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Analysis of the mandibular pheromone of living honeybee queens using non-destructive sampling techniques

By

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Supervisor
Prof. E.R. Rohwer

Co-Supervisors
Prof. R.M. Crewe
Dr T.C. Wossler

Submitted in partial fulfillment of the requirements for the degree of

Master of Science
Chemistry

In the Faculty of Natural and Agricultural Sciences
University of Pretoria
Pretoria

Declaration

I, Monyadiwa Martha Masemene, declare that the dissertation submitted for the degree MSc in Chemistry at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Monyadiwa M. Masemene

…… ……
Signed Date

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ABSTRACT

Honeybee queens produce a number of pheromones that influence the behaviour and physiology of worker bees. The mandibular gland secretion of queens, the major pheromone source, suppresses the formation of emergency queen cells, worker reproduction and coordinates the social organisation of the

colony.

A study of analytical procedures for honeybee queen mandibular gland pheromone was undertaken, with the aim of doing multiple analyses of the same individual over a period of time. Attention was given to developing new non-destructive sampling methods that would help to characterize signal changes.

This study involves the characterisation of non-destructive sampling devices that are highly selective and sensitive towards extraction of mandibular pheromone. Two polymer based sampling techniques, solid phase micro extraction and silicone rubber tubing, compatible with gas chromatography were studied. A solvent extract, of mandibular pheromone was analysed by gas chromatography (GC) and employed as a tested reference method for the two newly developed techniques.

Direct sampling with solid phase micro extraction fibres at the glandular openings at the base of the mandibles is a non-destructive method that met our objectives. Mandibular gland secretions from living honeybee queens were sampled with polar and non-polar fibres. Non-polar fibres were saturated with Bis(trimethylsilyl)triflouroacetamide (BSTFA) prior to mandibular pheromone extraction. Treatment of the polymer devices with derivatising agent enhances extraction of polar components of the mandibular pheromone. BSTFA saturated non-polar fibres with a low-polarity column gave consistent results compared to polar fibres with a mid-polar column.

The results confirmed that the solid phase micro extraction technique is a sensitive and non-destructive method that can ideally be used to analyse insect secretions particularly in tracking temporal changes in the secretion composition during an individual's life.

Silicone rubber tubing consisting of polydimethylsiloxane was explored as an alternative sampling technique for pheromones from living individuals. Prepared One cm long silicone rubber tubing was saturated with BSTFA prior to mandibular pheromone extraction to enhance extraction of polar components. Preliminary studies done on mandibular pheromone standards sampled with this method showed promising results. However, queen mandibular secretion analyses were characterized by low recovery of pheromonal compounds.

The new polymer based techniques that we employed isolated the mandibular pheromones from living honeybee queens directly from the mandibles. The pheromonal components of the mandibular gland secretion were successfully analysed.

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CHAPTER 1

INTRODUCTION

The honeybee colony

Honeybees are social insects that live in large family groups called colonies (Winston & Slessor, 1992). A single fertile female, the queen, is the only egg layer and regulates colony activities. Members of the colony are the queen, the drones and the worker bees. The individuals have special tasks and activities, all of which produce a well co-ordinated, integrated colony. This colony integration and coordination depend largely on the pheromones secreted by the queen (Plettner *et al*., 1995).

The honeybee queen

There are two female castes in a honeybee colony; the queen and several thousand workers. The queen is normally the only sexually productive female in the colony (Winston & Slessor, 1992; Plettner *et al*., 1995). Queen determination occurs through selective feeding of female larvae. The nurse honeybees decide which larvae to rear as a queen by feeding them differentially with royal jelly (Corona *et al*., 1999).

Some of the queen pheromone compounds are not volatile enough to be perceived from a distance and thus the workers need to contact the queen to become aware of less volatile pheromones (Velthuis, 1985; Free, 1987). To achieve this, the queen secretes an array of pheromones that attract the workers to form a royal court or retinue around her (Velthuis & Els, 1964; Slessor *et al*, 1989; Kaminski *et al*., 1990; Naumann *et al*., 1991; Winston & Slessor, 1997). Retinue bees pick up queen pheromone as they groom, lick and antennate her, see figure 1.1. These attendants or messenger bees then move around the colony transferring the pheromone by way of worker-to-worker contacts through mouthpart and antennal contacts as well as depositing some pheromones on the comb (Velthuis & Es, 1964; Free, 1987; Naumann *et al*., 1991; Winston & Slessor, 1997).

Figure 1.1. The royal court or retinue formed around the queen as workers are licking and antennating her.

Pheromones

Social insects, such as honeybees, rely on pheromones for colony organization, defence, control of reproduction and division of labour (Plettner *et. al*., 1996). A pheromone is a mixture of chemicals secreted to the outside from the exocrine gland of an individual and received by the second individual of the same species (Free, 1987). Pheromones are multicomponent blends that carry chemical messages, which facilitate communication among members within a colony (Free, 1987). Signal economy has given rise to a single pheromone having more than one function by either eliciting an immediate behavioural response (releaser function) or initiating physiological changes (primer function) in the recipients (Brockmann *et al*., 1998). For example the major component of queen mandibular pheromone 9-oxodecenoic acid (9-ODA), inhibits the ovarian activation of workers (Velthuis, 1970; Hepburn et al., 1991), attracts drones during mating and elicits retinue behaviour (Crewe & Velthuis 1980, Velthuis 1985; Slessor *et al*., 1988; Plettner *et al*., 1996).

It is apparent that compounds that convey information over a distance between organisms must be reasonably volatile and capable of inducing a response at very low concentrations. Some of the pheromones are volatile chemicals that can evaporate and easily diffuse through the air where they can be detected by other insects at a distance. Highly volatile components with low molecular weights are transmitted in air, while the heavier less volatile pheromones as previously mentioned, are adsorbed on the body surface and are passed by direct bodily contact or during food transfer (Free, 1987; Naumann *et al*., 1991).

Mandibular glands

Mandibular glands are well developed exocrine glands found in both honeybee queens and workers, but are almost completely reduced in the drones (Plettner *et al*., 1995). The mandibular glands are located in the bee's head at the base of each mandible (see figure 1.2) and are the source of the mandibular gland pheromone. The mandibular glands are relatively uniform in structure throughout the castes but differ in their chemical constituents (Winston, 1987; Plettner *et al*., 1995). The most common compounds secreted in the mandibular glands are fatty acids, alcohols, esters and hydrocarbons (Velthuis, 1985; Plettner *et al*., 1995, 1996).

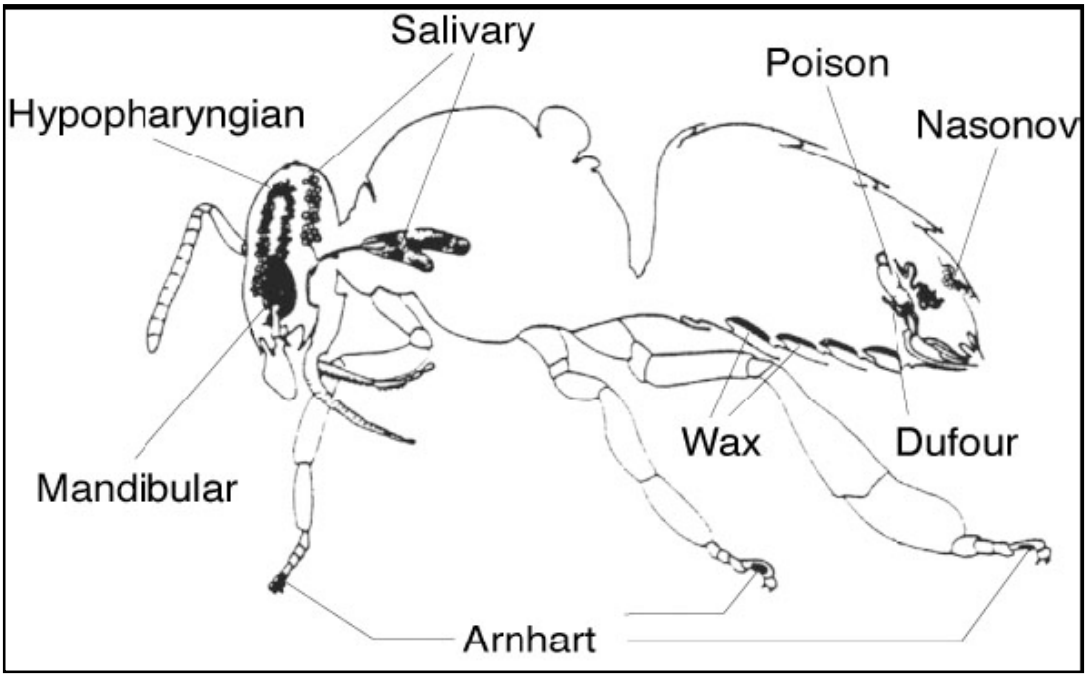


Figure 1.2 Gland systems in worker bees (from Winston, 1987)

Mandibular gland secretions

The most thoroughly studied pheromones of the queen honeybee are the mandibular gland pheromones because they are considered as the major source of pheromones that regulate social behaviour (Winston *et al*., 1982; Velthuis, 1985; Crewe, 1988 Plettner *et al*., 1995, 1996). The differences in mandibular gland components between honeybee castes have attracted scientific interest because of their functional differences and structural similarities.

Composition of mandibular gland secretions

Mandibular gland secretion is mainly composed of fatty acids ranging from 6-10 carbon atoms (Velthuis, 1985; Plettner *et al*., 1995, 1996). Synthesis of some of the compounds is not caste specific while others appear to be unique to a specific caste (Plettner *et al*., 1995). Crewe and Velthuis (1980) demonstrated that queenless *Apis mellifera (A.m.) capensis* honeybee workers could synthesize the 'queen substance' 9-oxodec-2-enoic acid (9-ODA) in their mandibular glands. The potential of these workers to imitate the queen signal demonstrated caste plasticity in the biosynthesis of mandibular gland components. Queens similarly can synthesise worker substance 10-hydroxydec-2-enoic acid (10-HDA) (Winston & Slessor, 1997). The biosynthetic capability of the two castes is apparently similar, but differs in biosynthetic selectivity (Crewe & Velthuis, 1980; Crewe, 1988; Plettner *et al*., 1996; Winston & Slessor, 1997).

The biological activity of queen mandibular gland pheromone depends mostly on a five-component blend (figure 1.3) of aliphatic acids 9-ODA and (+/-)-9-hydroxydec-2-enoic acid (9-HDA), and two phenols methyl *p*-hydroxybenzoate (HOB) and 4-hydroxy-3-methoxyphenylethanol (HVA) (Slessor *et al*., 1989, 1992; Naumann *et al*., 1991; Engels *et al*., 1996; Plettner *et* al., 1996; Moritz *et al*., 2001). 9-ODA is the major component of the queen secretion and 9-HDA is the second most abundant constituent and is produced in the form of two isomers. Worker mandibular gland secretions are characterised by the presence of two hydroxy acids, 10-HDA and 10-hydroxydecanoic acid (10-HDAA) (Crewe & Velthuis, 1980; Plettner *et al*., 1995, 1996; Moritz *et al*., 2001).

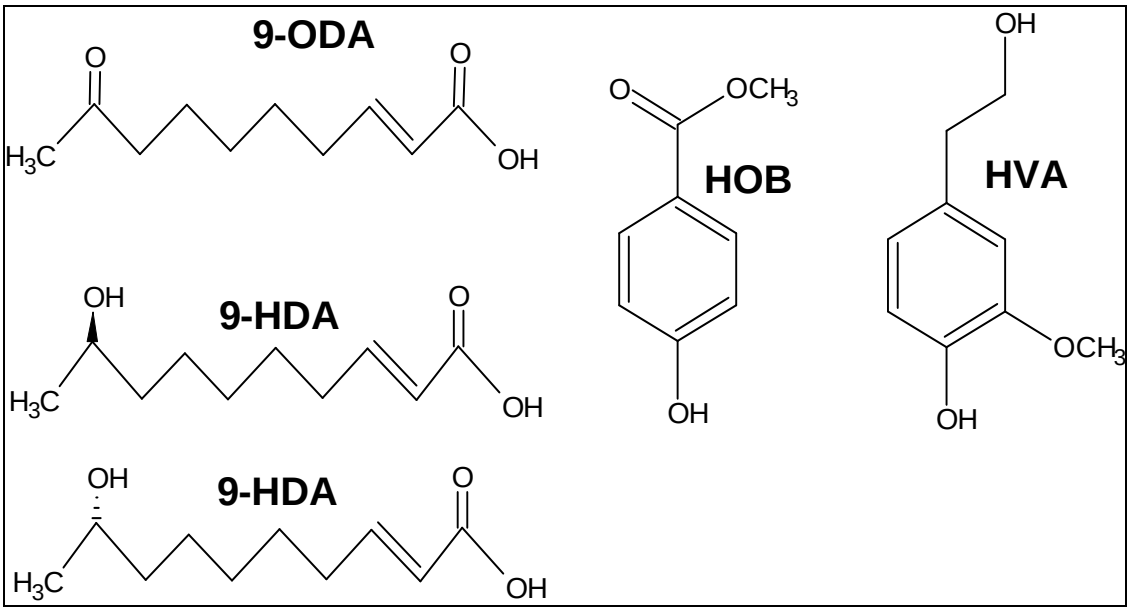


Figure 1.3. Biologically active components of the queen mandibular gland pheromone. (9-ODA= (*E*)-9-keto-2-decenoic acid; 9-HDA=(R, E) (-)-9-hydroxy-2-decenoic acid; HOB=Methyl *p*-hydroxybenzoate; HVA=4-Hydroxy-3-methoxyphenylethanol).

Functions of mandibular gland secretions

Queen mandibular gland secretions function as a releaser pheromone in attracting workers to form retinues, eliciting swarm clusters, attracting mates and inhibititng queen rearing (Velthuis & Es, 1964; Crewe & Velthuis, 1980; Slessor *et al*., 1989; Naumann *et al*., 1991; Winston & Slessor, 1997). However, besides having releaser effects, mandibular pheromones also have primer effects in influencing worker physiology, colony organization and division of labour (Free, 1987; Winston & Slessor, 1997; Moritz *et al*., 2001). The relative quantities of individual components present in the queen mandibular pheromone may vary but all five components are needed to elicit a full range of worker responses (Plettner *et al*., 1996; Winston & Slessor, 1997). However 9-ODA alone is sufficient to attract drones for mating (Crewe & Velthuis 1980, Velthuis 1985; Plettner *et al*., 1996).

Sampling techniques

A principal challenge in pheromone research is to improve the ways in which pheromones can be sampled for analysis since pheromones are secreted in minute quantities (Jones, 1999). The principle of sampling processes is to allow partitioning of the analyte between the extracting phase and the sample matrix followed by desorption of extracts into an analytical instrument for analysis. Since mandibular gland pheromones are important in honeybee communication, the sampling techniques used for the analysis of their chemical components are of

interest to us. Solvent-free sampling avoids possible contamination of the sample and therefore is ideal for isolation of minute biological samples such as exocrine secretions (Pawliszyn, 1990; Maile *et al*., 1998). Two different non-destructive polymer based techniques; solid phase micro-extraction and silicone rubber tubing, were tested to isolate components of the mandibular gland pheromone in this study. Non-polar solid phase micro-extraction and silicone rubber tubing techniques involve the trapping of analytes onto a polymer extracting phase, polydimethylsiloxane (figure 1.4). To enable the fibres to extract fatty acids in mandibular gland secretion the fibres were saturated with derivatising agent by exposing their extracting phase to the vapour of BSTFA {(bis-trimethylsilyl) triflouroacetamide} at room temperature.

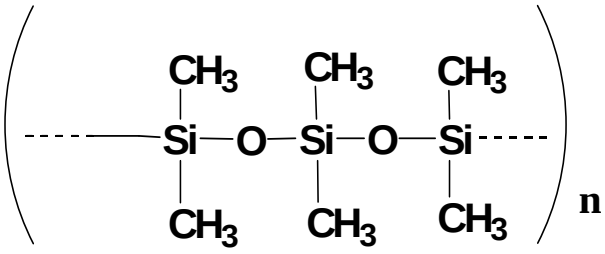


Figure 1.4 Polydimethylsiloxane structure, the stationary phase in both silicone rubber tubing and non-polar solid phase micro extraction fibres (Bartelt 1998; Pawliszyn, 1995).

Analytes present in trace amounts such as chemical components of the mandibular pheromone need to be enriched before the analysis step so that they can be obtained in detectable amounts. The choice of the sampling procedure is determined by the aim of the study. The sampling procedure depends on a number of aspects such as the sample matrix, availability of analytical instruments, sensitivity, selectivity and reliability of the sampling method.

Gas chromatography

Gas chromatography (GC) is a valuable technique for the separation of a mixture into its component compounds. The compounds must be volatile and thermally stable at high temperatures. The volatile character of pheromones makes gas chromatography an ideal technique to characterise, identify and quantify them. The introduction of capillary columns with high resolving capacities has aided pheromone research with complex mixtures of compounds adequately separated (Crewe, 1988; Maile *et al*., 1998; Jones & Oldham, 1999). The most common conventional GC detection method used in pheromone analysis is the flame ionisation detector (FID). FID has high sensitivity and linear response over a wide concentration range therefore it makes it ideal for studying compounds encountered in pheromone research (Jones & Oldham, 1999).

Derivatisation

Gas chromatography separates volatile compounds that are thermally stable at high temperatures, while those that are thermally labile decompose during analysis. Some pheromone components have high molecular weights or contain polar functional groups that cannot be readily analysed by gas chromatography. These components when analysed with gas chromatography either tail badly, or are not sufficiently volatile or are strongly attracted to the stationary phase and therefore never get detected (Pawliszyn, 1990; Jones & Oldham 1999; Maile *et al.*, 1998).

Non-volatile or thermally unstable compounds need to be treated with suitable derivatising agents in order to produce a volatile and thermally stable derivative (figure 1.5). Silylation with BSTFA is the most widely used derivatisation procedure for enhancing gas chromatographic performance of polar components of the mandibular gland (Crewe, 1988; Slessor *et al.*, 1989). It involves the replacement of an acidic hydrogen on the polar compound (**A,** see figure 1.5) with an akylsilyl group trimethylsilyl [$Si(CH_3)_3$]. Silyl derivatives (**B,** see figure 1.5) are generally less polar, more volatile and thermally stable hence easier to chromatograph (Pawliszyn, 1990)

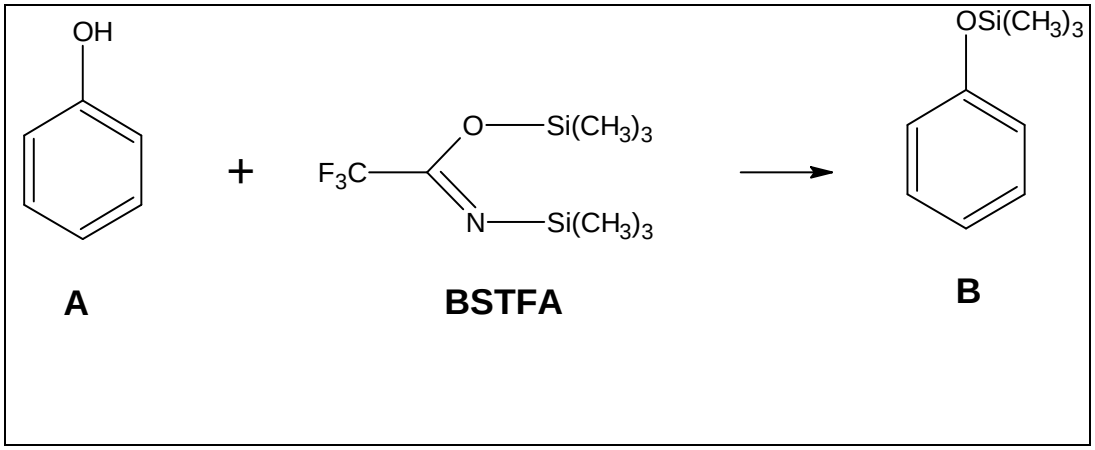


Figure 1.5 Derivatisation steps illustrating the blocking of active hydrogen in polar compounds by BSTFA. A = phenol, B = trimethylsilyl derivative of phenol.

Derivatisation for gas chromatographic separation is used for a variety of reasons but the principal ones are:

1. To increase volatility and to reduce polarity of compounds such as acids, phenols and some alcohols;

2. To reduce thermal degradation of the sample by increasing their thermal stability;

3. To enhance extraction efficiency,

4. To improve separation and to reduce tailing of polar compounds.

Why study honeybees?

Honeybees play a significant role in agriculture. They are estimated to pollinate a large percentage of deciduous fruits, vegetables, legumes and oil seed crops. Many species of wild pollinators have disappeared from the land as their habitats have been destroyed or altered by humans. Consequently, the honeybee has become the chief pollinator of many of the wild plants that remain; its ecological value in this regard is significant. In South Africa *Apis mellifera capensis* and *Apis mellifera scutellata* are used for crop pollination.

Motivation of the study: *Apis mellifera capensis* invasion

There are two existing honeybee races in South Africa, *Apis mellifera capensis* and *Apis mellifera scutellata* (Hepburn & Crewe, 1990; Wossler *et al.*, 2000). *Apis mellifera capensis* are Cape honeybees commonly known as the *"capensis"* bees. Relocation of the *"capensis"* bees from their natural site along the south-west and south coast of South Africa resulted in *"capensis"* bees invading neighbouring colonies of the African honeybee, *A. m. scutellata.* Once the colony has been invaded the *"capensis"* bees start to lay eggs and reproduce, the host queen is lost, colony productivity declines and eventually the invaded colony slowly dies (Hepburn & Allsopp, 1994; Beekman *et al*., 2000). Worker bees produce chemical signals that are different from those of queen in quality and quantity. However *"capensis"* workers can produce queen-like signals in the

absence of the queen (Hepburn & Crewe, 1990; Crewe, 1988; Hepburn, 1992; Allsopp & Crewe, 1993; Winston & Slessor, 1997). This development is very rapid when the *"capensis"* workers are placed in the company of *scutellata* (Hepburn & Crewe, 1990; Allsopp & Crewe, 1993). The invasion of the colonies by *"capensis"* bees is a major problem facing commercial apiculture.

This study focused on the queen mandibular secretion since these pheromones are essential in regulating various aspects of colony organization and integration. The reproductive dominance of the queen is also mediated through the mandibular gland pheromones. Examining the characteristics of the invaded queens' mandibular secretions will offer insights into the pheromone milieu of the host colony. Studies testing the relationship between invasion and queen mandibular pheromone needed to be done with new sampling techniques that would allow repetitive sampling of the same individual over a period of time, such that mandibular pheromone analysis could be done before, during and after invasion. Knowledge and better understanding of queens' mandibular secretions can help researchers and beekeepers to apply synthetic pheromone to control the *"capensis"* invasion while enhancing queen survival, crop pollination, commercial queen rearing and successful beekeeping. The conservation of honeybees, both wild and managed, is in our best interest.

AIMS AND APPROACH

The mandibular gland pheromone is one of the most important pheromones in the honeybee's life and small changes in its chemical composition can have an effect on the behaviour of worker bees towards the queen (Jones, 1999). An understanding of the chemical changes that occur in the queen's mandibular gland secretion when she ages, gets mated as well as certain colony effects like swarming and more recently, the invasion by *"capensis"* bees is necessary. A full understanding of the complex chemistry of the mandibular secretion will require analysis of its volatile emission before, during and after such activities to be monitored and studied.

Early isolations of queen mandibular gland pheromones were done by dissecting the heads of a number of queens and extracting the secretion with organic solvents (Crewe, 1988; Slessor *et al*., 1989; Maile *et al*., 1998; Jones, 1999). These classical methods of solvent extraction can lead to contamination by the solvents that will make it difficult in distinguishing between compounds produced by the bees versus those introduced with the solvent (Birch, 1982).

This study was aimed at providing an alternative to existing classical methods by utilising new non-destructive sampling techniques. The main aim of the study was to explore sampling techniques that can sample the mandibular gland secretion

from living honeybee queens over a period of time. The sampling technique had to be characterised by a number of criteria:

1. Gentle, non-invasive sampling of insects (honeybees) for collection and retention of mandibular pheromones.

2. Provision for multiple extractions of the same individual so that temporal changes in the mandibular gland pheromone can be tracked.

3. Compatibility with gas chromatographic analysis.

4. Satisfactory sample recovery.

The presentation of this dissertation is as follows;

In chapter 1, I briefly introduced the honeybee and discussed the importance of mandibular gland secretions. I also introduced the sampling and analytical techniques to be used in the subsequent chapters.

Solid phase micro extraction is reviewed as a sampling technique for mandibular pheromone analysis in chapter 2. The development of an alternative sampling technique for the analysis of mandibular pheromone using silicone rubber tubing is discussed in chapter 3. Chapter 4 discusses the reliability of the new techniques with reference to solvent extraction methods. Chapter 5 is a general

discussion of the performance of the sampling techniques used to sample mandibular gland pheromone.

CHAPTER 2

ANALYSIS OF THE MANDIBULAR GLAND PHEROMONES OF LIVING HONEYBEE QUEENS USING SOLID PHASE MICRO EXTRACTION TECHNIQUES

SUMMARY

The current method of choice for the isolation of an insect pheromone is by rinsing the insect's glands or the whole body in organic solvent. This method has various drawbacks such as contamination and long preparation time. The main objective of the study was to use a non-destructive sampling technique to analyse the chemical components of the mandibular glands from living honeybee queens. Direct sampling with solid-phase micro-extraction fibres at the glandular openings at the base of the mandibles is a non-destructive method that met our objectives.

Mandibular gland secretions from living honeybee queens were sampled with polar and non-polar fibres. Non-polar fibres were saturated with BSTFA prior to extraction from the mandibles. Mid-polar and low-polar columns were used for analysis. Factors that were known to affect the performance of solid-phase

micro-extraction were optimised using mandibular gland standards. In the present study, I report a new technique based on a sorbent coated fused silica fibre which we successfully applied to the analysis of mandibular gland pheromones.

INTRODUCTION

Pheromone signals in social insects serve social functions like alarming members against predators, recognising colony members, attraction of mates, etc (Free, 1987; Malosse *et al*., 1995; Plettner *et al*., 1996 Winston & Slessor, 1997). Queen mandibular pheromone has a major influence on the organization of honeybee societies but our understanding of mandibular pheromone chemistry has been hampered by shortcomings in analytical techniques that are borrowed from other fields of analysis (Crewe, 1988).

Solid phase micro-extraction is a potential solvent free technique for the study of airborne insect pheromones (Pawliszyn, 1997, Bartelt, 1997). Collection of volatiles using solid phase micro-extraction more accurately characterises the ratios of compounds secreted and present in the vapour phase. However, different affinities (attraction) and chemical properties of the compounds for the adsorbent during collection and concentration may alter the ratios (Malosse *et al*., 1995).

Importantly solid phase micro-extraction adsorbs only those analytes that are actually released because it extracts only those compounds secreted onto the surface of the cuticle. Through touching and rubbing of the mandibles the sample is directly collected on the fibre. The solid phase micro-extraction process has two steps i.e. partitioning of the analyte between the coating and the sample matrix, followed by desorption of extracts into an analytical instrument. In the first step the fibre is exposed to the sample and target analytes partition from the sample matrix into the coating (Berg, 1992; Pawliszyn, 1997). After extraction, the fibre with the concentrated analytes is inserted into the gas chromatograph for desorption where separation of components takes place.

The selection of solid phase micro-extraction fibre coatings is based primarily on polarity and volatility differences of the target analytes. Since solid phase micro-extraction is intrinsically more sensitive to less volatile compounds it can be an advantage in insect behavioural research because highly active, heavier compounds such as pheromones are secreted at very low levels. Solid phase micro-extraction has been used to investigate chemical communication in several insect species (Malosse, *et al*., 1995; Jones & Oldham, 1999; Sledge *et al*., 20300, Crewe *et al*., 2004).

Mandibular gland extracts of queens and workers are characterised by several classes of compounds but are mainly composed of phenols and C6 –C10 aliphatic acids and diacids (Crewe & Velthuis, 1980; Velthuis, 1985; Plettner *et*

al., 1995, 1996). Since most compounds of the queen mandibular pheromone contain polar functional groups, which are not readily analysed by GC because they tend to adsorb onto the column, Crewe & Velthuis, (1980) introduced a derivatization technique that advanced chemical analysis of mandibular gland extracts.

The chemical structure and nature of the compounds to be separated will determine the choice of derivatising reagents that can be used for gas chromatographic analysis. There are two possible ways to perform derivatisation on solid phase micro-extraction fibres (SPME). Derivatisation can be carried out directly in the fibre coating of the solid phase micro-extraction device by exposing a fibre containing the derivatising reagent to the sample, whereby the fibre coating acts as an organic medium for the microreaction. An alternative way is to perform postderivatisation following extraction of target analytes on the fibre (Lin *et al*., 1995; Lin & Pawliszyn, 1997).

The objective of this work was to isolate chemical components of both the synthetic mandibular pheromone and the mandibular pheromone of living honeybee queens using a non-destructive and selective technique; solid phase micro-extraction (Pawliszyn, 1997).

MATERIALS AND METHODS

Silylation of glassware

All the glassware used in this work was initially deactivated with dichlorodimethylsilane (DMDCS) (Supelco, Bellefonte) solution before being used. The glass surface was washed with dichlorodimethylsilane for 15 seconds, rinsed twice with toluene (Merck, Germany) and three times with methanol. The glass was dried under a nitrogen stream. This treatment reduces adsorption of analytes on the glass surface (Bartelt, 1997). Adsorption of analytes on glass surface is a potential cause of quantitative errors and a complete loss of analytes at trace level. It was previously reported that glass without silanised surfaces gave low or erratic results (Bartelt, 1997). The disadvantage of this glass deactivation is that the silicone contaminants can impair the analysis if liberated from the glass surface. After silanisation the glassware was used immediately.

Preparation of synthetic mandibular gland pheromone

A mandibular gland standard was prepared by accurately weighing approximately 1 mg of all listed chemicals in Table 2.1. into 4 ml of dichloromethane. The standard mixture was kept in a refrigerator at low temperatures until ready for analyses.

Table 2.1. Chemical compounds used to prepare the mandibular pheromone standard. The masses given are for a particular standard mixture used in this study.

Chemical compounds	**mg**
9-Oxo-2-decenoic acid	1.048
10-Hydroxydecanoic acid	1.062
Methyl *p*-hydroxybenzoate	1.080
10-Hydroxy-3-methoxyphenylethanol	0.996
Oleic acid	1.100
Hexadecanoic acid	1.068
Dodecanoic acid	1.109
Benzoic acid	1.034
Octanoic acid	1.042
Tetradecane	1.092
Palmitoleic acid	1.072

SPME fibres

To investigate the effect of fibre coatings two different types of fibre coatings (mixed and non-polar) were evaluated for mandibular gland pheromone analysis. The mixed phases fibres were Carboxen-polydimethylsiloxane (Carboxen), and Carbowax-divinylbenzene (CW/DVB); and the non-polar fibres were polydimethylsiloxane, of different thickness (7 µm and 100 µm), all supplied by Supelco. SPME fibres were conditioned according to the supplier's (Supelco, Bellefonte) manual before use. Blanks were run between analyses to check that fibres are still working efficiently and that there were no traces of memory effects. The fibres were thermally cleaned daily after use.

Polydimethylsiloxane (PDMS) fibres

This is a non-polar phase and it is usually used to extract non-polar compounds. It can extract polar analytes if extraction conditions are optimised, for example by adding a derivatisation agent. Two PDMS films of different thickness (100 µm and 7 µm) were used to extract mandibular gland pheromones of honeybee queens. PDMS fibres have coatings that are able to withstand high injector temperatures up to 300°C (Alpendura, 2000). Five virgin queens were sampled with 100 µm and 4 virgin queens were sampled with 7 µm PDMS fibre.

Fibres with mixed phase coatings

Mixed phases of 75 µm Carboxen-PDMS and 65 µm Carbowax-divinylbenzene coated on fibres were used to extract underivatised mandibular pheromone. Carboxen fibres are primarily used for extraction of volatiles at trace level and CW / DVB is used for extraction of more polar volatiles such as alcohols. The mixed phase fibres were used to complement PDMS fibres. One fibre from each coating was used. 4 virgin queens were sampled with each fibre.

Headspace on-fibre derivatisation using non-polar fibres (PDMS fibres)

Fibres to be used for on fibre derivatisation were prepared by placing 1 ml BSTFA (Sigma, Germany) into an 8 ml sample vial sealed with caps and Teflon-coated septa see figure 2.1 below. BSTFA was used without further purification. Headspace loading of the derivatizing agent into the fibre coating was performed by piercing the septum with the solid phase micro-extraction needle and exposing the polydimethylsiloxane (PDMS) fibres to the vapour of BSTFA at room temperature for five minutes. The BSTFA impregnated fibre was subsequently used for extraction of the mandibular pheromone standard or queen mandibular pheromone with resultant on-fibre derivatisation.

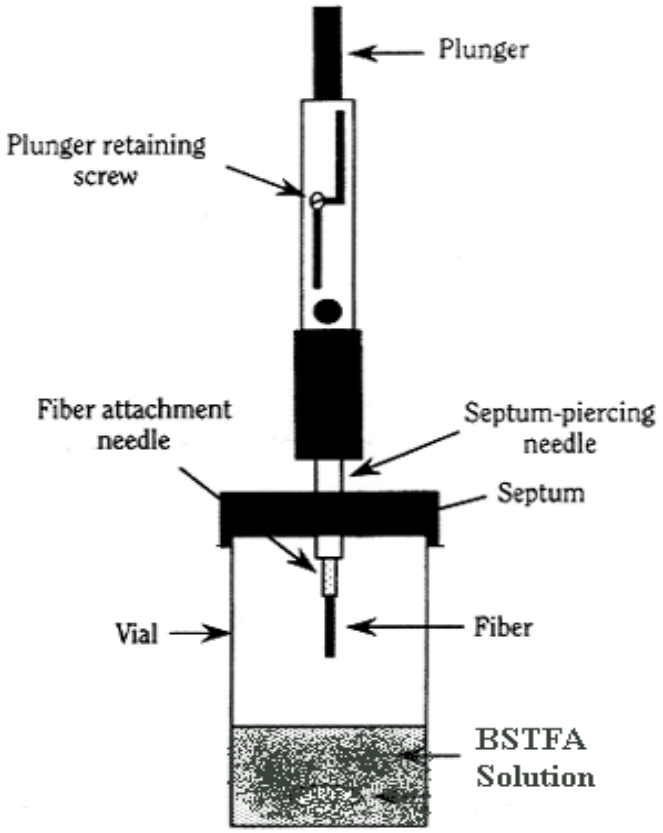


Figure 2.1 Apparatus used to prepare for headspace derivatisation of PDMS-coated fibers (Supelco catalogue).

Extraction of mandibular pheromone

SPME fibres were used to extract synthetic pheromone and queen mandibular pheromone as indicated below.

a) Synthetic pheromone

A sample of 10 µl of the mandibular pheromone standard solution was placed onto a silanised glass slide. The solvent was allowed to evaporate completely leaving the solutes deposited on the slide. The surface of the slide coated with synthetic pheromone was rubbed with a fibre for three minutes. The fibre with the concentrated analytes was then desorbed for five minutes in the hot injector of the gas chromatograph for analysis.

b) Queen mandibular pheromone

The virgin queens were reared in our laboratory and kept with a few worker bees in wooden Liebefeld cages. The bees were given fresh sugar water on a daily basis and pollen (Hässlers, Cullinan). Prior to trapping of the mandibular pheromone, the whole body of the queen was trapped in a plastic tube to immobilise her during the manipulation. A solid phase micro-extraction fibre was rubbed on the mandibles of a living queen for exactly five minutes. Followed by

five minutes desorption in the hot injector of the gas chromatograph for analysis.

Optimum extraction times for both synthetic and queen mandibular pheromones were determined by varying the extraction time from 1 to 5 minutes. Optimal 3-minute extraction times were used for synthetic pheromone while living queen mandibular pheromones were sampled for 5-minute extraction times. Each application (synthetic pheromone and queen mandibular pheromone) required a different optimal sampling time. Since pheromones are secreted in low concentrations it was necessary to extract the queen for a longer period of times in order to obtain good recovery of the compounds of interest. All the extractions were performed at room temperature.

Before the analysis of queen mandibular pheromone several types of solid phase micro extraction adsorbents were evaluated using the prepared mandibular pheromone standard as a reference.

Chromatographic analyses

Analysis of samples was accomplished on two gas chromatographs with columns of different polarities. The non-polar GC column stationary phases are generally preferred for the analysis of insect pheromones because of their thermal stability

and long lifetimes. However polar phases are sometimes used if the separation obtained with the non-polar phases is not satisfactory (Jones & Oldham, 1999). In this study, a low-polarity column was used for the analysis of both derivatised and underivatised queen mandibular pheromone samples while a mid-polar column was used for the analyses of underivatised samples.

System 1 (low-polarity column)

Analyses of the derivatised mandibular pheromone were carried out on a Hewlett-Packard 5890 gas chromatograph coupled with a flame ionisation detector and equipped with a capillary column coated with cross-linked 5% diphenyl / 95% dimethyl polysiloxane (HP 5) (30 m x 0.25 mm x 0.25 µm film thickness). Helium was used as carrier gas at a flow rate of 1 ml / min. The GC was programmed as follows: the initial oven temperature was 60˚C for 5 minutes, then heated at 25˚C / minute to 120˚C and then programmed at 3˚C / minute to 300˚C and held at 300˚C for 5 minutes. The detector temperature was 300˚C and the injection port temperature was set at 270˚C for PDMS fibres and set at 230˚C for both mixed phase fibres. The fiber was desorbed in the splitless mode for 5 minutes. Chromatograms were recorded and peak areas quantified with HP ChemStation software®.

System 2 (mid-polarity column)

A Hewlett-Packard 5890 gas chromatograph fitted with a flame ionisation detector was used. Professor B.V Burger of the Laboratory for Ecological Chemistry at Stellenbosch University in South Africa prepared a mid-polar column (40 m x 0.32 mm) coated with OV-1701-OH (14% cyanopropylphenyl / 86% dimethyl polysiloxane) at film thickness of 0.375 μm that was used. The initial temperature was set at 40˚C for 5 minutes, heated at 4˚C / minute to 260˚C and then held at this temperature for 10 minutes. The injection port was set at 250˚C and the detector at 300˚C. Desorption of the Carboxen and CW/DVB fibres into the injection port was carried out at 250˚C for 5 minutes in a splitless mode. Chromatograms were recorded and peak areas quantified with an HP 3396 Series II integrator.

Chemical Analysis

Identification of individual components in the samples was accomplished by comparing their retention times with those of standard compounds.

Data analysis

One–way ANOVA (Analysis of variance) was used to determine whether there were any significant differences among different SPME fibres used to sample

mandibular gland pheromone. It was further used to determine whether different extraction times affected the amount of chemical extracted with the 100 µm PDMS fibre. 10 runs were done with 100 µm, 11 runs with 7 µm and 8 runs were done with Carboxen and CW / DVB.

RESULTS AND DISCUSSION

The mandibular pheromone compounds that are known to be biologically active in the honeybee colony were chosen as target analytes during the development of this method.

Fibre coating

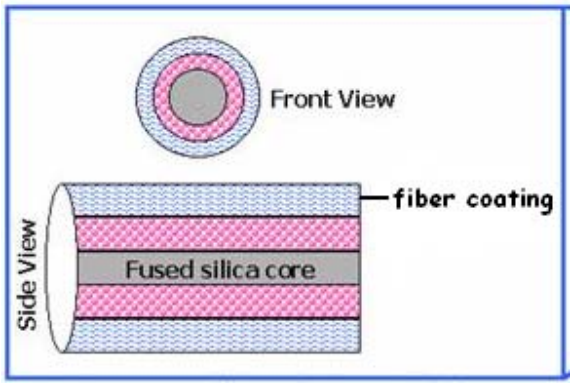


Figure 2.2 Cross-sectional view of SPME fiber used in extraction of volatile compounds.

The heart of solid phase micro-extraction sampling is the coating on the fibre.

This is where analytes are concentrated and derivatised during extraction see figure 2.2. Theoretically, analytes with polar functional groups are expected to have a high affinity for polar fibres while non-polar molecules will be trapped by non-polar fibres. The mandibular gland pheromone analytes have both polar and non-polar functional groups. The performance of the four types of solid phase fibres were compared based on the recovery of compounds in a standard mixture of the mandibular gland pheromone (figure 2.3). The quality of the fibre depends on the manufacturer and at times the performance is different from batch to batch (Malosse, *et al*., 1995; Pawliszyn 1997; Lin & Pawlisyn, 1997, Jones & Oldham, 1999).

One-way analysis of variance showed a significant difference between the different fibres at 5% since $F_{calc} > F_{crit}$ (DF: 3; P = 0.012; $F_{calc} = 4.71$; $F_{crit} = 3.098$). $F_{calc} > F_{crit}$ implies that the four means of the different fibres demonstrated differences in their ability to extract various chemical components of the mandibular gland mixture. Although the mixed phase coatings Carboxen and CW / DVB should have been best suited for the extraction of the polar components of the mandibular pheromone, the results obtained were not satisfactory and they were found to be unsuitable for the analysis of underivatised mandibular pheromones on a low-polarity column (figure 2.4). The major components of the mandibular gland pheromone (C10 acids) on the polar fibres were clearly not separated on the HP-5 column that was used.

The BSTFA treated 100 µm PDMS fibre performed better than all other fibres and gave consistent results. The 100 µm PDMS, with its universality of sorption characteristics for most compounds, was therefore selected for further analysis. The thickness of the fibre coating seemed to exert an influence on extraction recoveries because 7 µm PDMS did also not yield as consistent results with satisfactory recoveries as the 100 µm PDMS (figure 2.5). BSTFA treated 100 µm PDMS fibre is best suited for the extraction of all target analytes investigated, the extraction yields of mandibular pheromone components extracted with 100 µm PDMS are many times higher than with other fibres examined in this test.

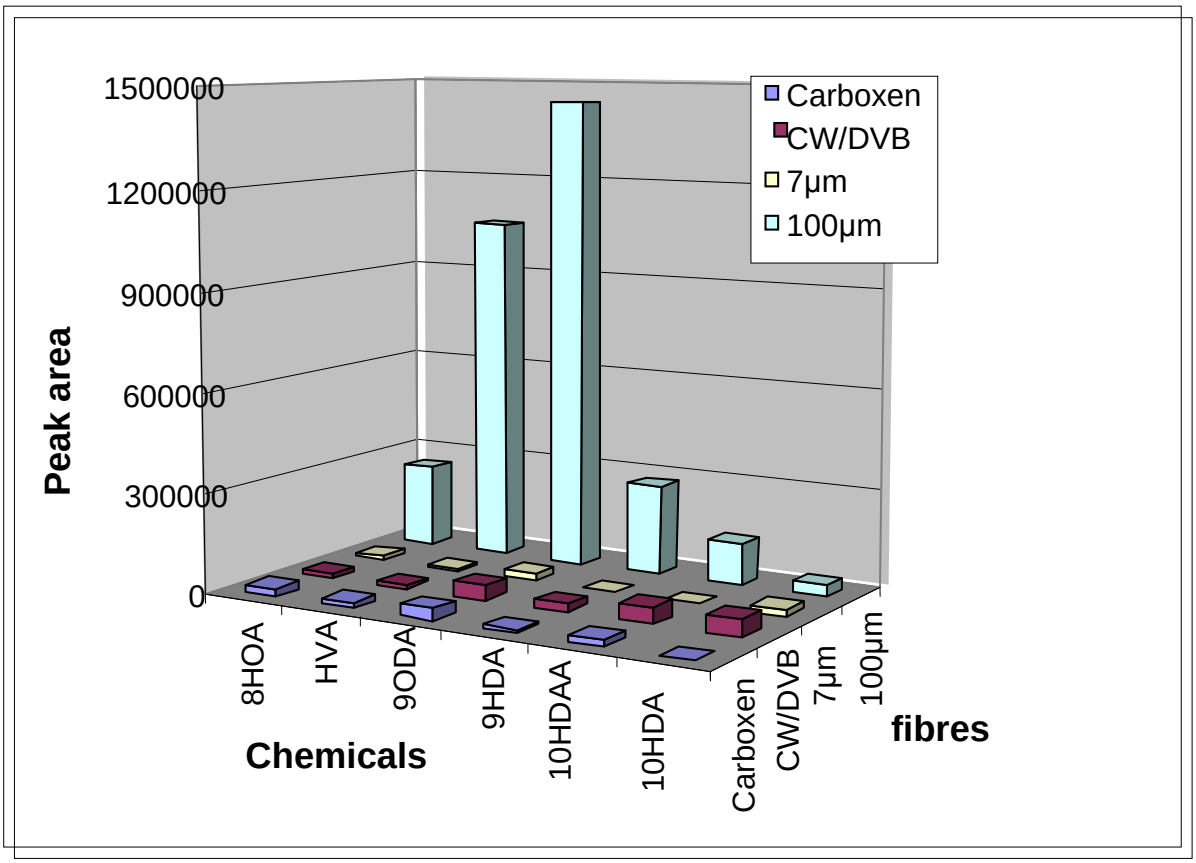
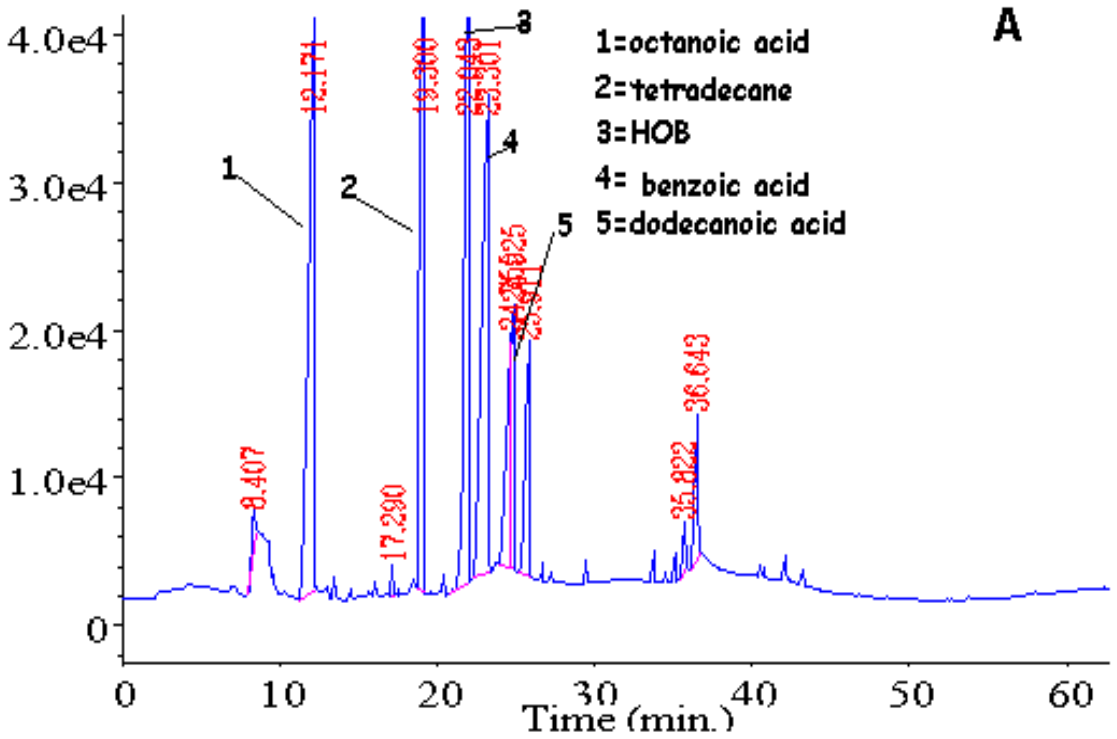


Figure 2.3 Comparison of different adsorbent efficiencies of mandibular pheromone standard analysed on a low-polar HP-5 column. The 7 µm and the 100 µm PDMS fibres were BSTFA-derivatised. The data are presented as the mean value of the peak areas. Ten runs were done with 100 µm PDMS film, 11 runs with 7 µm PDMS film and 8 runs were done with Carboxen and CW / DVB fibre. 8-Hydroxy octanoic acid (8-HOA), 9-Oxo-2-decenoic acid (9-ODA), 4-hydroxy-3-methoxyphenylethanol (HVA), Hexadecanoic acid (HDA), 10-hydroxydecanoic acid (10-HDAA), 10-hydroxydecenoic acid (10-HDA).


A
1=octanoic acid
2=tetradecane
3=HOB
4= benzoic acid
5=dodecanoic acid
4.0e4
3.0e4
2.0e4
1.0e4
0
0
10
20
30
40
50
60
Time (min.)
8.407
12.171
17.290
19.300
36.643

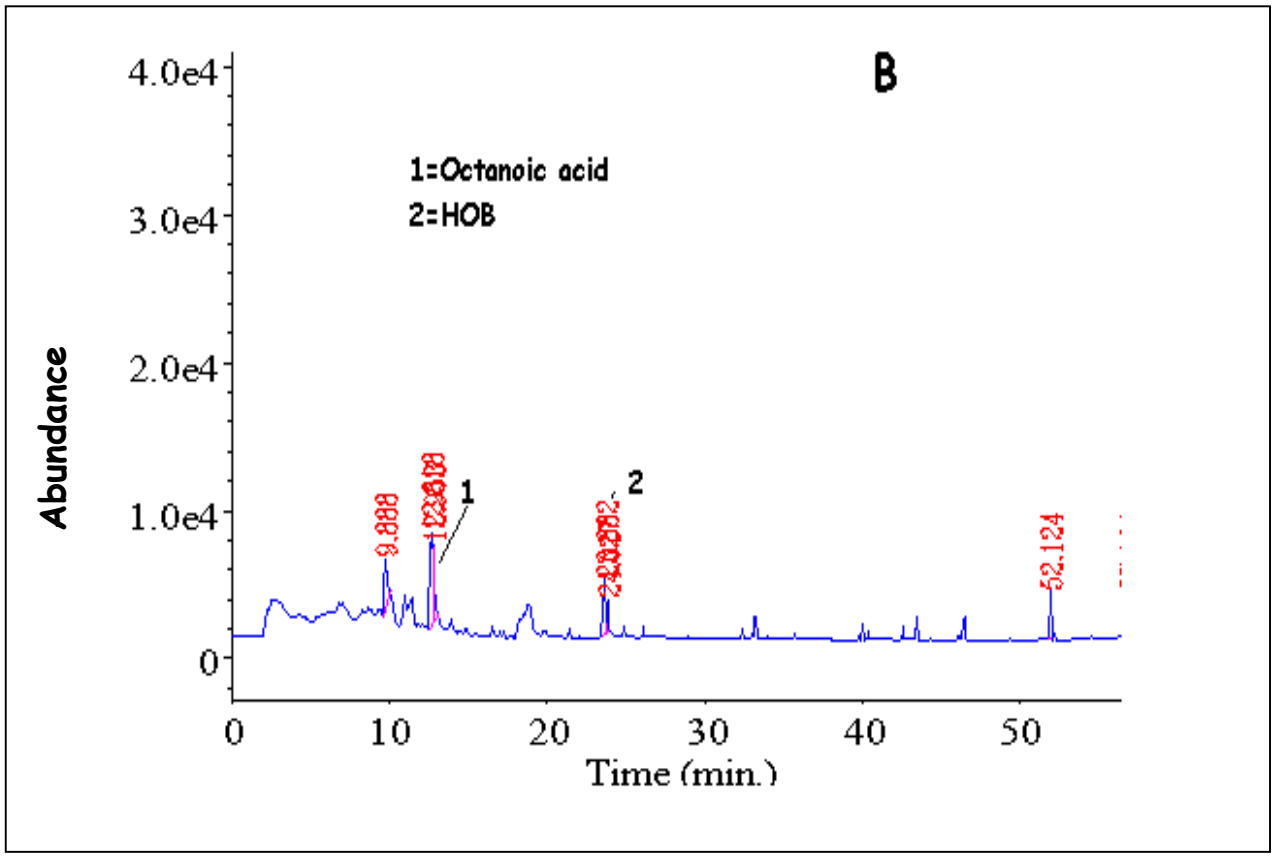


Figure 2.4 Chromatograms A and B of the underivatised mandibular pheromone standard solution extracted with mixed phased fibres and analysed on the HP-5 column. The chromatograms show none of the major C10 acids present in the mandibular gland pheromone standard. A: CW/DVB fibre, B: Carboxen fibre. Methyl *p*-hydroxybenzoate (HOB).

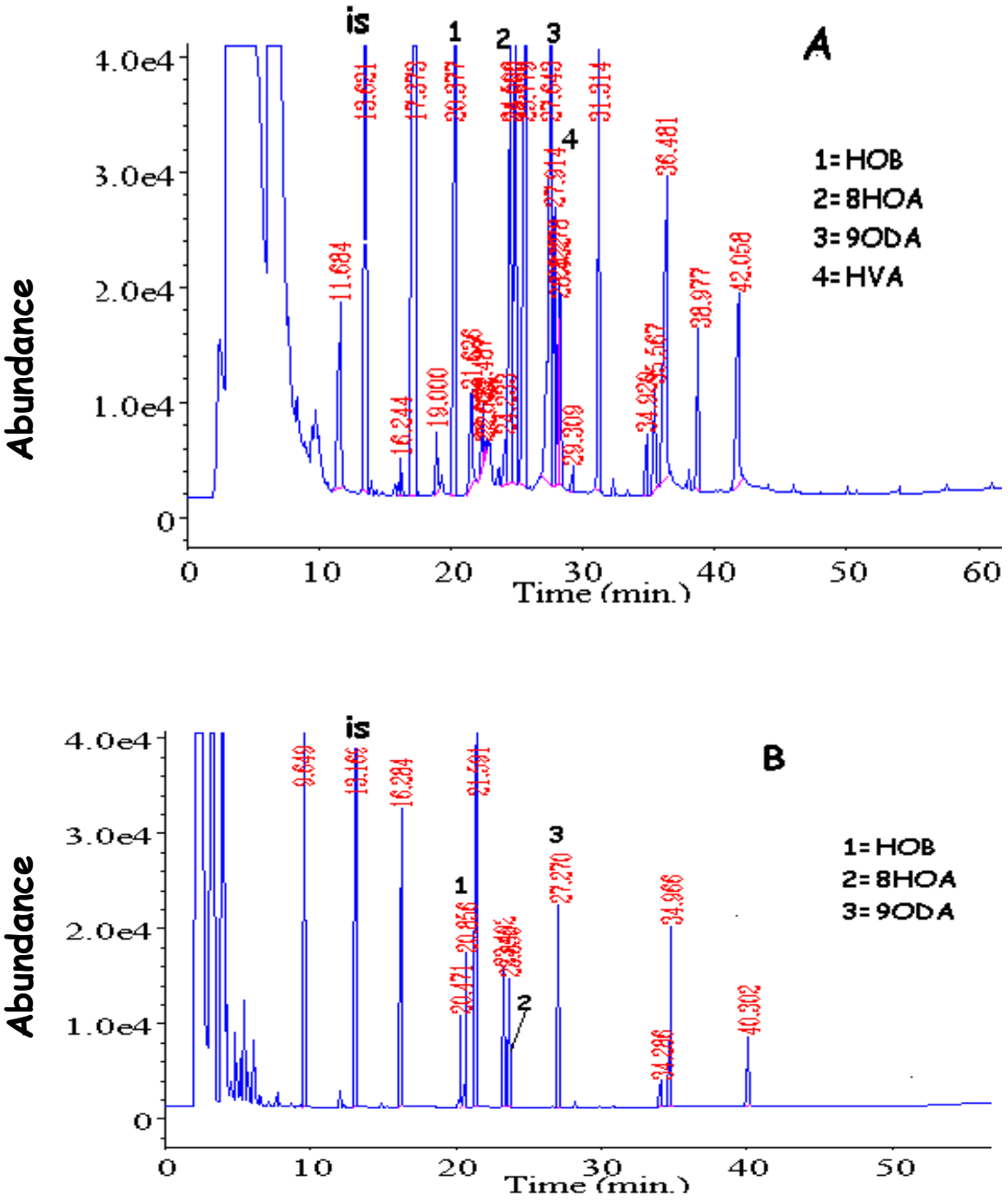


Figure 2.5 Chromatograms of mandibular pheromone standard extracted with BSTFA treated non-polar fibres (100 µm PDMS fibre = A; 7 µm PDMS fibre =

B) and analysed on a low polarity (HP-5) column. Tetradecane was used as internal standard and labeled (is) on the chromatogram. Methyl *p*-hydroxybenzoate (HOB), 9-Oxo-2-decenoic acid (9-ODA), 4-hydroxy-3-methoxyphenylethanol (HVA), 8-Hydroxy octanoic acid (8-HOA)

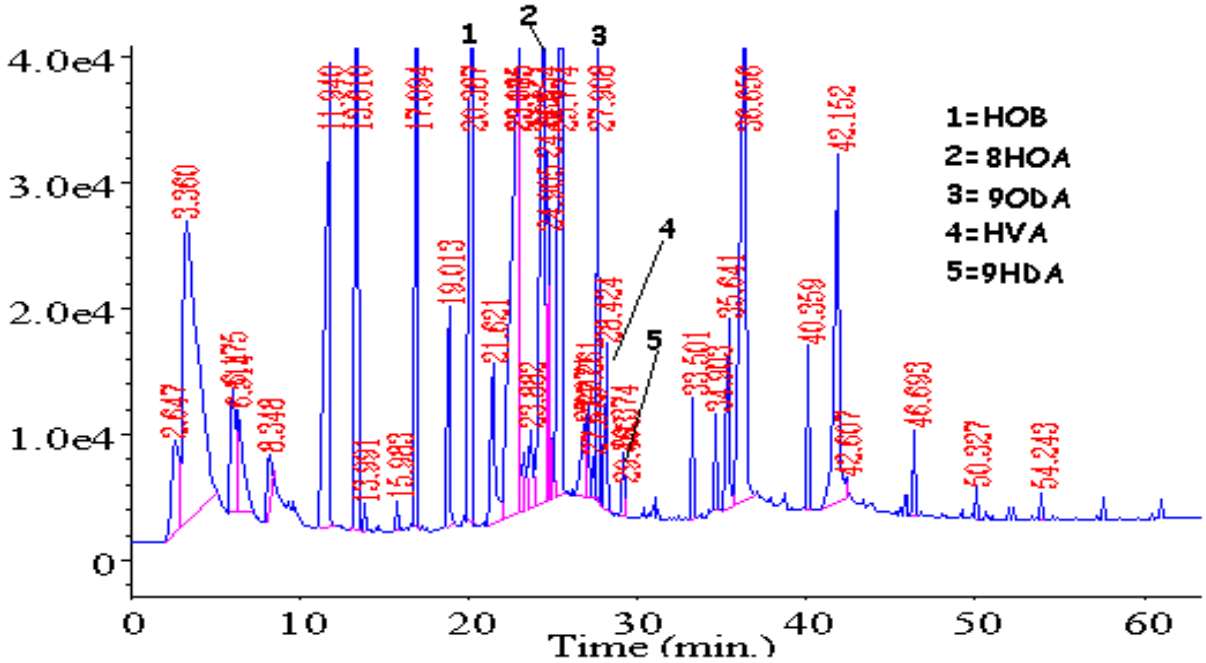


Figure 2.6 A chromatogram of queen mandibular pheromone extracted from a living mature queen with a BSTFA treated 100 µm PDMS fibre and analysed on a low polarity (HP-5) column. Methyl *p*-hydroxybenzoate (HOB), 9-Oxo-2-decenoic acid (9-ODA), 4-hydroxy-3-methoxyphenylethanol (HVA), (+/-)-9-hydroxydec-2-enoic acid (9-HDA), 8-Hydroxy octanoic acid (8-HOA)

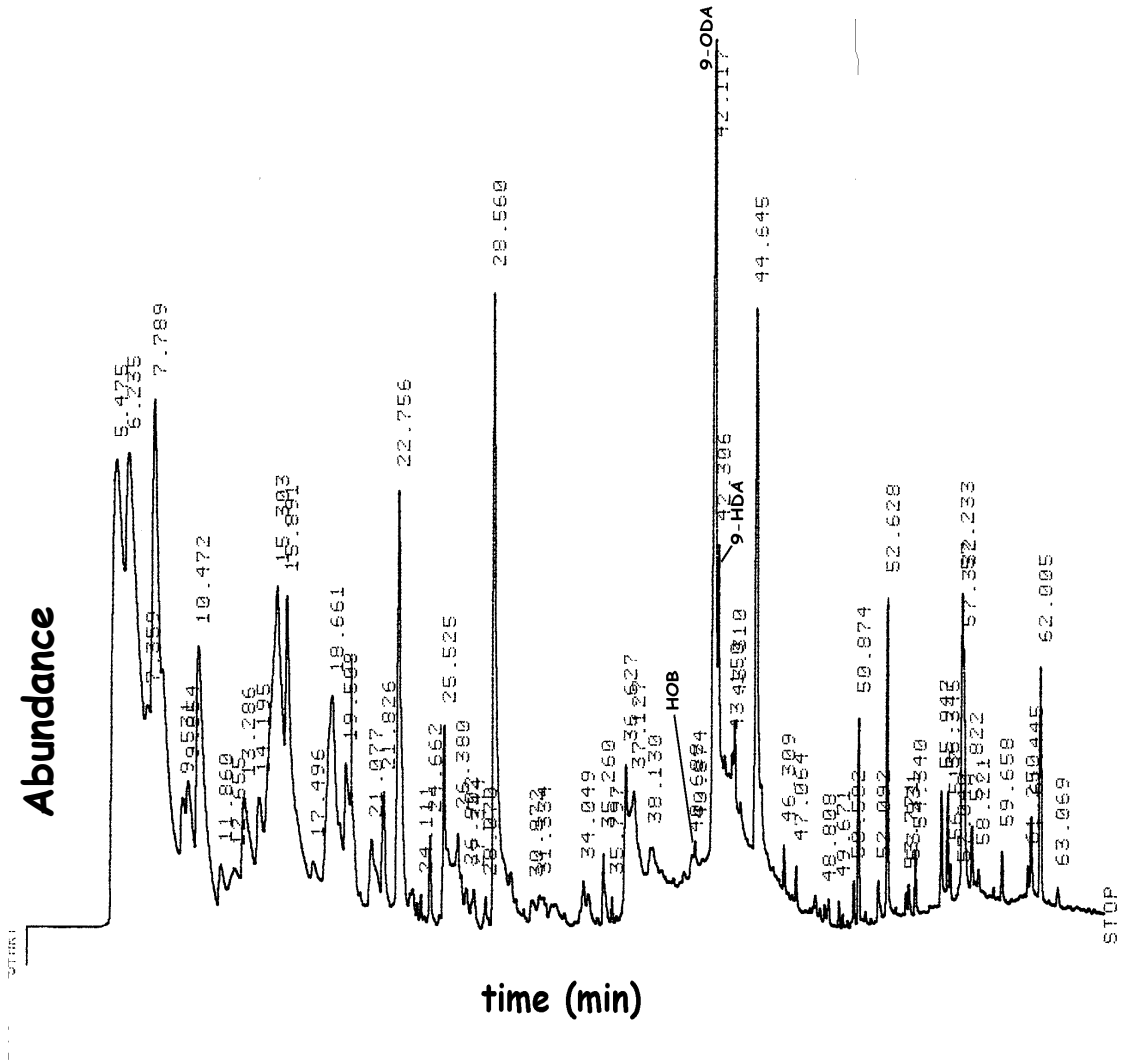


Figure 2.7 Chromatogram of underivatised queen mandibular pheromone sampled with Carboxen fibre and analysed on the mid-polar column. Methyl *p*-hydroxybenzoate (HOB), 9-Oxo-2-decenoic acid (9-ODA), (+/-)-9-hydroxydec-2-enoic acid (9-HDA).

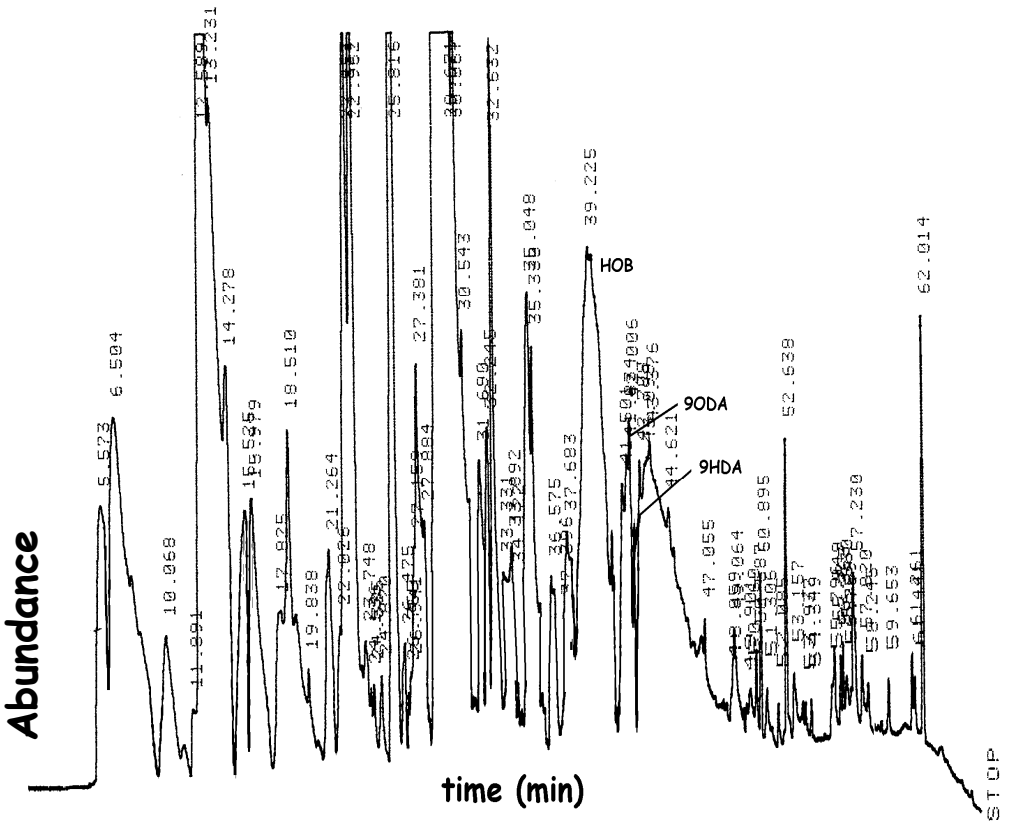


Figure 2.8 Chromatogram of queen mandibular pheromone sampled with polar CW/DVB fibre and analysed on the mid-polar column. Methyl *p*-hydroxybenzoate (HOB), 9-Oxo-2-decenoic acid (9ODA), (+/-)-9-hydroxydec-2-enoic acid (9-HDA).

Although it was possible to recover mandibular gland components using fibres with mixed phase coatings and to run these on a mid-polar column, the sensitivity of the analysis was much lower than that obtained with BSTFA treated non-polar fibres with the HP-5 column. The release of analytes from fibres needs to be investigated because irreversible sorption will also affect the results. Comparing Figure. 2.4 and figure. 2.7 demonstrate that the mixed phase fibres are extracting the major C10 acids which appear when analysed on the mid-polar column. The more polar CW/DVB fibre clearly extracts a greater amount of secretion from the mandibular gland, unfortunately not readily separated on the mid-polar column, see for example the large amount of HOB in Fig 2.8. The improved extraction efficiency of this fibre could probably be used to good effect with a more polar separation column that would produce symmetrical peaks for the highly polar analytes.

The best separations of mandibular pheromone components were obtained on the low-polar column using non-polar 100 µm PDMS fibres treated with BSTFA (figure 2.5 A and 2.6).

Extraction time profile

The fused silica fibre was exposed to the sample matrix of mandibular pheromone standard which allowed partitioning of analytes into the fibre coating until equilibrium was reached. The timing of extraction commenced immediately

after the fibre was introduced to the sample matrix. Figure 2.9 shows the extraction time profile of selected queen mandibular pheromone compounds from a standard mixture using the BSTFA treated 100 µm polydimethylsiloxane fibre. Different sampling times ranging from (1 min, 3 min, 4 min, and 5 min) were studied by monitoring the peak areas that were produced. The extraction time profile was studied by monitoring the peak area as a function of exposure time. Figure 2.9 shows the dependence of amount of the analyte extracted as a function of time. Some of the mandibular pheromone compounds did not equilibrate with the fibre within a reasonable time. Since components of a multicomponent mixture such as mandibular gland pheromone differ in chemical properties (some are relatively heavier and less volatile whereas others are lighter and volatile) they will therefore reach equilibrium at different times. Compounds with low diffusion coefficients have long equilibration times (Alpendura, 2000). The amount of HVA extracted does not show much change with increasing time. Some compounds like 9-HDA fluctuate with time and may need many hours or even days to equilibrate with the fibre coating. Considering that the experiment was applied to living insects (honeybees), the extraction/contact time could not be extended beyond five minutes, since longer periods of confinement can lead to the death of the bee.

One-way ANOVA applied at the 5% level demonstrated that the amount of chemicals extracted show no significant difference as extraction time is increased (F_{calc}= 3.648; df: 3; p = 0.057) i.e. the amount of HOB extracted at 1min does not

differ significantly from the amount extracted at 5 min. However, the extraction yields of the various compounds per extraction time do differ significantly from each other (F_{calc} = 9.548; F_{crit} = 3.098; df: 3; p = 0.037) i.e. HOB extracted at one minute differ from HDA, 9-ODA and HVA extracted at one minute. The same applies to other extraction times and chemicals.

Maximum extraction yields were obtained for most target analytes in 5 minutes. As a consequence of these results, in later experiments extraction was done for five minutes because at five minutes the recovery of all compounds is maximal.

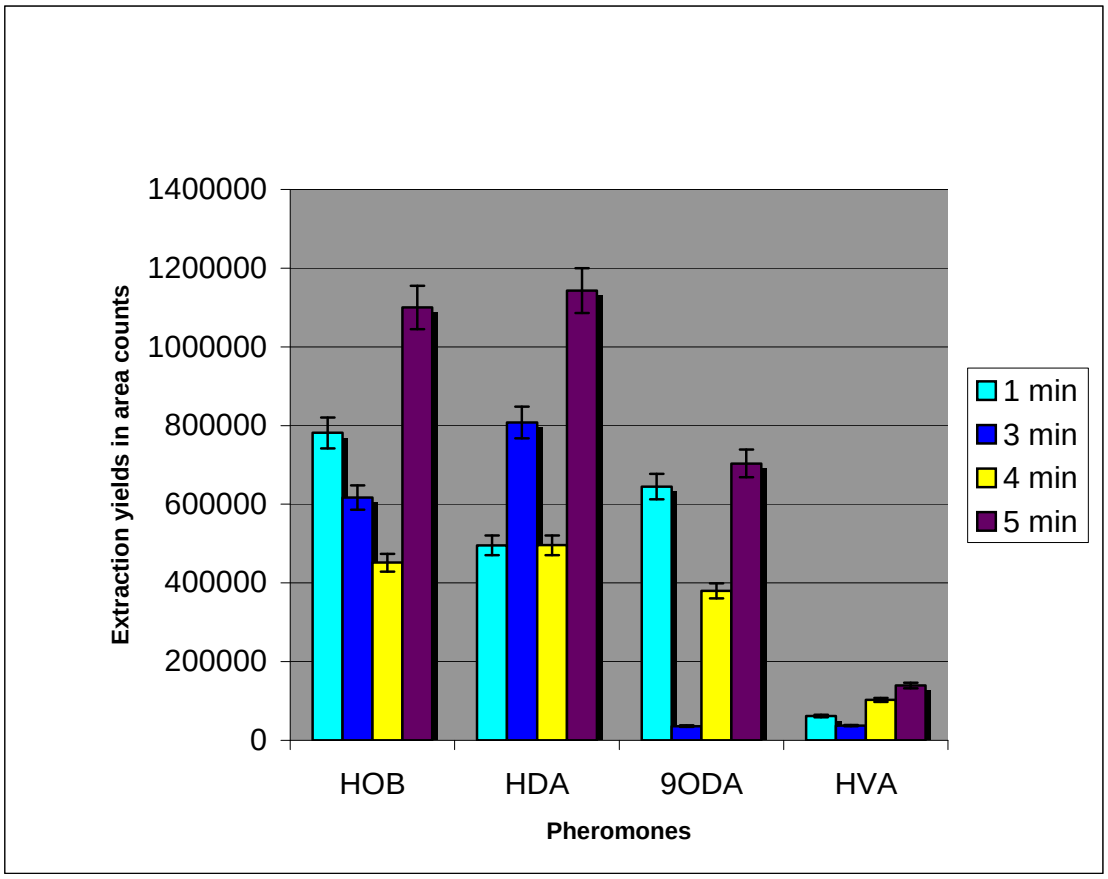


Figure 2.9 Amount of mandibular pheromone standard components extracted

after different time exposures with derivatised PDMS fibre. Means±SE are used to determine the time profile of the mandibular compounds adsorbed by 100 µm PDMS fibre (n=11). Amount of chemicals extracted show no significant difference as extraction time is increased ($F = 3.648$; $df = 3$; $p = 0.057$). Methyl *p*-hydroxybenzoate (HOB), 9-Oxo-2-decenoic acid (9-ODA), 4-hydroxy-3-methoxyphenylethanol (HVA), hexadecanoic acid (HDA).

Effect of on-fibre derivatisation

Gas chromatographic analysis of volatile, non-polar compounds may be carried out without derivatising the sample. In the case of polar compounds this may also be achieved by the use of polar columns e.g in figure 2.8 it was possible to analyse the mandibular gland pheromone without derivatisation. However, it is difficult to separate polar compounds by gas chromatography without derivatisation as can be seen in figure 2.8 (Crewe & Velthuis 1980, Pawlisyn 1990, Maile *et al*., 1998). The chromatography of intractable polar compounds such as fatty acids has been facilitated by derivatisation (Crewe & Velthuis, 1980; Crewe, 1988).

Derivatising agents however tend to contain materials that could damage or contaminate the fibre coating if the fibre is directly exposed to the agent. In our study, if the PDMS fibre touched the liquid BSTFA the whole coating would

dissolve. For this reason headspace loading of the fibre (figure 2.1) was used to protect the coating from damage.

On fibre derivatisation is a single step for converting polar analytes (A) into corresponding derivatives (B) prior to their analysis by GC-FID (see figure 1.5). The derivatising reagent, BSTFA, is loaded onto the polydimethylsiloxane fibre coating, typically a non polar sorbent, the loaded fibre is then exposed to the sample matrix for extraction. The analytes are simultaneously extracted and derivatised in the fibre. The derivatives formed from the reaction of BSTFA and extracted analytes are then introduced into the gas chromatograph for analysis. Recovery of compounds that were derivatised on fibre on a low-polarity column was very high (figure 2.5 and 2.6). The absence of BSTFA hinders the detection of mandibular pheromone in a dramatic way (figure 2.4B). BSTFA is used to enhance the extractability of polar compounds with non-polar fibres and to improve detection of analytes.

Desorption

Compounds of low volatility must be desorbed at a temperature which is sufficiently high in order to ensure sufficiently high vapour pressure of each component in the sample. High temperatures can however damage the coating and lead to decomposition of the samples thus producing inconsistent results. The optimum desorption time was determined by varying the desorption time of fibres from 2 minutes to 5 minutes in the GC injector at 270°C for PDMS fibres

and 230°C for mixed fibres. Carryover testing was undertaken by injecting a blank fibre after the mandibular gland pheromone standard was run to verify that there were no memory effects from the previous analysis. Carryover of mandibular gland pheromone volatiles was not observed with 5 minute desorption times with all fibres.

CONCLUSION

A prepared pheromone standard was used to analyse the performance of various solid-phase micro-extraction methods. Method optimisation was done with a mandibular pheromone standard mixture because this made calibration easier to carry out. Sampling from living insects where a more complex condition exists makes it difficult to duplicate the results for calibration. Queen mandibular pheromone is characterised by the presence of several classes of substances, the most prominent of which are fatty acids (Crewe & Velthuis, 1980; Velthuis, 1985; Plettner et al., 1995, 1996). Among the complex mixture of volatiles emitted from queen mandibular pheromone, a few compounds were of particular interest to us because they are known to have biological activity in honeybee life. The compared chromatograms run on both mid-polarity (figure 2.7 and 2.8) and low-polarity (figure 2.5) columns from living queens revealed 9-ODA as a major peak, which is a common characteristic of the honeybee queen mandibular secretion.

Solid phase micro-extraction reduces analysis time by combining sampling, extraction, concentration, and injection into a single process (Pawlizyn, 1997). This work has explored a range of solid phase micro-extraction methods used for insect semiochemical analysis. The pheromones were isolated directly from five live queens in a reproducible way. SPME provides a sampling technique that is sensitive enough to make insect pheromone analysis accessible to routine instrumental analysis.

This is an equilibrium sampling method and can be used to accurately analyse target analytes in a sample matrix through proper calibration. Several factors can influence the amount of the analytes extracted by the fibre: The volume of the coating that is the size or thickness of the fibre, coating characterisation and modification of the sample such as derivatisation. Zhang and co-workers (1994) revealed that the derivatising reagent in the fibre coating could act as the concentrator, isolator and reactor to the analytes contained in the sample. These factors need to be considered for optimal use of the SPME method. An initial comparative trial of fibre coatings using the synthetic mandibular pheromone allowed us to select 100 µm PDMS fibres for further analyses.

This technique was effective in analysing volatiles from the mandibular gland pheromone of living honeybee queens by gas chromatography. The advantages of this method were its high sensitivity and non-destructive nature. The use of solid phase micro-extraction to study mandibular pheromones will also give an

indication of what is being secreted because sampling by solid phase micro-extraction will only extract what has been secreted onto the surface of the mandibles, as opposed to sampling by solvent where everything in the gland is extracted. In the future the technique could be applied to track changes in the glandular secretion of an individual over its lifetime.

The practical shortcoming of the sampling system employed here is the fragility of the fibres because movement of the mandibles can break them. Once the sample has been extracted it has to be analysed immediately because SPME fibres cannot be stored for later analysis and this makes it impractical to sample a large number of individuals simultaneously, they can only be sampled consecutively.

CHAPTER 3

SILICONE RUBBER TUBING, AN ALTERNATIVE NON DESTRUCTIVE METHOD FOR ANALYSIS OF THE MANDIBULAR GLAND PHEROMONES OF LIVING HONEYBEE QUEENS

SUMMARY

A new technique using silicone rubber tubing was developed for routine analysis of pheromones. The silicone rubber tubing was tested as an effective and alternative sampling technique for the isolation and concentration of volatiles from mandibular gland secretions of living honeybee queens. Silicone rubber tubing utilises a polydimethylsiloxane membrane to extract analytes from the sample matrix. In this study, BSTFA treated hollow tubing with thin rubber walls is exposed to the sample matrix and target analytes partition into the walls of the tubing. The saturation of BSTFA onto silicone rubber tubing makes the tubing more efficient for the extraction of polar analytes. Extracted analytes are then chemically desorbed or eluted from the tubing using an organic solvent, dichloromethane. The extract was then concentrated under a stream of nitrogen gas and chromatographically analysed. The ability of this technique to sample mandibular pheromone is clearly demonstrated by positive results obtained with the mandibular standard sample. The technique offers the advantage of isolating the mandibular pheromone directly from their site of release.

INTRODUCTION

Classical methods for analysis of glandular pheromones involve extraction by organic solvents (Jones, 1999) but recently new technologies such as SPME have attracted attention for sampling (Pawliszyn, 1995). Volatile trapping is important to study freshly secreted pheromones from insects (Jones, 1999). Silicone rubber tubing is an extraction technique that uses a polymer-tubing wall to extract analytes. Silicone rubber tubing is the most commonly used extracting material (Ortner & Rohwer, 1996) because a wide range of organic compounds are soluble in the polymer, while water and interfering ions are not. This provides the basis for the separation of desired analytes from the bulk sample.

An example of a nonporous membrane is silicone rubber, which on a macroscopic scale is a homogeneous phase of polymer polydimethylsiloxane. Silicone rubber is a polydimethylsiloxane polymer in the rubbery state that can be considered as a viscous liquid (Ortner & Rohwer, 1996). This viscous liquid acts as an extracting organic phase during partitioning of analytes whereby targeted analytes permeate into the membrane at a given rate. The driving force for the partitioning process is the difference in concentration of the analytes between the inner and outer walls of the tubing (Pawliszyn, 1995). Silicone rubber tubing is one of the few good nonporous membranes and has high permeability for small hydrophobic molecules, increasing selectivity on extraction (Pawliszyn, 1995).

The silicone rubber tubing extraction procedure consists of two simultaneous processes: partitioning of analytes between extracting phase (tube walls) and sample matrix followed by desorption of analytes from the membrane by means of a stripping solvent. The hollow tubing provides a higher surface area to volume ratio for the stripping solvent offering a more efficient desorption of target analytes (Pawliszyn, 1995). In principle, silicone rubber tubing offers advantages mentioned above but one problem could be keeping the tubing stable, which could subsequently limit its application.

The silicone rubber tubing is made up of polydimethylsiloxane and this means that the tubing is non-polar, therefore having lower absorption capabilities toward polar analytes. To improve silicone rubber tubing extraction efficiency of very polar analytes and making it more suitable for the isolation step, appropriate derivatisation procedures must be used prior to extraction. Sometimes it is useful to use silicone rubber tubing that has been coated with stationary phases that will selectively react with the compound of interest to form a more stable product.

In this chapter, we extended the analysis of mandibular gland pheromones from living honeybee queens using a non-destructive silicone rubber tubing technique that also allows for successive extraction of pheromone components from the same individual. Silicone rubber tubing allows for sampling using a procedure that is related to SPME (Crewe et al, 2004).

MATERIALS AND METHODS

Experimental procedure

Silicone rubber tubing preparation and construction

One centimetre long pieces of silicone rubber tubing (Technical Product Inc., Georgia, USA, 0.012 mm I.D X 0.025 mm O.D) were prepared. The tubing was conditioned by washing in dichloromethane and baked for 3 hours at 300ºC in an oven to get rid of impurities. Immediately after conditioning, the tubing was loaded with derivatising agent by the headspace method at room temperature.

1 ml BSTFA was poured into a sample vial and glass wool was used to keep the silicone rubber tubing suspended over the derivatising agent. The derivatising agent saturated the silicone rubber walls where the analytes could be simultaneously extracted and derivatised to non- polar analogues, which would be ready for analysis. A clutch pencil was used as the silicone rubber-tubing holder (figure 3.1).

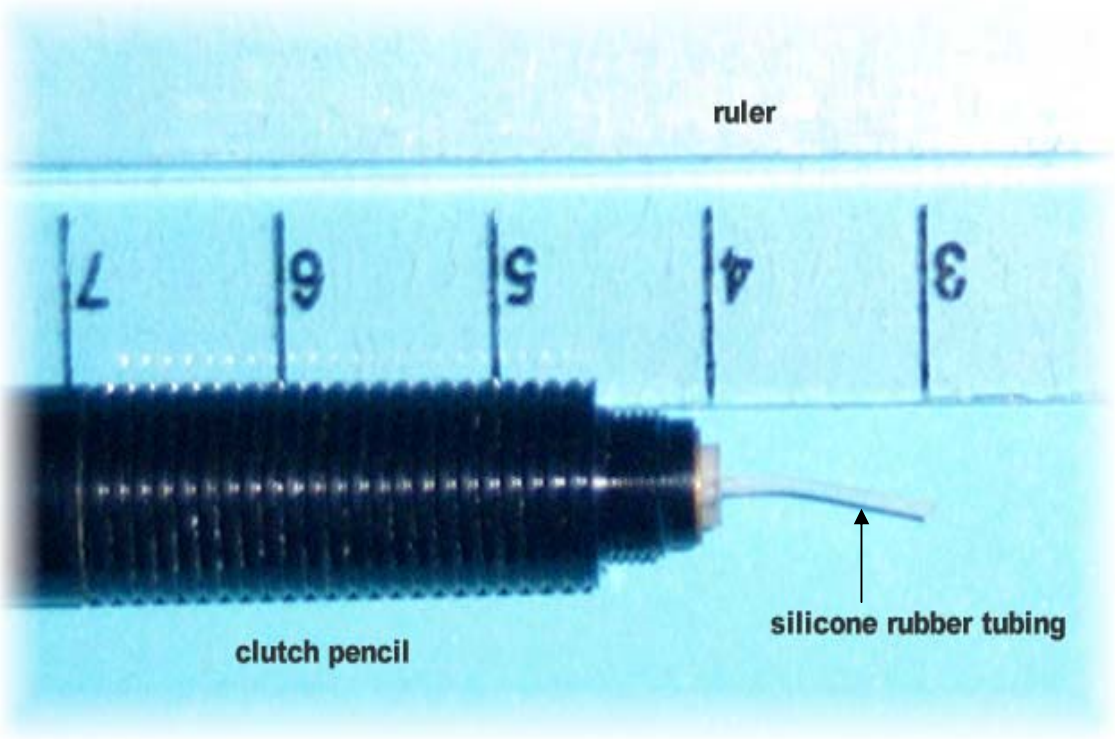


Figure 3.1 A sampling device constructed from a short piece of silicone rubber tubing and clutch pencil

The length of the piece of silicone rubber tubing (1 cm) was determined by making it equivalent to the length of the solid phase micro-extraction fibres previously used for the analysis of mandibular pheromone in chapter two. The clutch pencil holder provides complete flexibility in the length of the tubing exposed to the sample. The silicone rubber tubing is placed directly into the sample matrix whereby target analytes are concentrated onto the tube walls, which acts as the liquid phase. Specific target components of the mandibular pheromone permeate through the tube walls.

Sampling mandibular pheromone with silicone rubber tubing

The tubing was thermally and chemically treated prior to use to minimise contamination. BSTFA was used for *in situ* derivatisation to ensure efficient trapping of honeybee mandibular pheromone. Concentrated analytes on the silicone tubing were chemically desorbed from the tubing with dichloromethane. The tubing was left at least overnight in dichloromethane, because the solvent had to strip all analytes from the tubing so that they could be analysed.

a) Synthetic pheromone

About 10 µl of the prepared mandibular pheromone standard (preparation of standard is outlined in chapter 2) was placed on a silanised glass slide and left to evaporate. Silicone rubber tubing impregnated with BSTFA was rubbed on the slide for 5 minutes. The tubing loaded with analytes was immersed into 200 µl dichloromethane contained in a sample vial. The vials were sealed and kept in a refrigerator at 4ºC overnight before the extracts were analysed. When ready for analysis the mixture was concentrated under a nitrogen stream. A tetradecane solution was added as an internal standard (see table 2.1 for detailed composition of mandibular pheromone standard). 1 µl of the extract was injected in the splitless mode for analysis.

b) Queen mandibular pheromone

Derivatised silicone rubber tubing was rubbed on the mandibles of the living virgin honeybee queen for 5 minutes aiming at the mandibular gland opening. Silicone tubing loaded with mandibular gland components was immersed in 200 µl dichloromethane. Three queens were sampled with this method. The sample was further prepared as for the samples of the synthetic mixture.

Chromatographic analysis

Gas chromatography of queen and synthetic pheromone extracts was carried out on a HP 5890 Series II instrument equipped with a flame ionisation detector. The column (HP-5, 30 m x 0.25 mm) was maintained at 60ºC for 1 minute and then programmed from 60ºC to 120ºC at 25ºC / min and then at 3ºC / min to 300ºC and held for 5 min at this temperature. The injection port was kept at 250ºC and the detector temperature was 320ºC.

Chemical Analysis

Tentative identification of individual target components in the samples was accomplished by comparing their retention times with those of standard compounds.

RESULTS AND DISCUSSION

a) Synthetic pheromone

Detailed preparation of mandibular pheromone standard is outlined in chapter 2 (see table 2.1).

When the mandibular pheromone standard was analysed all chemicals that constitute the pheromone were detected at good levels (figure 3.2). This demonstrated the potential of the technique to sample mandibular gland pheromones.

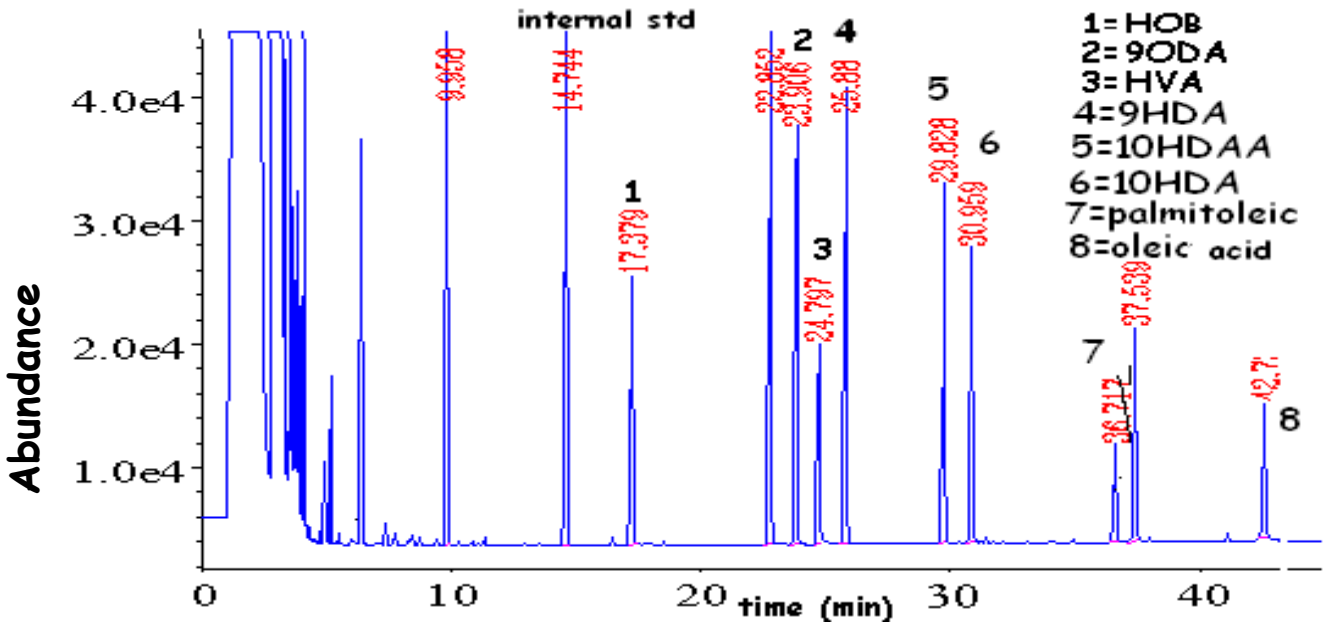


Figure 3.2.The chromatogram of mandibular pheromone standard sampled with BSTFA loaded silicone rubber tubing which achieved *in situ* derivatisation. Methyl *p*-hydroxybenzoate (HOB), 9-Oxo-2-decenoic acid (9-ODA), 4-hydroxy-3-methoxyphenylethanol (HVA), 9-Hydroxydec-2-enoic acid (9-HDA), 10-Hydroxydecenoic acid (10-HDA), 10-Hydroxydecanoic acid (10-HDAA).

b) Queen mandibular pheromone

The mandibular secretion of living honeybee queens was detected only in minute quantities (figure 3.3). A cluster of peaks eluted at approximately 28 minutes was at very low intensities. The cluster of the hydroxy acids ranging from 9-HDAA to 10HDA were identified in figure 3.3. A number of the chemical components of the mandibular pheromones could not be detected possibly because they were isolated in concentrations below the detection limit of the gas chromatograph.

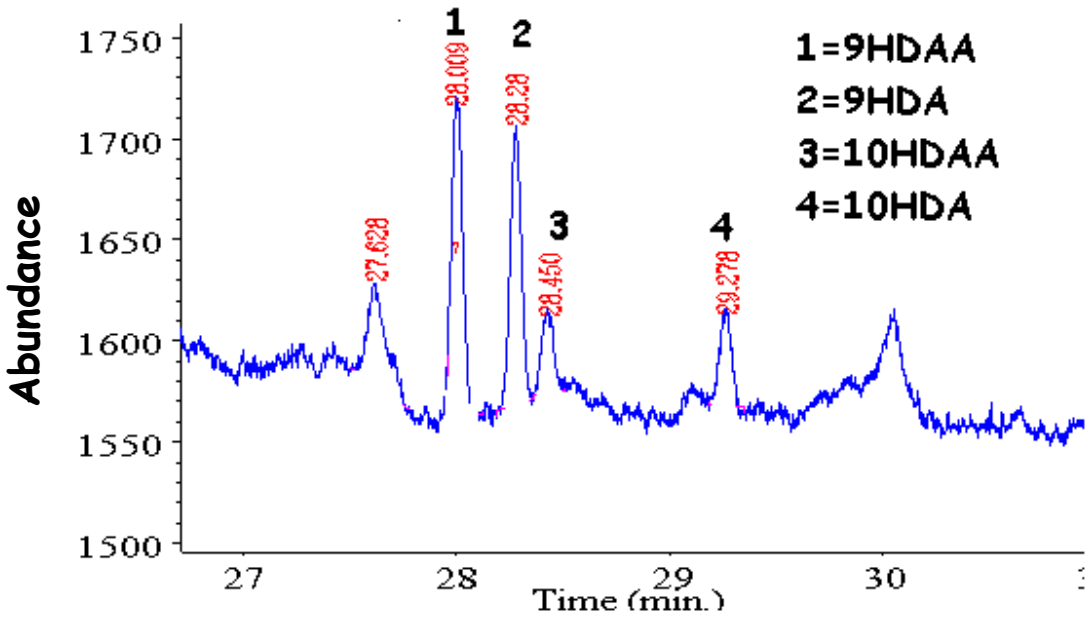


Figure 3.3. The chromatogram of mandibular pheromone of living honeybee queen sampled with derivatised silicone rubber tubing analysed on HP-5. 9-Hydroxydecanoic acid (9-HDAA), 9-Hydroxydec-2-enoic acid (9-HDA) 10-Hydroxydecanoic acid (10-HDAA), 10 Hydroxydecenoic acid (10-HDA).

Queen mandibular pheromone sampled with silicone rubber tubing yielded peaks that were very small. In figure 3.2 the concentrations of target analytes (prepared standard mixture) was present in higher concentrations than secreted queen pheromone in figure 3.3. Hence standard mixture analysis resulted in better recovery than the queen secretions. The sensitivity of the tubing can be adjusted by changing the thickness of the stationary phase because we observed that addition of organic solvents (dichloromethane and BSTFA) to the tubing actually makes it swell.

CONCLUSION

This collection technique has proven very useful in isolating the mandibular gland pheromone standard of honeybees. The advantage of the technique is that samples can be collected in the field and sealed directly in sample vials and analysed later. The non-destructive nature of silicone rubber tubing allows repeated sampling of the same individual, something that was previously not possible.

The solvent selected for stripping analytes from the tubing should be one that removes the largest amount of analytes because the analytes may sometimes be strongly attached to the tubing (Pawliszyn, 1995). The effectiveness of the stripping solvent to desorb analytes from the tubing depends on the affinity of the target analytes for the stripping solvent (Pawliszyn, 1995). Alternative desorption

methods, such as direct insertion of the tubing into the injector can be applied to improve recovery yields. Desorption could also be carried out using available desorbers that can be connected to the gas chromatograph such as thermal desorbers.

Using silicone rubber tubing as a sampling technique for pheromones is still in the developmental stage. The most exciting aspect of this technique is that it is wide open to innovative approaches in the attempt to analyse pheromone production. The results obtained with the pheromone standard (figure 3.2.) are very promising and represent the potential success of silicone rubber tubing in future applications. In 2004 Crewe, Moritz and Lattorff were able to trap and analyse mandibular pheromone using silicone rubber tubing.

The technique uses organic solvents to desorb analytes, which is an undesirable limitation of the method. Addition of organic solvents to tubing made it swell and the swelling may change either the porosity and /or the chemical activity of the tubing (Pawliszyn et al., 1990). Although the trapping of the pheromones is solventless, the subsequent work up involves solvent extraction which could not be avoided because of the number of samples generated in a short space of time.

The samples needed to be stored before analyses to allow sufficient time for desorption of analytes from silicone rubber tubing into dichloromethane. During

storage the sample is exposed to possible contamination. Volatile components may be lost during storage. The low level of recovery experienced with queen samples is a serious problem that needs to be addressed by considering alternative methods of collection.

CHAPTER 4

TRIED AND TESTED METHOD FOR THE ANALYSIS OF MANDIBULAR PHEROMONE: SOLVENT EXTRACTION

SUMMARY

Solvent extraction is a procedure that has been a useful tool in studies on the isolation and identification of mandibular pheromones for many years. The mandibular gland pheromones of queens were analysed with solvent extraction followed by gas chromatographic analysis. Two columns of different polarities were employed and it was possible to analyse the mandibular pheromone underivatised on a polar column. The presence of previously identified compounds in queen mandibular glands was confirmed.

INTRODUCTION

Pheromones are important to the survival of social insects because of their dependence on pheromones to convey information among colony members (Free, 1987). Queen mandibular pheromone provides important signals for nest mate interactions (Kaminski et al., 1990). The classical application of solvent extraction for the chemical analysis of mandibular pheromone has been the proven method of analysis. Solvent extraction is still an important, common and

very useful technique used for the analysis of honeybee mandibular pheromone (Crewe & Velthuis, 1980; Crewe, 1988; Jones et al., 1999).

Solvent extraction has been used for compounds having relatively wide ranges of polarity and molecular weight (Ortner, 1999). Solvent extraction involves separation of glandular contents by partitioning of its components into an organic solvent. The glandular contents equilibrate with an organic solvent whereby analytes partition themselves between the sample matrix and the organic solvent according to their relative solubilities.

The efficiency of an extracting solvent depends on the affinity of the target analytes for the extracting solvent. Additives can influence the performance of the method. The addition of acid or base controls the pH to fractionate samples into neutral, basic or acidic fractions. The addition of a derivatising reagent was used to enhance detection and separation of components in the GC (Crewe & Velthuis, 1980; Crewe, 1988). Solvent extraction methods use larger volumes of solvent than needed for chromatographic analysis. Large solvent volumes used for extraction dilute the sample hence excess solvent is evaporated to concentrate the sample prior to analysis. The use of solvent is identified as a possible source of contaminants, and this may impair the results. The major drawback of solvent extraction is that the experimental subject is killed and subsequent changes in secretion quality or quantity cannot be monitored. Consequently if one needs to study changes in the secretion of an insect over

time, then improved methods of successive sampling from the same individual are necessary.

The aim of this work was to use a solvent extraction technique for the analysis of queen mandibular pheromone, as the reference method to the other newly developed techniques that were employed to sample mandibular gland pheromone.

MATERIALS AND METHODS

Experimental procedure

Extraction of mandibular pheromone

a) Mandibular pheromone standard

1 µl of the prepared mandibular pheromone standard (preparation of standard pheromone is detailed in chapter 2) and 1 µl BSTFA was injected into the gas chromatograph for analysis. Tetradecane and octanoic acid were added as internal standards.

b) Queen mandibular pheromone

Eleven honeybee queens were reared in our laboratory at University of Pretoria and kept in cages with a few worker bees in an incubator at ±35°C.

The heads of the queens were cut off and extracted in 200 μl dichloromethane. The extract was stored in the refrigerator at low temperatures (about 5°C) until needed for analysis. Solvent evaporation was used to pre-concentrate analytes in the organic solvents. The queen's head was removed from the vial and the 200 μl solvent was evaporated to about ±5 μl under a gentle stream of nitrogen. For the derivatised samples a mixture of 1 μl queen mandibular extract and 1 μl BSTFA was injected for analysis. For underivatised samples 1 μl of queen mandibular extract was injected for analysis. Tetradecane and octanoic acid were used as the internal standards for both analyses.

Chromatographic Analysis

Two GC-FID (HP 5890) systems fitted with columns of different polarities were used. There are significant advantages in analysing compounds on different stationary phases. Some peaks may be unresolved on one phase and be well resolved on the other phase (Jones *et al*., 1999).

The stationary phase influences the rate of migration of a component through a column. Chromatographic separation of analytes is achieved by the interaction of these analytes with the column coating. As the sample flows with the mobile phase through the stationary phase each component of the sample establishes a unique interaction with the stationary phase. Those components that have stronger interactions with the stationary phase will be retained longer hence

eluted last from the column. It is unlikely that two compounds with the same retention times on one stationary phase will have the same retention times on different stationary phases.

System 1 (derivatised sample)

A Hewlett-Packard 5890 gas chromatograph coupled with a flame ionisation detector and equipped with a capillary column coated with cross-linked 5% phenylmethyl-silicone (HP-5) (40 m x 0.32 mm) was used. The initial temperature was 60˚C for 1 min, programmed at 25˚C / min to 120˚C and again at 3˚C / min to 300˚C and held at this temperature for 5 min. The injection port was set at 250˚C and the detector at 300˚C. Chromatograms were recorded and peak areas quantified with HP ChemStation software®.

Low polarity gas chromatographic columns tend to adsorb polar compounds onto the column wall and preventing them from reaching the detector in a well defined peak (Maile et al., 1998). Most of the mandibular pheromone components are polar therefore derivatisation was required prior to analysis to facilitate their elution and detection on a low-polarity column (HP-5).

System 2 (underivatised sample)

A Hewlett-Packard 5890 gas chromatograph coupled with a flame ionisation detector was used. Professor B.V Burger of the Laboratory for Ecological Chemistry at Stellenbosch University in South Africa prepared a mid-polar column (40 m x 0.32 mm) coated with OV-1701-OH at film thickness of 0.375 μm that was used. The initial temperature was set at 40˚C for 1 min and programmed to 260˚C at a rate of 4˚C / min and held at this temperature for 10 minutes. The injection port was set at 250˚C and the flame ionisation detector at 300˚C. Chromatograms were recorded and peak areas quantified with a HP 3396 Series II integrator.

Chemical Analysis

The components of the extracts were identified by comparison of retention times with those of standard compounds.

RESULTS AND DISCUSSION

Engels and co-workers (1997) managed to identify more than 100 compounds in the extracts of honeybee queens. For this reason, analyses in this study were restricted to those compounds which are known to be biologically significant (see figure 1.1).

The mandibular pheromone dichloromethane extracts of virgin and mature queens were analysed using a low-polarity column and a mid-polarity column. A

successful separation of the derivatised honeybee queen mandibular extracts was achieved using GC-FID fitted with a low-polarity column. In the virgin secretion only 9-ODA and 9-HDA were detected at very low concentrations. This method of analysis demonstrated that the mature queen mandibular pheromone in figure 4.1 and virgin mandibular pheromone in figure 4.2 differ only in quantity of mandibular pheromone secretion. The variations in queen mandibular secretions related to age have been reported previously (Crewe, 1982; Slessor et al., 1990). The mature queen mandibular secretion in figure 4.3 had all target analytes in high amounts with 9-ODA dominating the mandibular secretion.

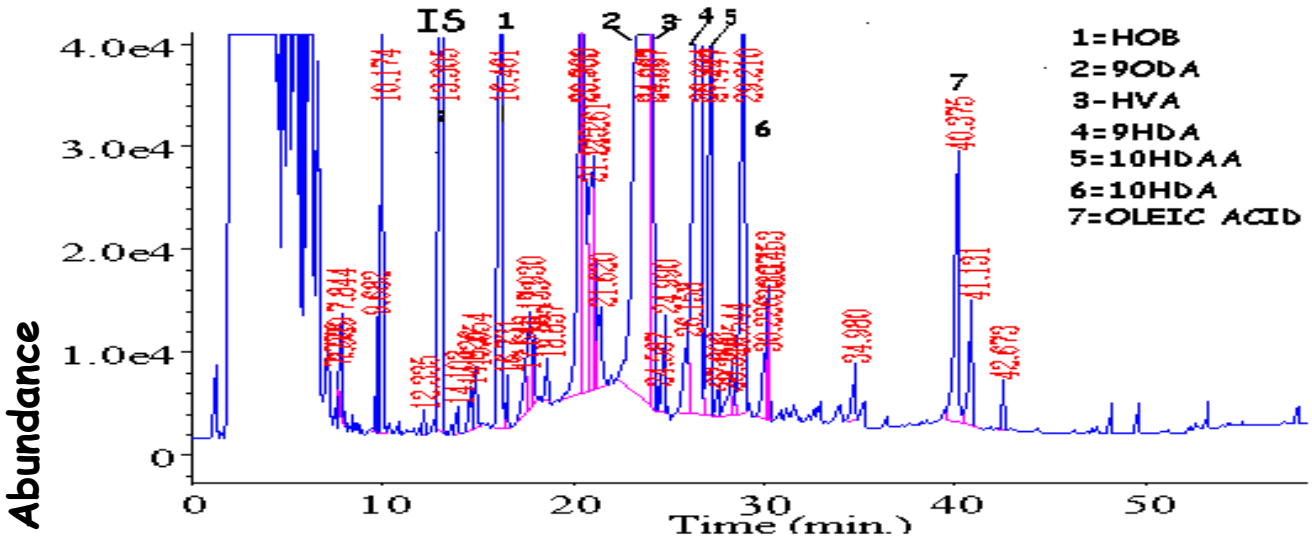


Figure 4.1. A chromatogram of derivatised mature queen pheromone extracted in dichloromethane and analysed on the HP-5 column. Methyl *p*-hydroxybenzoate (HOB), 9-Oxo-2-decenoic acid (9-ODA), 4-hydroxy-3-methoxyphenylethanol (HVA), 10-hydroxydecanoic acid (10-HDAA), 10-hydroxydecenoic acid (10-HDA)

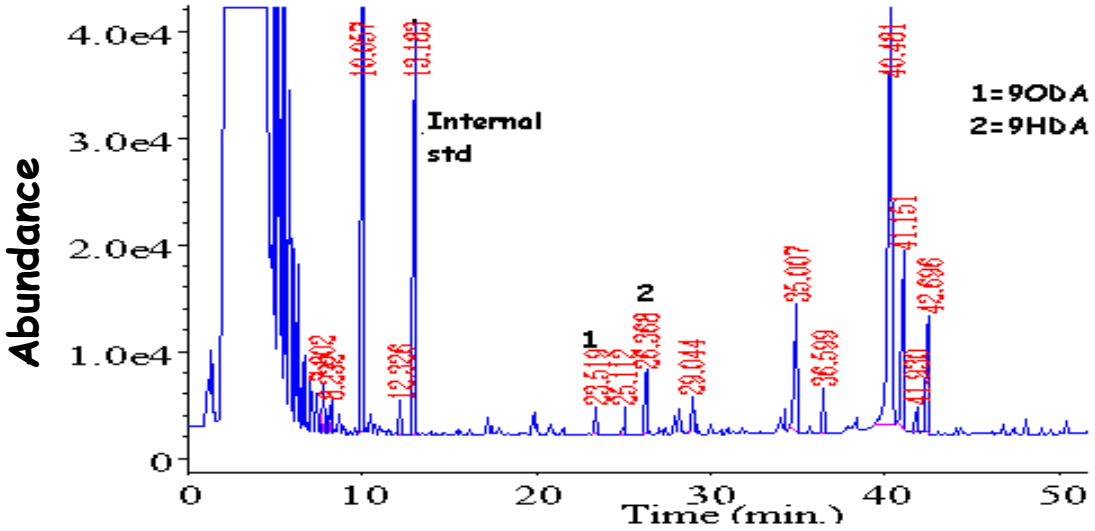


Figure 4.2. A chromatogram of a derivatised virgin queen pheromone extracted in dichloromethane and analysed on the HP-5 column. 9-Oxo-2-decenoic acid (9-ODA), 9-hydroxydec-2-enoic acid (9-HDA).

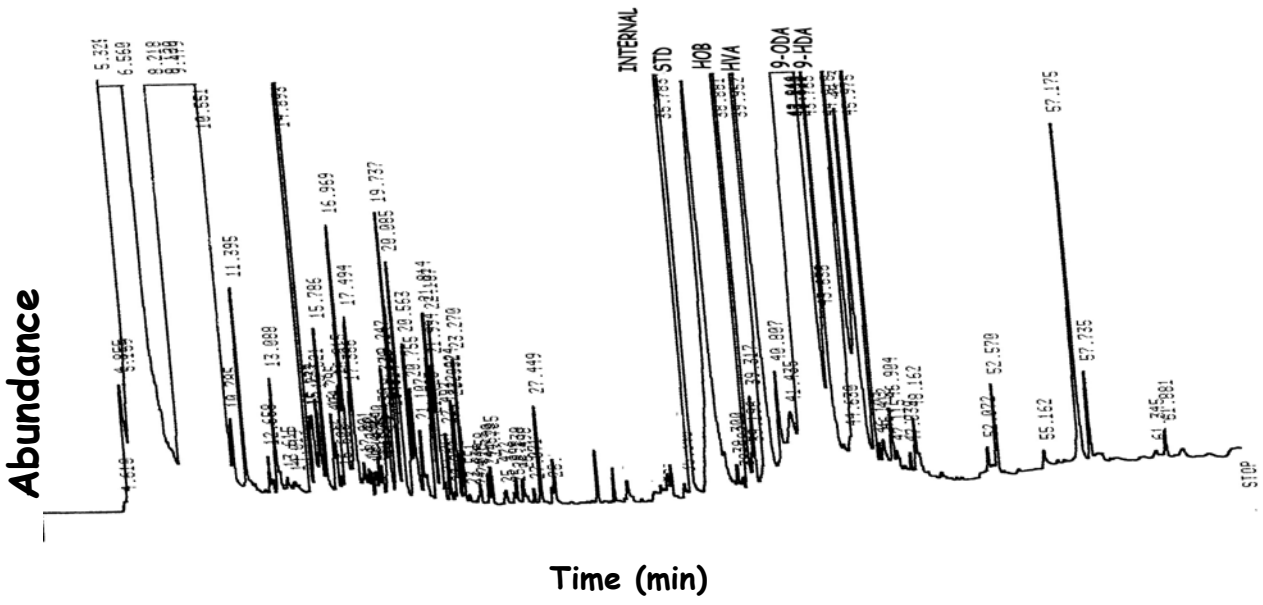


Figure 4.3. A chromatogram of mature queen pheromone extracted in dichloromethane and analysed on the mid-polar OV-1701-OH column. Methyl *p*-

hydroxybenzoate (HOB), 9-Oxo-2-decenoic acid (9-ODA), 4-hydroxy-3-methoxyphenylethanol (HVA), 9-hydroxydecenoic acid (9-HDA).

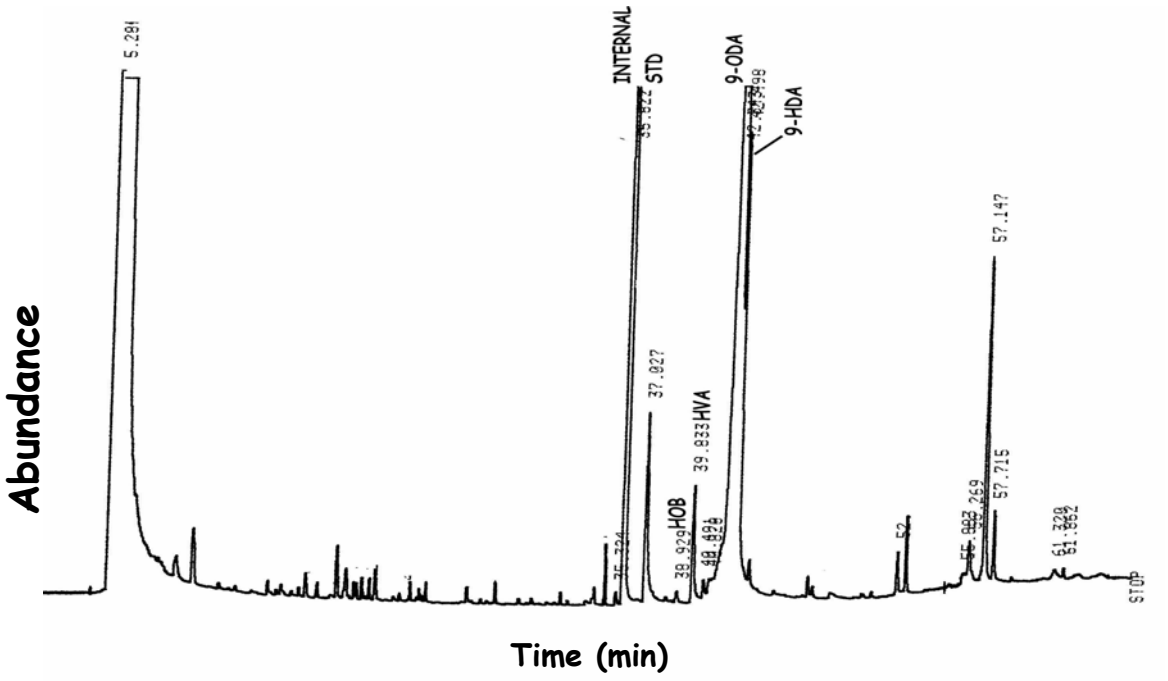


Figure 4.4. A typical chromatogram of virgin queen pheromone extracted in dichloromethane and analysed on the mid-polar OV-1701-OH column. Methyl *p*-hydroxybenzoate (HOB), 9-Oxo-2-decenoic acid (9-ODA), 4-hydroxy-3-methoxyphenylethanol (HVA), Hexadecanoic 9-hydroxydecenoic acid (9-HDA)

A successful separation of the mandibular pheromone was also achieved by equipping the GC-FID with a mid-polar column. The extracts of queen mandibular gland were analysed without using a derivatising reagent. All target analytes were detected in mature queen secretion (figure 4.3) in high quantities. The virgin queen secretion had HOB and HVA in very small amounts while 9-

ODA and HDA dominated the secretion. These identified compounds represent just a fraction of the compounds present in mandibular gland extract.

The main advantage of using the polar column is that the extracts do not need to be derivatised as was the case with the HP-5 column. The mature queen pheromone exhibited a number of components in its secretion while virgin mandibular secretion had fewer components with 9-ODA dominating the secretion.

Analyses of mandibular pheromone on mid-polar and low-polar column revealed 9-ODA as the major peak in both virgin (figure 4.2 and 4.4) and mature queen secretions (figure 4.1 and 4.3). The presence of 9-ODA as the major peak component of queen mandibular secretion has been reported in the past (Velthuis, Crewe, 1988; Slessor et al., 1990; Plettner, 1995; Winston, 1998). Elution of 9-ODA as an asymmetric broad peak in both columns presented a problem in peak quantification of other compounds HVA and 9-HDA. In the derivatised sample 9-ODA co-eluted together with HVA (figure 4.1) on the HP-5 column while the underivatised 9-ODA co-eluted with 9-HDA on the mid-polar column (figure 4.3 and 4.4). Co-elution of compounds gives inaccurate results where quantitative analysis is required.

CONCLUSION

The possibility of using the polar column to analyse the mandibular pheromone without derivatisation is a step forward in the elimination of the use of solvents. The difference in pheromone secretion quantities can be related to age and status of the queen as illustrated in this study. The mandibular gland secretion of honeybee queens is dominated by the presence of 9-ODA.

CHAPTER 5

GENERAL DISCUSSION

The analysis of pheromones in social insects has gained much interest over the years due to the importance of pheromones in regulating colony functioning (Slessor *et al*., 1990). Sampling of pheromones has been based on classical methods such as solvent extraction, from Porapak Q, or activated charcoal, however these methods are time consuming and require the use of toxic organic solvents for complete extraction of analytes. With new regulatory restrictions on solvents and their hazardous nature, new sampling techniques are phasing out solvent based techniques (Pawliszyn, 1995). The development of simple, inexpensive sampling methods are useful for routine monitoring and evaluating pheromone secretion in living insects.

Analysis of queen mandibular pheromones was undertaken with the emphasis on developing non-destructive sampling methods that would allow repetitive sampling of the same individual over a period of time. The developments include the use of sensitive, non-destructive, environmental friendly sampling methods based on a polymer-extracting phase that allows repetitive sampling of the same individual.

In this study two non-destructive sampling methods that do not require the death of the individual being sampled have been proposed for gas chromatographic analysis of mandibular gland pheromones. This chapter discusses the three methods used in sampling mandibular gland pheromone and their optimisation.

Solventless extracting methods using solid phase micro-extraction and silicone rubber tubing were evaluated for their efficacy in trapping compounds secreted on the surface of the mandibles. Purity is usually high in that compounds are directly analysed without tedious clean-up steps. With these methods an extracting phase is directly exposed to the sample for a well-defined period of time. The opportunity to extract directly from the glandular opening with the new techniques avoids possible contamination hence more reliable analyses are obtained.

The solid phase micro-extraction results are comparable to those obtained with standard solvent extraction methods. In both methods 9-ODA was present in abundance. Analytical attention was restricted to components of the mandibular pheromones that are known to have semiochemical significance.

The concept of using adsorbent material to extract trace components was developed by Pawliszyn, 1990 and Arthur *et al*, 1992. A sorbent with a strong affinity for organic compounds will retain and concentrate target compounds from a very dilute sample matrix. The new sampling techniques can be directly

inserted into the volatile source thereby minimising loses and simultaneously maximising sensitivity. An extent of selectivity is required for any sample preparation method. With selective extraction techniques there is less sample preparation required, however solvent extraction is a tried and tested method that has been useful in the isolation of pheromones for more than three decades.

Sample preparation methods should have good analytical performance with high selectivity and be applicable to various matrices. An ideal sampling method should be easy to use, inexpensive, rapid, highly selective towards target analytes and compatible with a range of analytical instruments (Pawliszyn, 1995).

Facts like whether the pheromone is released continuously or stored and only released when essential need to be assessed. Sampling with polymer-based techniques, glandular secretions were isolated directly from their sites of release at glandular opening. The methods do not require complete removal of analytes from the sample matrix because it is an equilibrium process whereby volatiles were separated from the matrix by extraction. In both methods there is partitioning of the analytes between the extracting phase and the sample matrix.
All methods identified the presence of the major component of the queen mandibular pheromone 9-ODA, in solid phase micro extraction and solvent extraction it was identified in abundance. Solid phase micro extraction generated good chromatograms that maintained stable baseline throughout the run, figure 2.4. Different sampling methods yielded different quantities of compounds

available in the queen mandibular gland. Relative amounts of chemicals emitted convey specific information to the receivers about the identity and physiological state of the emitter (Slessor, 1998).

The reasons for the observed difference between silicone rubber tubing (figure 3.3) and other methods used are not well understood. One possibility could be that the sampling time of five minutes is not long enough for the analytes to equilibrate with the tubing. The poor chromatograms obtained using silicone rubber tubing suggests that target analytes present could not be detected. In order to sample detectable pheromones one can increase the extraction time. With our experiment it was not practical for one to extract beyond five minutes because we did not want to increase the mortality of the bees.

The sampling procedure must be reproducible and give concentration factors that permit the detection limit of the gas chromatographic system to be reached (Alpendura, 2000). Most methods involve some sort of sample pre-treatment that is often a significant part of the overall procedure when evaluating an analytical method. A method with a minimum amount of sample pre-treatment and that produces results of acceptable accuracy and reliability is often given preference over the other methods.

Table 5.1. Comparison of the different sample preparation techniques used in this study: solvent extraction, SPME, and silicone rubber tubing.

Technique and principle	Drawbacks
Solvent extraction	
1. Head dissection	Kills the insect
2. Extraction in CH_2Cl_2	Use of organic solvents
3. Evaporation of excess solvent	Possible loss of volatiles
4. Re-dissolve the residue	Contamination due to solvents
5. 1 µl injection for analysis	
SPME	
1. Conditioning of fibre	Quality of fibres differs from batch to batch
2. Fibre derivatisation	Needs calibration
3. Extraction and enrichment	Memory effects
4. Thermal desorption for analysis	Thermal shock to labile analytes
	Bleeding of the fibre coating
Silicone rubber tubing	
1. Conditioning	Time consuming and use of organic solvents
2. Derivatisation	Needs calibration
3. Extraction	Use of organic solvents
4. Chemical desorption in glass vial	Contamination
5. Concentration of the organic solvent	Possible loss of analytes

6.	1 µl injection for analysis	Low recoveries

Silicone rubber tubing offers an advantage in that sampling can be done in the field away from the laboratory and stored for long periods without any special precautions. It is also very cost effective in that the pieces of tubing are significantly cheaper than SPME fibres. Silicone rubber tubing has increased permeability due to its 'openness'. The 'openness' of the tubing accounts for high diffusion rates exhibited by the tubing. Because silicone rubber tubing is still under study, we believe that it should be used in conjunction with other sampling techniques.

Solvent extraction is a well-established technique that we used as the reference method to test the reliability of the new non-destructive methods. Solvent extraction as a sampling technique is the simplest to apply and does not require method optimisation. However analysis by this method suffers serious drawbacks and is being phased out by the newly developed environmentally friendly methods. Though the method has shortcomings it is still recognized as a powerful technique in analysing glandular contents (Jones, 1999). The problem when using solvent extraction is that enrichment by evaporation is time consuming and often suffers contamination from solvents and that the volatile components of the mixture may be lost in the procedure.

General expansion of solid phase micro extraction and silicone rubber tubing applications is limited by the availability of appropriate instrumentation and coatings. If the mid-polar column that was used to analyse mandibular pheromone underivatised in chapter 4 could be used with one of these new techniques that has the right coating that can extract the pheromone without derivatisation then analysis of pheromone would be completed without any solvent contribution.

The methods were successful in isolating the mandibular gland pheromones. However differences in efficiency and sample recovery between the methods are well demonstrated by the results obtained, the increased sensitivity realised with solid phase micro-extraction in chapter two is noteworthy. The reasons for the profile differences between the methods could be:

1. Differences in extraction efficiency of the techniques,

2. Differences in physical structure of the technique, fibres are fused silica with a liquid phase coating while silicone rubber tubing is open tubing,

3. Non-reproduciblity in the desorption procedures.

The successful isolation of mandibular pheromones from living honeybee queens using the non-destructive methods proved that the techniques are very effective

in analysing emitted volatiles from living individuals without prior manipulation of the sample source. Being able to track daily secretions of mandibular pheromones would be advantageous to the beekeepers and researchers as they could track any changes that occur before, during and after '*Capensis* invasion'.

FUTURE APPLICATIONS

An ideal extraction method should be very simple, rapid and highly selective towards target compounds. The technique would require little or no sample preparation and offer an online analysis. With the new techniques, sampling and sample preparation steps are combined and this makes onsite analysis and process monitoring of pheromones possible. The new techniques like SPME and silicone rubber tubing can be used to study seasonal and short-term changes in the secretions of individuals. Daily pheromone secretion of queens (or any insect) can be followed and studied to monitor any changes in the pheromone bouquet to establish a signal or behavioural link.

The development and use of a wide range of selective and sensitive polydimethylsiloxane extracting techniques remains an active research area in our laboratory (Crewe, 2004). Currently we are exploring the use of silicone rubber tubing for remote sampling of honeybees. Once the analytes are trapped on the silicone rubber tubing they are sufficiently stable to allow their

transportation from the field to the laboratory. The greatest improvement over the classical methods hinges on sample preparation and isolation in the field where the sample (honeybee) is in its natural environment. The technique will enable the study of pheromones in the normal insect colony. The experimental applications are not restricted to sampling caged bees. We believe this work can help to reveal the dynamics of the complex chemical nature of pheromone emission and composition.

ABBREVIATIONS

ANOVA	Analysis of variance
BSTFA	Bis(trimethylsilyl)triflouroacetamide
CH_2Cl_2	Dichloromethane
DVB	Divinylbenzene
DMDCS	Dichlorodimethylsilane
GC	Gas chromatography
GC-FID	Gas chromatograph fitted with flame ionisation detector
GC-MS	Gas chromatography-mass spectrometry
HDA	hexadecanoic acid
9-HDA	(+/-)-9-hydroxydec-2-enoic acid
10-HDAA	10-hydroxydecanoic acid
10-HDA	10-hydroxydecenoic acid
8-HOA	8-Hydroxyoctanoic acid
HOB	methyl *p*-hydroxybenzoate
HVA	4-hydroxy-3-methoxyphenylethanol
9-ODA	9-oxodec-2-enoic acid
PDMS	Polydimethylsiloxane
SPME	Solid phase micro-extraction

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