was preferred due to easy accessibility, low toxicity and properties when mixed with water. Adams and Hall (2003) have stated that in one study, 80% ethanol was used as a preservative for larvae killed in hot water, causing an expansion in the larvae of both *C. vomitoria* and *L. sericata*. The same group determined that 95% ethanol also showed the same effect.

Previous studies on ethanol have focused on the investigation of the effect of ethanol as a preservative solution, which this study extends by analysing the effects of preservative solutions on the hydrocarbon structure of insects.

It is not a common practice to kill larvae directly in ethanol solution. Larvae that have been killed and preserved in this way have shown indications of significant deterioration, and they are in very bad condition subsequent to experimentation (Adams & Hall, 2003). It is not suggested in any literature study that live larvae are put directly into the preservative solution to kill. Hot water is preferred as a standard killing method for insects with a fairly wide area of legal forensics, from criminal investigators to forensic pathologists, because hot water largely prevents autolysis by destroying digestive enzymes and gut flora (Day & Wallman, 2008). Adams and Hall (2003) stated that submersion in water at a minimum of 80°C for at least 30 seconds was the best killing method for larvae. At the same time, in a criminal investigation, a thermos of near-boiling hot water (to be used for making coffee) if often the most logistically expedient and effective real-life application in entomological forensic investigations.

The larvae killed in hot water are swollen and reach their maximum length (Figure 5-1). During the time they are preserved in solution, they also change their length depending on the effect of the preservative solution on the cuticle. The change in cuticle structure affects both the length of the larvae and the species identification and age determination by the hydrocarbon analysis method. Tantawi and Greenberg (1993) found that different concentrations of the same solution lead to swelling or shrinking of larvae in very different quantities. For example, the *P. terraenovae* larvae were shrunk by 24.4% in 90% EtOH while shrunk by only 17.3% in 70% EtOH. This means that different concentrations of the same preservative solution affect the cuticle structure in different ways.

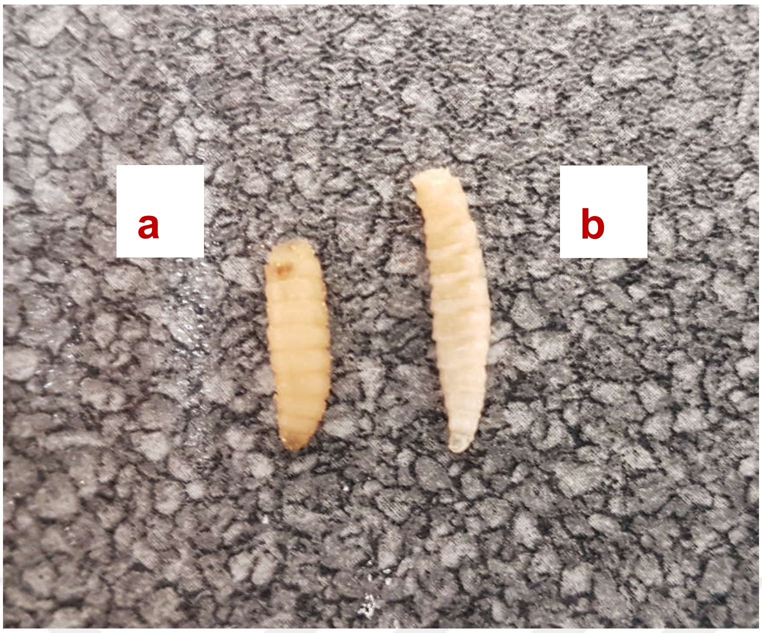


Figure 5-1 Comparison of the length of post-feeding instar larva of *C. vomitoria* a) alive and b) killed in near-boiling hot water

The extraction step was completed using a larva for each vial for hydrocarbons analysis of the preserved larvae in the ethanol solution at the selected concentrations. Looking at the GC chromatograms in Figure 4-1, it is seen that the concentrations of extracts from the larvae are too low for analysis. At the same time, it is also understood that there is a high amount of contamination in the extracts. Both of these factors played a role in determining the hydrocarbon structure, and have led to the inability to obtain results.

The colours of the larvae that are not waiting in the preservation solution quickly darken, indicating the beginning of degradation. Darkened larvae cannot be used to identify species using conventional methods or hydrocarbon analysis. The darkened larvae become hardened and morphological criteria that are necessary for species identification become invisible.

When the physical states of the larvae preserved in the ethanol solution in the three different concentrations used in the experiment setup were compared, it was seen that the larvae preserved in the 80% ethanol solution began to blacken on Day 7, and the number of the black larvae had seriously increased by Day 14. The colours of the larvae preserved in the 60% ethanol solution also started to turn brown from cream on Day 7. On Day 14, the number of larvae with brown colour increased and darkened larvae were observed. However, it was observed that the number of darkened larvae was lower when compared to other preservative solutions containing 80% alcohol.

The colour of the preserved larvae in 40% ethanol solution was maintained for seven days, and on Day 14 only a small number of brownish colours of the larvae were observed. Compared to the physical states of the larvae preserved in the preservative solutions for preliminary conditions, the result is that the reduced alcohol content provides more preservation. If we only commented on the physical state of the larvae in the preservative solutions, we could say that 40% ethanol-containing solution provides more preservation than the others.

5.2 Effects of Optimised Hydrocarbon Extraction

The concentration in the preliminary analysis was insufficient; this problem was solved by increasing the number of larvae used for extraction. In addition, column chromatography was used for contamination arising from the extraction. Column chromatography is one of the most popular methods used for separation processes. It is preferred because it is easy to prepare and use. The silica gel inside the column prevents contamination by retaining polar compounds, thereby reducing the contamination concentration in the extraction.

5.3 Effects of 70% EtOH on *C. vomitoria* Larvae

Due to the inadequate concentration problem experienced in the preliminary set, a new optimized set of experiments was decided. Unlike the ratios used in the previously prepared set of preservative solutions, 70% ethanol, which is widely used in the literature, was preferred for this set. This is germane to observation of the effect of this commonly used ratio on the hydrocarbon structure. By increasing the number of larvae used for extraction and adding column chromatography, a chromatogram sufficient to determine the hydrocarbon structure was obtained.

To examine the effect of preservative solutions on the hydrocarbon structure of the larvae, a control group was prepared without preservation methods or killing in near-boiling hot water. Post-feeding instar *C. vomitoria* larvae were killed directly in the hexane. A variety of alkane, alkene and branched methyl structures ranging from carbon chain lengths C16-C33 were identified on the GC chromatogram obtained after extraction. The dominant class of hydrocarbons in *C. vomitoria* is n-alkanes, but it contains a greater number of alkenes and methyl branched hydrocarbons. The profile is dominated by distinctively long chain methyl branched hydrocarbons (Moore *et al*., 2014)

After 4 hours, 8 hours, 24 hours and 96 hours, in order to be able to observe changes in the hydrocarbon structures of larvae preserved in 70% EtOH solution, the larvae were taken from the solution and subjected to hydrocarbon analysis. Several alkanes, alkenes and branched methyl structures, with carbon chain lengths ranging between C14–C31, were identified in the larvae of *C. vomitoria* that had remained for 4 and 8 hours in the preservative solution. Thus, no significant change in hydrocarbon structure was observed in the four hours subsequent to the initial four-hour period.

Various alkanes, alkenes and branched methyl structures, with carbon chain lengths ranging between C14–C31, were identified in the larvae preserved for 4 days in ethanol solution. When all the results are evaluated, 70% of the ethanol-containing preservative solution does not damage the hydrocarbon structure and the larvae of *C. vomitoria* can be used for hydrocarbon analysis.

The practical methodology required for hydrocarbon analysis with GC-MS is quite simple, and the extraction method is also very practical. It takes about 1.5 hours to prepare the hydrocarbon extractions of larvae for GC analysis. Any GC-MS instrument can be used for analysis and the chromatogram from each sample can be used to distinguish hydrocarbon profiles.

The results presented in this article were conducted in a controlled laboratory environment. However, field tests are required to look at the effects of environmental conditions on the stability of hydrocarbons. As a result, further studies are needed to determine the maximum time that larvae can preserve in the ethanol solution without any change in hydrocarbon structures of larvae. A further study can examine the effects of ethanol as a preservative solution on hydrocarbon species analysis and age estimation.

6 CONCLUSION

The results presented are very important within the field of forensic entomology, which is used to determine the estimated time of death after a forensic event. The time taken to move the entomological evidence collected from the crime scene to the laboratory environment directly affects the PMI estimates. More accurate results are obtained with the recommended preservation methods, by preventing the deterioration of evidence during laboratory delivery. In addition, with hydrocarbon analysis technique, it is possible to obtain both PMI estimation and species identification within a short period of time without waiting for the completion of the life cycle.

Our aim in this study was to examine the effects of different ratios of ethanol solution on hydrocarbon structure as a preservative. For this purpose, the ethanol solution was used at both very low and high concentrations (40%, 60%, 70% and 80%). The reason for choosing hot water as the killing method is to provide parallelism and ease of use in fieldwork.

This study has implications for many research directions, such as identification of species and uses for age determination for larvae preserved in ethanol. As a result of our study, it is observed that larvae preserved in the ethanol solution are still able to maintain and analyse the hydrocarbon structure even on Day 4. The results obtained are promising for the field of forensic entomology, in that hydrocarbon analysis can be combined with preservation methods, and in terms of providing ease of use for practical application in forensic entomology.

REFERENCES

Adams, Z. J. & Hall, M. J. (2003) Methods used for the killing and preservation of blowfly larvae, and their effect on post-mortem larval length. *Forensic Science International,* **138**, 50-61.

Aggarwal, A. (2005) Estimating the postmortem interval with the help of entomological evidence. *Anil Aggrawal's Internet Journal of Forensic Medicine and Toxicology,* **6**.

Amendt, J., Campobasso, C. P., Gaudry, E., Reiter, C., LeBlanc, H. N. & Hall, M. J. (2007) Best practice in forensic entomology—standards and guidelines. *International journal of legal medicine,* **121**, 90-104.

Amendt, J., Campobasso, C. P., Goff, M. L. & Grassberger, M. (2010) *Current concepts in forensic entomology*. Springer.

Amendt, J., Krettek, R. & Zehner, R. (2004) Forensic entomology. *Naturwissenschaften,* **91**, 51-65.

Amendt, J., Richards, C., Campobasso, C. P., Zehner, R. & Hall, M. J. (2011) Forensic entomology: applications and limitations. *Forensic science, medicine, and pathology,* **7**, 379-392.

Ames, C. & Turner, B. (2003) Low temperature episodes in development of blowflies: implications for postmortem interval estimation. *Medical and veterinary entomology,* **17**, 178-186.

Benecke, M. (2001) A brief history of forensic entomology. *Forensic Science International,* **120**, 2-14.

Blankaart, S. (1690) Schauplatz derer Raupen‖, Wurm und Maden. *Leipzig*.

Boehme, P., Spahn, P., Amendt, J. & Zehner, R. (2013) Differential gene expression during metamorphosis: a promising approach for age estimation of forensically important *Calliphora vicina* pupae (Diptera: Calliphoridae). *International journal of legal medicine,* **127**, 243-249.

Bourel, B., Callet, B., Hédouin, V. & Gosset, D. (2003) Flies eggs: a new method for the estimation of short-term post-mortem interval. *Forensic science international,* **135**, 27-34.

Brown, W., Morton, R., Lacey, M., Spradbery, J. & Mahon, R. (1998) Identification of the geographical source of adults of the Old World screw-worm fly, *Chrysomya bezziana* Villeneuve (Diptera: Calliphoridae), by multivariate analysis of cuticular hydrocarbons. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology,* **119**, 391-399.

Brown, W., Morton, R. & Spradbery, J. (1992) Cuticular hydrocarbons of the Old World screw-worm fly, *Chrysomya bezziana* Villeneuve (Diptera: Calliphoridae). Chemical characterization and quantification by age and sex. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry,* **101**, 665-671.

Byrd, J. H. & Castner, J. L. (2009) Forensic entomology: the utility of arthropods in legal investigations. CRC press.

Campobasso, C. P., Di Vella, G. & Introna, F. (2001) Factors affecting decomposition and Diptera colonization. *Forensic science international,* **120**, 18-27.

Campobasso, C. P. & Introna, F. (2001) The forensic entomologist in the context of the forensic pathologist’s role. *Forensic Science International,* **120**, 132-139.

Catts, E. P. (1992) Problems in estimating the postmortem interval in death investigations. *Journal of Agricultural Entomology,* **9**, 245-255.

Catts, E. P. & Goff, M. L. (1992) Forensic entomology in criminal investigations. *Annual review of entomology,* **37**, 253-272.

Davies, L. & Ratcliffe, G. (1994) Development rates of some pre-adult stages in blowflies with reference to low temperatures. *Medical and veterinary entomology,* **8**, 245-254.

Day, D. M. & Wallman, J. F. (2008) Effect of preservative solutions on preservation of *Calliphora augur* and *Lucilia cuprina* larvae (Diptera: Calliphoridae) with implications for post-mortem interval estimates. *Forensic science international,* **179**, 1-10.

Drijfhout, F. P. (2009) Cuticular hydrocarbons: a new tool in forensic entomology. In *Current Concepts in Forensic Entomology*, pp. 179-203. Springer.

Duke, L. D. (2003) Effects of amitriptyline and nortriptyline on time of death estimations in the later postmortem interval using insect development. Theses (School of Criminology)/Simon Fraser University.

Figarola, J., Skoda, S. R., Berkebile, D. R. & Foster, J. E. (2001) Identification of screwworms, *Cochliomyia hominivorax* (Coquerel)(Diptera: Calliphoridae), with a monoclonal antibody-based enzyme-linked immunosorbent assay (MAb-ELISA). *Veterinary parasitology,* **102**, 341-354.

Frederickx, C., Dekeirsschieter, J., Brostaux, Y., Wathelet, J.-P., Verheggen, F. & Haubruge, E. (2012) Volatile organic compounds released by blowfly larvae and pupae: New perspectives in forensic entomology. *Forensic Science International,* **219**, 215-220.

Gennard, D. (2007) Forensic entomology- an introduction, identifying flies that are important in forensic entomology, Wiley, England.

Gołębiowski, M., Cerkowniak, M., Boguś, M. I., Włóka, E., Dawgul, M., Kamysz, W.*,* & Stepnowski, P. (2013) Free fatty acids in the cuticular and internal lipids of *Calliphora vomitoria* and their antimicrobial activity. *Journal of insect physiology,* **59**, 416-429.

Goodrich, B. (1970) Cuticular lipids of adults and puparia of the Australian sheep blowfly *Lucilia cuprina* (Wied.). *Journal of lipid research,* **11**, 1-6.

Grassberger, M. & Reiter, C. (2001) Effect of temperature on *Lucilia sericata* (Diptera: Calliphoridae) development with special reference to the isomegalen-and isomorphen-diagram. *Forensic Science International,* **120**, 32-36.

Lane, R. P. (1975) An investigation into blowfly (Diptera: Calliphoridae) succession on corpses. *Journal of Natural History,* **9**, 581-588.

Lord, W. & Burger, J. (1983) Collection and preservation of forensically important entomological materials. *Journal of Forensic Science,* **28**, 936-944.

Marchenko, M. (2001) Medicolegal relevance of cadaver entomofauna for the determination of the time of death. *Forensic science international,* **120**, 89-109.

Martin, S. J. & Drijfhout, F. P. (2009) How reliable is the analysis of complex cuticular hydrocarbon profiles by multivariate statistical methods. *Journal of chemical ecology,* **35**, 375-382.

McDonagh, L., Thornton, C., Wallman, J. F. & Stevens, J. R. (2009) Development of an antigen-based rapid diagnostic test for the identification of blowfly (Calliphoridae) species of forensic significance. *Forensic Science International: Genetics,* **3**, 162-165.

McNair, H. M. & Miller, J. M. (2011) *Basic gas chromatography*. John Wiley & Sons.

Mendonça, P. M., dos Santos-Mallet, J. R., de Mello, R. P., Gomes, L. & de Carvalho Queiroz, M. M. (2008) Identification of fly eggs using scanning electron microscopy for forensic investigations. *Micron,* **39**, 802-807.

Midgley, J. M. & Villet, M. H. (2009) Effect of the killing method on post-mortem change in length of larvae of *Thanatophilus micans* (Fabricius 1794)(Coleoptera: Silphidae) stored in 70% ethanol. *International journal of legal medicine,* **123**, 103-108.

Moore, H. E. (2012) Analysis of cuticular hydrocarbons in forensically important blowflies using mass spectrometry and its application in post mortem interval estimations (PhD thesis). *Keele University*.

Moore, H. E., Adam, C. D. & Drijfhout, F. P. (2013) Potential use of hydrocarbons for aging *Lucilia sericata* blowfly larvae to establish the postmortem interval. *Journal of forensic sciences,* **58**, 404-412.

Moore, H. E., Adam, C. D. & Drijfhout, F. P. (2014) Identifying 1st instar larvae for three forensically important blowfly species using “fingerprint” cuticular hydrocarbon analysis. *Forensic science international,* **240**, 48-53.

Pechal, J. L., Moore, H., Drijfhout, F. & Benbow, M. E. (2014) Hydrocarbon profiles throughout adult Calliphoridae aging: A promising tool for forensic entomology. *Forensic science international,* **245**, 65-71.

Richards, C., Crous, K. & Villet, M. (2009) Models of development for blowfly sister species *Chrysomya chloropyga* and *Chrysomya putoria*. *Medical and veterinary entomology,* **23**, 56-61.

Richards, C. S., Rowlinson, C. C. & Hall, M. J. (2013) Effects of storage temperature on the change in size of *Calliphora vicina* larvae during preservation in 80% ethanol. *International journal of legal medicine,* **127**, 231-241.

Rodriguez, W. C. & Bass, W. M. (1983) Insect activity and its relationship to decay rates of human cadavers in East Tennessee. *Journal of Forensic Science,* **28**, 423-432.

Roux, O., Gers, C. & Legal, L. (2006) When, during ontogeny, waxes in the blowfly (Calliphoridae) cuticle can act as phylogenetic markers. *Biochemical systematics and ecology,* **34**, 406-416.

Roux, O., Gers, C. & Legal, L. (2008) Ontogenetic study of three Calliphoridae of forensic importance through cuticular hydrocarbon analysis. *Medical and veterinary entomology,* **22**, 309-317.

Sharma, R., Garg, R. K. & Gaur, J. (2015) Various methods for the estimation of the post mortem interval from Calliphoridae: A review. *Egyptian Journal of Forensic Sciences,* **5**, 1-12.

Shean, B. S., Messinger, L. & Papworth, M. (1993) Observations of differential decomposition on sun exposed v. shaded pig carrion in coastal Washington State. *Journal of Forensic Science,* **38**, 938-949.

Slone, D., Gruner, S. & Allen, J. (2005) Assesing error in PMI prediction using a forensic entomological computer model. *Rapport technique, US Department of Justice*.

Smith, K. G. (1986) *A manual of forensic entomology*, British Museum (Natural History), London.

Sukontason, K., Sukontason, K. L., Piangjai, S., Boonchu, N., Kurahashi, H., Hope, M., & Olson, J. K. (2004) Identification of forensically important fly eggs using a potassium permanganate staining technique. *Micron,* **35**, 391-395.

Sukontason, K. L., Sribanditmongkol, P., Chaiwong, T., Vogtsberger, R. C., Piangjai, S. & Sukontason, K. (2008) Morphology of immature stages of *Hemipyrellia ligurriens* (Wiedemann)(Diptera: Calliphoridae) for use in forensic entomology applications. *Parasitology research,* **103**, 877-887.

Tantawi, T. & Greenberg, B. (1993) The effect of killing and preservative solutions on estimates of maggot age in forensic cases. *Journal of Forensic Science,* **38**, 702-707.

Tomberlin, J. K. & Benbow, M. E. (2015) Forensic entomology: international dimensions and frontiers. CRC Press.

Trabalon, M., Campan, M., Clement, J.-L., Lange, C. & Miquel, M.-T. (1992) Cuticular hydrocarbons of *Calliphora vomitoria* (Diptera): relation to age and sex. *General and comparative endocrinology,* **85**, 208-216.

Turner, B. & Wiltshire, P. (1999) Experimental validation of forensic evidence: a study of the decomposition of buried pigs in a heavy clay soil. *Forensic Science International,* **101**, 113-122.

Upton, M. S. & Norris, K. R. (1980) The collection and preservation of insects and other terrestrial arthropods. Australian Entomological Society.

Wells, J. & LaMotte, L. R. (1995) Estimating maggot age from weight using inverse prediction. *Journal of Forensic Science,* **40**, 585-590.

Wells, J. D. & Kurahashi, H. (1994) *Chrysomya megacephala* (Fabricius)(Diptera: Calliphoridae) development: rate, variation and the implications for forensic entomology. *Medical Entomology and Zoology,* **45**, 303-309.

Williams, H. (1984) A model for the aging of fly larvae in forensic entomology. *Forensic science international,* **25**, 191-199.

Wolff, M., Uribe, A., Ortiz, A. & Duque, P. (2001) A preliminary study of forensic entomology in Medellın, Colombia. *Forensic Science International,* **120**, 53-59.

Wyss, C. & Cherix, D. (2013) Traité d'Entomologie Forensique: Les insectes sur la scène de crime. PPUR Presses polytechniques.

Xu, H., Ye, G.-Y., Xu, Y., Hu, C. & Zhu, G.-H. (2014) Age-dependent changes in cuticular hydrocarbons of larvae in *Aldrichina grahami* (Aldrich)(Diptera: Calliphoridae). *Forensic science international,* **242**, 236-241.

Ye, G., Li, K., Zhu, J., Zhu, G. & Hu, C. (2007) Cuticular hydrocarbon composition in pupal exuviae for taxonomic differentiation of six necrophagous flies. *Journal of medical entomology,* **44**, 450-456.

Zhu, G., Ye, G., Hu, C., Xu, X. & Li, K. (2006) Development changes of cuticular hydrocarbons in *Chrysomya rufifacies* larvae: potential for determining larval age. *Medical and veterinary entomology,* **20**, 438-444.