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Cuticular Hydrocarbon Analysis in Forensic Entomology: A Review

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Blowflies are the first inhabitants of decomposing remains and are therefore of forensic relevance for post mortem interval estimations. Forensic entomology is becoming widely accepted as a branch of forensic science and is being utilized more within forensic casework. This wider use has driven an increase in research being carried out within the field, in particular, in less "classical" techniques such as DNA and chemical analysis in the form of cuticular hydrocarbon analysis. This short review will examine the research currently being studied in the area of cuticular hydrocarbon analysis of forensically important Diptera for species identification and ageing.

Introduction

Forensic entomology is the study of insects that utilize decomposing remains to aid legal investigation (Amendt *et al.* 2000; Moore 2013) and its main use has been the estimation of the minimum time since death or minimum post mortem interval estimation (minPMI) of human cadavers (Hart *et al.* 2008). Although the use of insects in criminal investigations has been recorded as early as the thirteenth century (Benecke 2001), it is only in the past few decades that this branch of forensic science has gained importance and is being utilized more by police forces for scenes of homicide and abuse (Amendt *et al.* 2000). Due to the abundance of insect activity associated with decomposing remains, they offer a variety of evidence (Moore 2013). Blow flies, among the first species to exploit dead remains (Benecke 2001), can provide information ranging from how long a person has been dead (Gennard 2007) to determining whether the deceased had ingested drugs prior to death (Hart *et al.* 2008).

The quality of this minPMI evidence, and the forensic entomologist's ability to estimate time since the first fly accessed the body is dependent on accurate insect species identification, their ability to determine the age of the oldest live colonising

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larvae and their possession of a thorough understanding of entomology, in terms of the ecology and the biology of insects and their environment (Hart *et al.* 2008).

The identification of larvae is usually completed by their collection from the dead host or surrounding area and their subsequent rearing to adult flies. While larvae can be difficult to identify by species morphologically, adult flies are more readily identified via published genital keys (Adams and Hall 2003). This species identification is important because different species have different life cycle timings therefore in order to utilize the correct developmental information, species identification is first required (Ames *et al.* 2006a). The time since colonization is then calculated, which is the estimate based on the age of the most developed insects present when the body is discovered. Current methods for ageing insect specimens involve measuring the length of the larvae or by dissecting any collected pupae, and comparing the measurements to published data in relation to temperature (Hart *et al.* 2008).

In recent years, there has been an increase in developments around DNA-based techniques (Ames *et al.* 2006b; Cainé *et al.* 2009; Harvey *et al.* 2003; Malgorn and Coquoz 1999; Ratcliffe *et al.* 2003; Sperling *et al.* 1994; Stevens and Wall 2001; Vincent *et al.* 2000; Wallman and Donnellan 2001; Wells and Sperling 2001) and chemical analysis research within forensic entomology with the aim of complimenting or enhancing current techniques used within this field of study.

This review will concentrate on the analysis of cuticular hydrocarbons (CHC) from Calliphoridae and summaries findings from studies that utilize this technique for the purposes of identification and ageing within the field of forensic entomology.

Cuticular hydrocarbons (CHC)

Cuticular hydrocarbons are present within the epicuticular wax layer of insect cuticles. This hydrophobic, flexible wax layer also consists of fatty acids and alcohols (Gibbs 1998; Moore 2013). The epicuticular lipids serve a variety of roles in different insects; within social insects, such as ants, their primary function is to protect against desiccation (Blomquist and Bagnères 2010), however research has also suggested that they are used for communication (Martin *et al.* 2008). As well as preventing desiccation, the cuticular lipid layer also serves as protection against micro-organisms (Gibbs and Crockett 1998). There are a vast number of different hydrocarbons present on the cuticles of insects, with many different possible combinations. This indicates that potentially each species of insect could have its own unique chemical profile, often referred to as a fingerprint (Everaerts *et al.* 1997; Martin *et al.* 2008; Moore 2013).

The cuticular hydrocarbons of insects are generally long linear molecules, typically ranging in chain lengths of C16 to C37 for Calliphoridae species (Howard and Blomquist 2005). They can be either saturated or unsaturated and can have one or more methyl groups attached to the carbon backbone of the chain. In their saturated form, *n*-alkanes consist of single bonded carbon atoms. In their unsaturated form there can be one (alkene), two (alkadiene) or three (alkatriene) double bonds present along the carbon chain (Moore 2013).

Cuticular hydrocarbon analysis is commonly carried out using Gas Chromatography – Mass Spectrometry (GC-MS) which is a well-established technique that pro-

vides separation, identification and characterization and is widely used within chemistry and forensic science laboratories world-wide (Martin and Drijfhout 2009) particularly in social insects. These findings are wholly or partly based on multivariate statistical methods such as discriminate analysis (DA).

Cuticular Hydrocarbon analysis for species identification

As mentioned, in order to utilize the correct life cycle timings for a PMI estimation, the identification of the insect specimen must first be established. Chemotaxonomy within the field of entomology has been around for many years and there are a number of studies on cuticular chemistry, especially concerning social insects such as ants (Akino *et al.* 2004; Guillem *et al.* 2012; Martin *et al.* 2008; Tissot *et al.* 2001), bees (Lavine and Vora 2005), termites (Haverty *et al.* 1997), and wasps (Bernier *et al.* 1998). Other insects have also been well studied such as cockroaches (Everaerts *et al.* 1997), grasshoppers (Chapman and Espelies 1995) and beetles (Lockey 1991, 1992; Page *et al.* 1997). Some of these publications have successfully demonstrated how the analysis of CHCs via the use of GC-MS can provide efficient species identification. A key study was that by Guillem *et al.* (Guillem *et al.* 2012) which proposed the use of chemotaxonomy to address the challenges of morphological identification between ant species. The two chosen ant species, *Myrmica sabuleti* and *M. scabrinodis* are morphologically very similar but by analysing their chemical profile via GC-MS, the presence or absence of two species-specific compounds meant that the two species were easily distinguishable.

Amongst the aforementioned insects, CHC analysis has also been used for identification purposes of Diptera (Urech *et al.* 2005) as a complimentary technique when the taxonomical identification is not possible. Ye *et al.* (2007) were the first to publish data from the empty puparial cases of six species of necrophagous flies from China: *Aldrichina graham*, *Chrysomya. megacephala, Lucilia sericata*, *Achoetandrus rufifacies*, *Boettcherisca peregrina* and *Parasarcophaga crassipalpis*. A discriminant model yielding eight cuticular hydrocarbon compounds was constructed from the initial extracted GC-MS profiles, allowing for complete separation of the pupal exuviae from the six species. The results were very promising and led to further investigations utilising this fingerprint chemical identification approach within the field of forensic entomology.

Identifying empty puparial cases using taxonomical methods can be challenging as many of the morphological features required are damaged due to the mechanism in which an adult fly emerges from the cases, meaning identification is much more difficult (Braga *et al.* 2013).

Braga *et al.* (2013) examined the puparial cases from four species of Sarcophagidae, *Peckia (Pekia) chrysostoma*, *Peckia (Pattonella) intermutans*, *Sarcophaga (Liopygia) ruficornis* and *Sarcodexia lambens.* They were able to differentiate between the specimens using their chemical profiles and analysing them using GC-MS and Bray-Curtis similarity index for cluster analysis. This shows great potential for analysing entomological evidence when only parts of the insect specimens are available for analysis. The results also highlight the strength of this technique when applied to a family of

Diptera that are notoriously difficult to identify using morphological techniques.

Research carried out by Musah *et al.* (2015) also examined the empty puparial cases but looked at four species of Calliphoridae, specifically; *Lucilia cuprina, L. sericata*, *Cochliomyia macellaria* and *Chrysomya rufifacies*. *Musca domestica* was also added to the dataset for comparison as this species had been previously sampled by one of the authors. Rather than the typical analytical technique of choice for CHC analysis, GC-MS, Musah *et al*. utilized the rapid ambient ionization technique of Direct Analysis in Real Time – Mass Spectrometry (DART-MS). DART analysis does not typically lend itself to hydrocarbon analysis since these compounds can be difficult to detect; especially the *n*-alkanes as they do not readily protonate. This makes the analysis of this type of compound much better suited to a high energy ionization technique such as Electron Ionization (in GC-MS) in order for them to become ionized. However, with an adapted sampling technique, *n*-alkanes were able to remain stable and hold their charge, enabling them to be detected with their O_2^- adducts for the first time using DART-MS (Moore 2013). The results showed clear chemical distinctions between the analysed species, yielding a high throughput method for species identification and classification. Work from this initial experiment was continued by looking at the effect of geographical location on the cuticular hydrocarbon profiles of the same species collected from Texas and Ohio. Empty puparial cases from *C. macellaria* were collected from the two regions in North America and analysed using DART-MS. The results showed small chemical differences within the cuticular hydrocarbon profiles and by applying Linear Discriminant Analysis (LDA), their distinct clusters could be visualized enabling differentiation between geographical location (Moore 2013).

Previous work by Yew *et al.* (2008)pheromones strongly influence social behaviors such as aggression and mate recognition. In Drosophila melanogaster, pheromones in the form of cuticular hydrocarbons play prominent roles in courtship. GC/MS is the primary analytical tool currently used to study Drosophila cuticular hydrocarbons. Although GC/MS is highly reproducible and sensitive, it requires that the fly be placed in a lethal solution of organic solvent, thereby impeding further behavioral studies. We present a technique for the analysis of hydrocarbons and other surface molecules from live animals by using direct analysis in real-time (DART investigated the pheromones in the form of cuticular hydrocarbons, which play a vital role in courtship for the fly species *Drosophila*. This work was carried out on awake, behaving adults using DART and they observed differences in the chemical composition between male and female profiles by detecting unsaturated hydrocarbons. However they did not gain clear, unambiguous mass spectra from the saturated alkanes (Musah *et al*. 2015).

As stated previously, the larval stages are usually difficult to identify to species level due to morphological similarities but they can be done in 3rd instar by experienced entomologists (Szpila 2010) but it can be done as early as the 1st instar stage (Szpila *et al*. 2013). However, it is still usual practice to rear the larvae to adult flies for identification. However this can be a time consuming process when involved in criminal investigations, as identification is the first key step before the correct life cycle timings for aging can be utilized. Chemotaxonomy results from 1st in star larvae of

L. sericata, *Calliphora vicina* and *Calliphora vomitoria* was published by Moore *et al.* (2014). During this study the authors observed that the species-specific chemical profiles allow for identification to be determined in the immature stages, saving valuable time.

Cuticular hydrocarbon analysis for ageing

Once identification has been established, the next step in order to calculate the PMI estimation is to age the insect specimens present. Cuticular hydrocarbons have also been utilized in entomology for ageing insects (Brown *et al.* 2000; Brown, Morton, and Spradbery 1992; Chen *et al.* 1990; Desena *et al.* 1999; Desena *et al.* 1999; Hugo *et al.* 2006; Tregenza *et al.* 2000). Trabalon *et al.* (Trabalon *et al.* 1992) examined the cuticular hydrocarbons of *C. vomitoria* with relation to age and sex using GC-MS and Principal Component Analysis (PCA) via liquid extraction from the leg of the adult flies. Usual practice is to extract from the whole insect specimen by submerging them in hexane for approximately 15 minutes. They were able to distinguish between young and old specimens due to the composition of the chemical profiles and they were also able to distinguish between males and females by examining the sex pheromones (alkenes).

Roux *et al.* (2008) were the first to carry out a complete ontogenetical study of three forensically important blowflies (*C. vicina, C. vomitoria* and *Protophormia terraenovae*) using Gas Chromatography with a Flame Ionization Detector (GC-FID), producing results that have shown the potential to utilize the hydrocarbons for a forensic entomological application. The discrimination analysis they carried out allowed for clear differentiations between the life stages (larvae, post-feeding larvae, puparia and adult fly).

Additional work from Zhu *et al.* (2006) also showed the potential to use hydrocarbons with a forensic application by being the first to analyse the larvae of *C. rufifacies* using GC-FID and GC-MS. The study presented results which allowed for ageing to be established by adopting the simple statistical analysis of taking the peak area of nonacosane (C29:H) and dividing it by the peak area of eight other selected compounds, giving a peak ratio compared to C29:H. This ratio was seen to significantly increase with age and was followed up by modelling the results using exponential, or power functions. However, the peak ratios can vary considerably between individuals and the method of the analyst selecting the other peaks for use with the ratio can be seen as being a rather subjective means of analysis.

Where a body is found to be highly decomposed and the only surviving entomological evidence remaining are the empty puparial cases, it would be very beneficial to be able to establish the identification as well as the age to allow time since colonization estimates to be determined. As previously mentioned, empty puparial cases are difficult to identify due to key morphological features being damaged as the adult flies eclose. Since the puparial cases are the hardened cuticle of the post-feeding larvae and are not living, they do not change morphologically over time which makes them impossible to age. However with the discovery of cuticular hydrocarbons analysis, researchers have been publishing some very promising results around ageing empty puparial cases. Zhu *et al.* (2007) investigated the weathering effects of hydrocarbons

extracted from the empty puparial cases of *C. megacephala*. During the study they used GC-MS to analyse the hydrocarbons and significant changes to the abundances of the compounds in relation to weathering time was observed. Interestingly, the abundance of the even numbered low molecular weight *n*-alkanes (C22:H, C24:H, C26:H) increased with time which could allow for an approximate age to be established up to 90 days. This was initially carried out under controlled conditions in the laboratory (Zhu *et al.* 2007) but was then repeated in a field experiment to validate the results (Zhu *et al.* 2013). Once again they reported that the chemical profiles extracted from the empty puparial cases of *C. megacephala* changed significantly during the 90 day weathering process and of the 106 compounds detected, 104 decreased significantly in abundance in correlation to the weathering time. However, in comparison to the lab data, they found the changes for the field study to be much greater, suggesting that cuticular hydrocarbons are significantly influenced by environmental factors. Zhu *et al.* (2017) continued field validation of empty puparial cases with a different species of forensic importance; *C. rufifacies*. This was another good example where they examined the effect of natural weathering for a characterization experiment in order to assess the chemical composition changes over time. They presented data for 50 days of weathering time with some in-depth statistical analysis which overall showed a decrease in the hydrocarbon abundances with time, modelled with a modified exponent function. As reported by Zhu in a previous paper (Zhu *et al.* 2007), they once again state that the weathering effects are correlated to the different cuticular hydrocarbon classes, with *n*-alkanes yielding relatively low weathering rates and methyl branched alkanes and alkenes showing relatively high weathering rates (Zhu *et al.* 2017). They also reported that the hydrocarbons weathered more rapidly in comparison to laboratory controlled datasets, again suggesting the importance of the influence of additional environmental factors on the stability of these cuticular compounds.

Further research on the estimation of age from puparial cases was carried out by Moore *et al.* (Moore *et al.* 2017) who published data from the empty puparial cases of *C. vicina* and *L. sericata* over a period of 9 months. They used GC-MS analysis and non-metric multidimensional scaling analysis (NMDS) permutational multivariate analysis of variance (PERMANOVA) and random forest models were applied to the hydrocarbon datasets to examine any chemical changes occurring in relation to time. This study was successful in establishing age differences and is the first of its kind to age empty puparial cases to this time frame. However, although these were preliminary results which showed great potential, it must be repeated in the field to reflect the weathering changes modelled by other researchers.

Two studies have specifically examined chemical changes with time within larvae, enabling the age to be established for *L. sericata*. These studies utilized the techniques of both PCA (Moore *et al.* 2013) and Artificial Neural Networks (ANNs) (Butcher *et al.* 2013). This was not the first time ANNs were used in the field of entomology but it does describe the first use of ANNs for the analysis of hydrocarbons of Diptera for PMI estimations (Butcher *et al.* 2013). Like PCA, ANNs are capable of identifying trends within datasets but they work on a training and learning basis. Once trained,

ANNs can reduce the analysis time required by clarifying novel data based on their knowledge of the domain that they have acquired during training (Moore 2013). The results showed significant promise for ageing the larval stages and this was replicated by a study from Moore *et al.* (Moore *et al.* 2016), successfully ageing *C. vomitoria* and *C. vicina* larvae.

Pechal *et al.* (2014) investigated the use of cuticular hydrocarbons for the chemical ageing of adult flies. The CHCs were examined for up to 30 days post-emergence of *C. macellaria* and *C. rufifacies*. Non-metric multidimensional scaling analysis and permutational multivariate analysis of variance were applied to the hydrocarbon dataset with significant differences detected amongst post-emergence age groups for each species. This was followed by a study carried out by Moore *et al.* (2017) on adult flies up to the same age (30 days) but focussing on *L. sericata*, *C. vicina* and *C. vomitoria* using ANN analysis. This was a proof of concept study which developed a method of determining the age of post-emergence adult blow flies by examining their chemical profiles. However, they found that self-organising map (SOM) was able to classify each of the adult fly ages with very promising results, achieving 100% classification accuracy in the case of the *L. sericata*, and at least 70% classification accuracy for *C. vicina* and *C. vomitoria* (Moore *et al.* 2017).

Cuticular hydrocarbon analysis is in its early stages of research within the field of forensic entomology and it is important that the cuticular chemistry of all forensically important Calliphoridae are studied so they can be used as a base line reference. Braga *et al.* (2016) analysed the CHC from male and female *Chrysomya putoria. They used GC-MS and* cluster analysis with Bray-Curtis measure of the relative abundances, demonstrating that it is possible to differentiate the ages of both sexes using CHC profiles for up to 5-day-old flies.

Conclusion

The research mentioned in this review briefly summarizes the work currently being carried out using cuticular hydrocarbons within the area of forensic entomology. There is a lot of potential for this work to be utilized for identification, especially in the immature life stages or with Diptera that is more difficult to identify using morphological criteria (e.g. Sarcophagidae). But its real potential becomes clear in relation to ageing. Ageing the post-feeding stage to the day is something that has always been challenging as the larvae do not change in size. Cuticular hydrocarbons shows good accuracy for this life stage as well as adult flies and empty puparial cases which currently cannot be aged using any other technique.

The next step for this field is to replicate the published lab studies and validate this technique by observing the chemical profiles when exposed to environmental factors as this has been reported to alter the ageing models.

About the authors

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Sue Shemilt is currently working in the fields of Chemical Ecology and Forensic Science at Keele University as both a PhD student and Teaching Fellow respectively. Her PhD is in the biosynthesis of cuticular hydrocarbons in British ant species, whilst her teaching areas focus on forensic imaging and photography.

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