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A survey of the levels of pesticides in bees, their colonies and forage

A thesis submitted by

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Abstract

Bees provide important and economically valuable pollination services to agriculture and other ecosystems. Recent global honeybee declines have been attributed to pesticides, which can ultimately affect the overall functioning and survival of a colony. Various routes of exposure include contaminated beeswax, pollen and nectar. This thesis presents work which examines the presence, accumulation and levels of pesticides found within a number of honeybee related matrices and bumblebee bodies, with four main aims to this study. Firstly, determine which pesticides are contained within beeswax from around the UK. Secondly, monitor which pesticides accumulate in beeswax over a two year period. Thirdly, measure residual neonicotinoid levels in oilseed rape (OSR) nectar and pollen samples. Finally, quantify the levels of thiamethoxam and metabolite clothianidin in bumblebees, following feeding trial exposure, as part of a collaborative study. Analysis was conducted using gas chromatography–mass spectrometry (GC-MS) and quadrupole time-of-flight liquid chromatography-mass spectrometry (Q-TOF LC/MS), utilising the Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) extraction method. Bumblebee samples were extracted using a refined in-house procedure. Results evidenced apiculturally applied chemical treatments to be most likely found in beeswax samples whilst tau-Fluvalinate was most persistent and likely to accumulate in the hive, including foundation wax not exposed to such treatment. Varied levels of neonicotinoids were identified in hand-collected OSR nectar samples; and there require a greater level of analysis, to further understand the implications of these results on UK bees. No quantifiable levels were detected in pollen. Bumblebee analysis determined possible levels of exposure to thiamethoxam during feeding. This thesis provides the first known attempt of identifying pesticide presence and accumulation within UK beeswax, in addition to the levels within UK OSR nectar and pollen. The findings may have wider implications on the beekeeping community. Also presented are various methodologies suitable for future research.

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"Time can't erase the magic of these magic moments…" – Perry Como

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List of abbreviations

ACN	Acetonitrile
Ar	Argon
β^-	Beta particle
BBKA	British Beekeepers Association
Br	Bromine
°C	[Degrees] Celsius
C18	[Chromatography column stationary phase] Octadecyl carbon chain
CI	Chemical ionisation
CID	Collision-induced dissociation
CUCARB	Graphitized carbon black
D (^{2}H)	Deuterium
Da	[Dalton] (the unit used to express molecular weight)
DC	Direct current
e^-	Electron
eV	Electron volt
ECD	Electron capture detection / detector
EFSA	The European Food Safety Authority
EI	Electron ionisation
EIC	Extracted-ion chromatogram
ESI	Electrospray ionisation
EU	European Union
F	Fluorine
FERA	Food & Environmental Research Agency
Fung	Fungicide
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
He	Helium
Herb	Herbicide
HPLC	High performance liquid chromatography
IGR	Insect growth regulator
Ins	Insecticide
IRM	Internal reference mass
K^+	Potassium ion
K	Partition coefficient
L	Litre
LC	Liquid chromatography
LC/MS	Liquid chromatography-mass spectrometry
LD_{50}	Lethal dose 50
L/min	litre per minute^{-1}
LOD	Limit of detection
LOQ	Limit of quantification
$[M]^+$	Molecular ion
$[M+H]^+$	Molecular ion plus proton
$[M+Na]^+$	Molecular ion plus sodium ion
$[M+K]^+$	Molecular ion plus potassium ion
MALDI	Matrix assisted laser desorption ionisation

MeOH	Methanol
$MgSO_4$	Magnesium sulphate
Min	Minute
ml	Millilitre
Mollus	Molluscicide
MRM	Multiple reaction monitoring
MS	Mass spectrometry/spectrometer
MS/MS	Tandem mass spectrometry
m/z	Mass-to-charge ratio
N_2	Nitrogen
Na^+	Sodium ion
N/A	Not applicable
Ng	Nanogram
^{63}Ni	Nickel (radioactive isotope)
NH_4HCO_2	Ammonium formate
NaOAc	Sodium acetate
ND	Not detected
NMR	Nuclear magnetic resonance imaging
NSBKA	North Staffordshire Beekeepers Association
Nut	Nutrient
PDCB	*para*-dichlorobenzene
pKa	[acid dissociation constant]
ppbv	Parts per billion by volume
ppmv	Parts per million by volume
ppm	Parts per million (mass accuracy)
PSA	Primary-secondary amine
QuEChERS	[Extraction method] Quick, Easy, Cheap, Effective, Rugged and Safe
Q-TOF	Quadrupole / time-of-flight
PER	Proboscis extension reflex
r^2	Regression coefficient
R	Mass resolution
RF	Radio frequency
SIM	Selective (single) ion monitoring
SPE	Solid phase extraction
SRM	Selected reaction monitoring
T (^3H)	Tritium
TIC	Total ion chromatogram/current
TE	Trace element
TOF	Time-of-flight
µg	Microgram
µl	Microlitre
UK	United Kingdom
USD	United States Dollar ($)

CHAPTER 1
Introduction

1.1 Overview

In its role as a pollinator the honeybee (*Apis mellifera*) plays a fundamental role in the functioning of nearly all existing terrestrial ecosystems, including those that are agriculturally dominated (Thompson, 2003). In 2007 the value of the honeybee to the UK agricultural sector, through increased output, was estimated to be worth between £165 million (Aston, Carreck, Ivor, Lovett, & Metcalf, 2009) and £200 million (Cuthbertson & Brown, 2009) per annum, whilst a total pollinator loss could cost around ~£400 million a year (Ross & Wentworth, 2010). The overall annual monetary value of bees globally is an estimated $212 billion (USD) (vanEngelsdorp & Meixner, 2010). Huge bee losses could threaten the supply of around 100 pollinated crop types (Dötterl & Vereecken, 2010), equating to 35 % of the global food production (Genersch, 2010). Natural biodiversity

could also be threatened, should the rate of pollination in wild flowers decline (Dötterl & Vereecken, 2010; Genersch, 2010).

Extensive honeybee declines, particularly during the past few decades, have been recorded across much of the world (Neumann & Carreck, 2010). The United Kingdom (UK) is also no exception to this and has seen a reduction in the number of honeybee hives by around 54 %, which is threatening the UK's ability to cope with changes in the demand for pollination services (Breeze, Bailey, Balcombe, & Potts, 2011). The current world decline of honeybees and other bee species has initiated significant research efforts in order to ascertain suitable explanations behind its occurrence (Genersch, 2010; Neumann & Carreck, 2010) as well as calls for action from Governments, land managers and conservationists (Ghazoul, 2005).

The honeybee can be considered a well-studied species. As of 2006 the honeybee became the first hymenopteran and only the fifth insect to have its genome fully mapped (Robinson *et al.*, 2006), whilst exploration into the understanding of honeybee communication was conducted by von Frisch in 1953 (von Frisch, 1954). Study of the honeybee need not be considered a recent thing, as previous work dates back to as early as 1609 (Butler, 1609). Yet despite our understandings into the honeybee, the reasons behind their decline are still not fully understood (Aston *et al.*, 2009); as no single factor has been identified as being responsible for all the simultaneous declines witnessed all over the world. Pesticides are believed to be one of the key drivers behind pollinator declines. Using sensitive analytical equipment, i.e. liquid chromatography – mass spectroscopy (Chapter 2), the presence of pesticides in honeybee hives (Chapters 3 and 4) and the environment (Chapter 5) can be determined.

The continued decline in bumblebee numbers has seen a surge in bumblebee related research. This includes studies into their susceptibility to pesticides as well as other efforts to re-establish various extinct UK species. Although this body of work will predominantly focus on the honeybee*,* Chapter 6 investigates the amount of two pesticides (thiamethoxam and clothianidin) found within individual buff-tailed bumblebee (*Bombus terrestris*) worker bees following feeding trials.

In order to understand how honeybees are affected by pesticides, it is important to consider their individual biology and hierarchy within a colony, their domestication, as well as the individual components of a hive.

1.2 Social bees

Within the UK there is a total of 276 bee species found in the *Apidae* Family, including many bumblebee species and the hugely recognised honeybee (all of which demonstrate varying levels of sociality), as well as over 220 species of solitary bee (Breeze *et al.*, 2011). Globally there are over 16,000 different species of bee (Michener, 2007), of which only a few thousand are considered to be 'social', whilst the remaining numbers are of a solitary nature. Social insects are thought to have evolved to adapt their morphology, physiology and behaviour in order to carry out such tasks which contribute to the overall success of a colony and not necessarily benefit the individual (Seeley, 1985). A colony is defined by two or more females, irrespective of their social relationship, cohabiting within a single nest (Michener, 2007). These females are distinctly identified as *(1)* a single queen, who is sexually reproductive and performs most or all of the egg laying within the colony; and *(2)* one or more workers, who are unmated and carry out duties such as brood care and

foraging for food supplies (Michener, 2007) amongst other duties. A characteristic found in social bees is that workers are able to produce enzymes which enable them to create a storable food reserve in the form of honey (Crane, 1990).

Those classified within Apidae are characterised by the presence of a pollen basket (corbicula) on their hind legs; this includes bumblebees (Bombini), stingless bees (Meliponinae) and of course the honeybee (Apini). However, the queen of both honeybee and stingless bee species no longer retains her corbicula (Butler, 1954). Figure 1.1 shows the taxonomic relationship between the honeybee (Genus: Apis) and the bumblebee (Genus: Bombus) both of the family Apidae, within the order of Hymenoptera. As stingless bees are predominately found in tropical and sub-tropical regions of the world (Bradbear, 2009) they shall not be covered any further in this thesis.

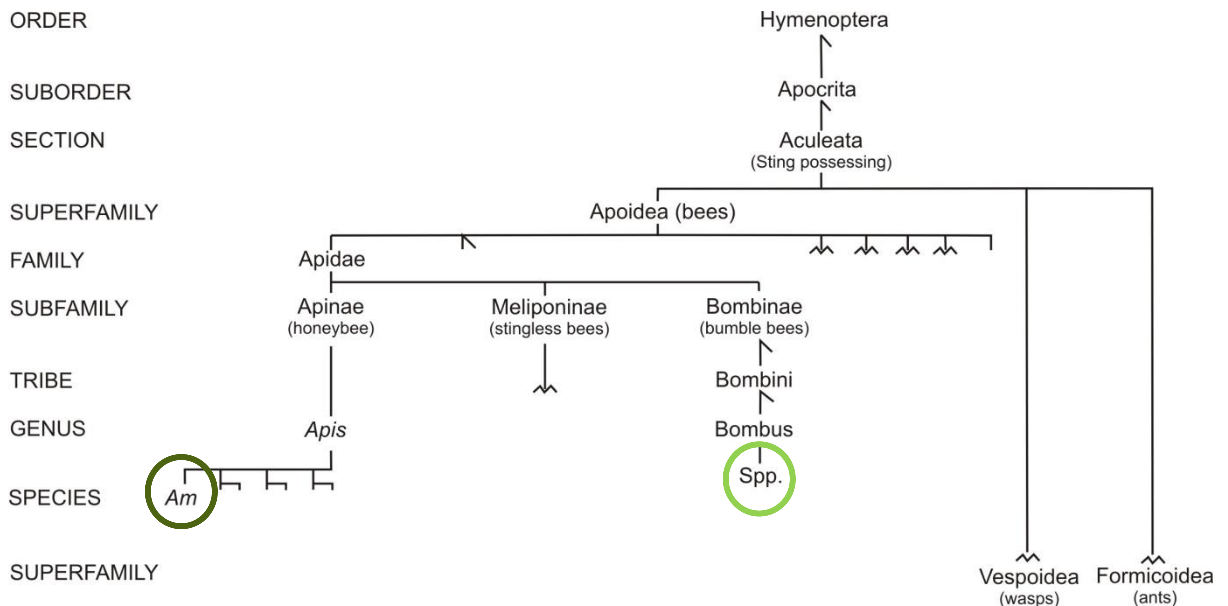

Figure 1.1: Diagram showing Taxonomy of insects found in Order Hymenoptera. An unnamed branch indicates one or more members which are not discussed in this thesis. *Am* - *Apis mellifera* (honeybee), spp – indicates multiple bumble bee species, including Bombus terrestris. Information redrawn and adapted from Crane (1990).

Nowadays there are five recognised species of honeybee, all classified within the genus Apis (Winston, 1991); yet of these, it is the western honeybee (*Apis mellifera*) that the laymen reader is probably more familiar with (Seeley, 1985). Several different races of the Apis species exist in Europe, Africa and the Orient (Alaux, Ducloz, Crauser, & Le Conte, 2010; Winston, 1991). Beekeepers will choose to keep a certain race of honeybee according to their respective differences in honey production, temperament, longevity and so on. For example, Italian bees (*Apis mellifera ligustica*) are often selected by amateur apiarists, due to their gentle and unaggressive nature (Head, 2010). In contrast, the Africanised honeybees (*Apis mellifera scutellata*) are particularly famed for their aggression (Crane, 1990) and are often referred to as 'killer bees' (Kevan, Clark, & Thomas, 1990).

1.3 Introduction to the honeybee

1.3.1 Development and colony hierarchy

Honeybees live in colonies of up to 80,000 bees with a single queen (Grunewald, 2010) making then highly eusocial (Michener, 2007). Having lost the predatory instinct of its sphecoid wasp ancestor, over 100 million years ago, the honeybee now chooses to collect provisions for its colony through the collection of pollen and nectar (Crane, 1990; Grunewald, 2010; Michener, 2007).

Morphologically, the queen and worker bees are different to one another (Michener 2000); despite being larger in size, some of the features of the queen are inferior or absent to those found in the worker bee. As the queen is not required to forage she lacks a corbicula and has a shorter proboscis (tube used to feed on nectar), meaning that the

queen is effectively unable to feed herself (Crane, 1990). The absence of wax glands also restricts a queen from building cells in which to deposit an egg (Crane, 1990). The queen however possesses a retractable sting, unlike the workers, meaning that she will generally not die after stinging (Crane, 1990). As a result of the colony's social evolution and structure, neither the queen nor a worker, away from a viable colony, are able to survive alone (Michener, 2007).

The queen controls the quantity of workers and drones within a colony, as her primary duty within the hive is to keep the colony populated through egg production. A virgin queen can make between one to five mating flights, where she can mate several times in mid-air by male bees called drones in regions known as congregation areas (Winston, 1991). The sole function of a drone is to mate (Dines, 1968) and following intercourse they die immediately (Seeley, 1985). The queen then returns to her hive where she is cared for by worker bees, who feed her mouth-to-mouth, till her death or supercedure (Winston, 1991). As shown in Figure 1.2, an unfertilised (haploid) egg will produce a drone, whilst a fertilised (diploid) egg will be female and will therefore be a worker or queen, depending on her upbringing (Winston, 1991). Approximately 90 % of a queen's offspring are female (Seeley, 1985).

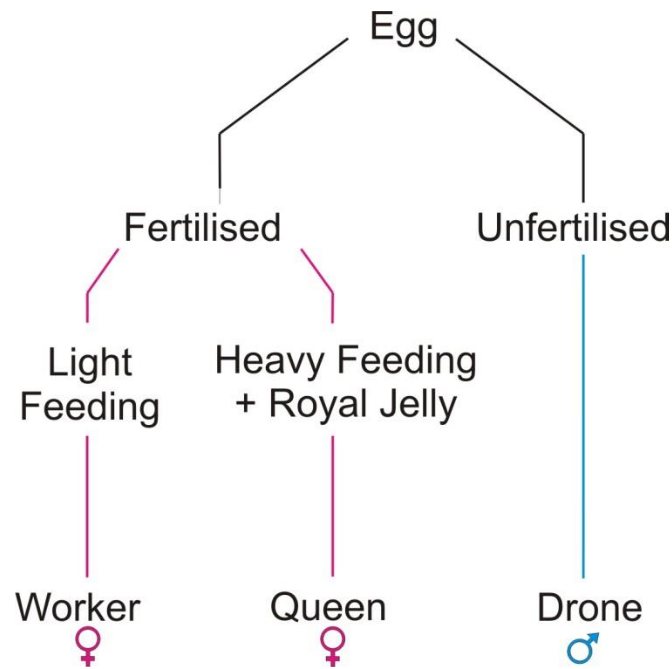

Figure 1.2: Determining factors for the variation between queen-laid eggs into workers, drones or queens. Redrawn and adapted from Winston (1991).

As presented in Figure 1.3, each caste (worker, drone and queen) goes through four major stages of development: egg, larva, pupa and adult. The time it takes to go from an egg to a fully emerged adult can take ~16 days for queen, ~21 days for workers and ~24 days for drones, although these times can vary depending on environmental conditions and nutrition (Winston, 1991).

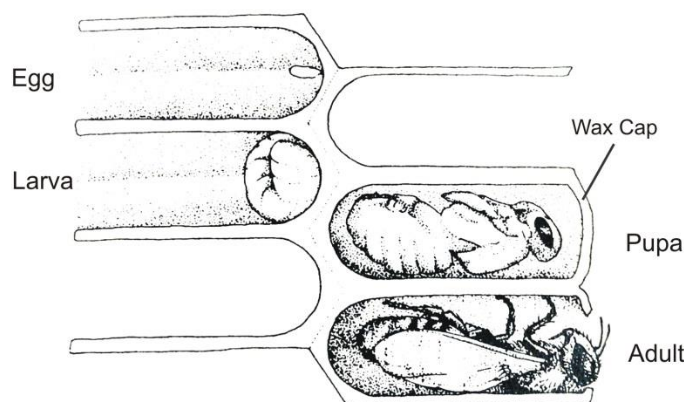

Figure 1.3: The major stages of honeybee development: egg, larva, pupa and adult. Adapted from Winston (1991).

Let us consider the development of a worker bee: once the egg has developed into a larva, at around 4 days, it is then tended for by young (nurse) bees. During the larval stage (days 4 – 8) the bee is fed a large amount food and grows rapidly in size and weight. Once at full size, the larvae are then capped within their cell (day 9), where they then spin a cocoon and pupate. The pupa will now begin to metamorphose into an adult (days 11 - 21). At day 21 the adult will then remove the wax cap from their cell and continue to develop for the next few days (Winston, 1991).

If a new queen is needed, a diploid egg would be set within or relocated from a worker cell to a larger queen cell. The need for a new queen can be due to a number of reasons, including injury, poor health or a reduction in the amount of pheromones she produces (Hepburn, 1986). During the larval stage, a specially administered worker secretion called royal jelly is given at a much higher rate to this designated queen larva than that of worker bee larvae; a low level of royal jelly results in the failed development of sex organs and thus creating normal workers (Dines, 1968). Ovarian development in adult workers is further supressed by the presence of a queen, who releases a pheromone known as queen substance to control worker behaviour, growth and reproduction (Hepburn, 1986).

A honeybee colony displays an impressive hierarchy of age related tasks amongst the worker bees; which, as the name suggests, conduct virtually all of the tasks within the nest (Winston, 1991). Upon emergence from their cell, worker bees immediately begin to perform in-nest tasks which are divided between workers based on individual age (polyethism) and associated muscular (Crane, 1990) and glandular development (Winston, 1991); which is presented in Figure 1.4.

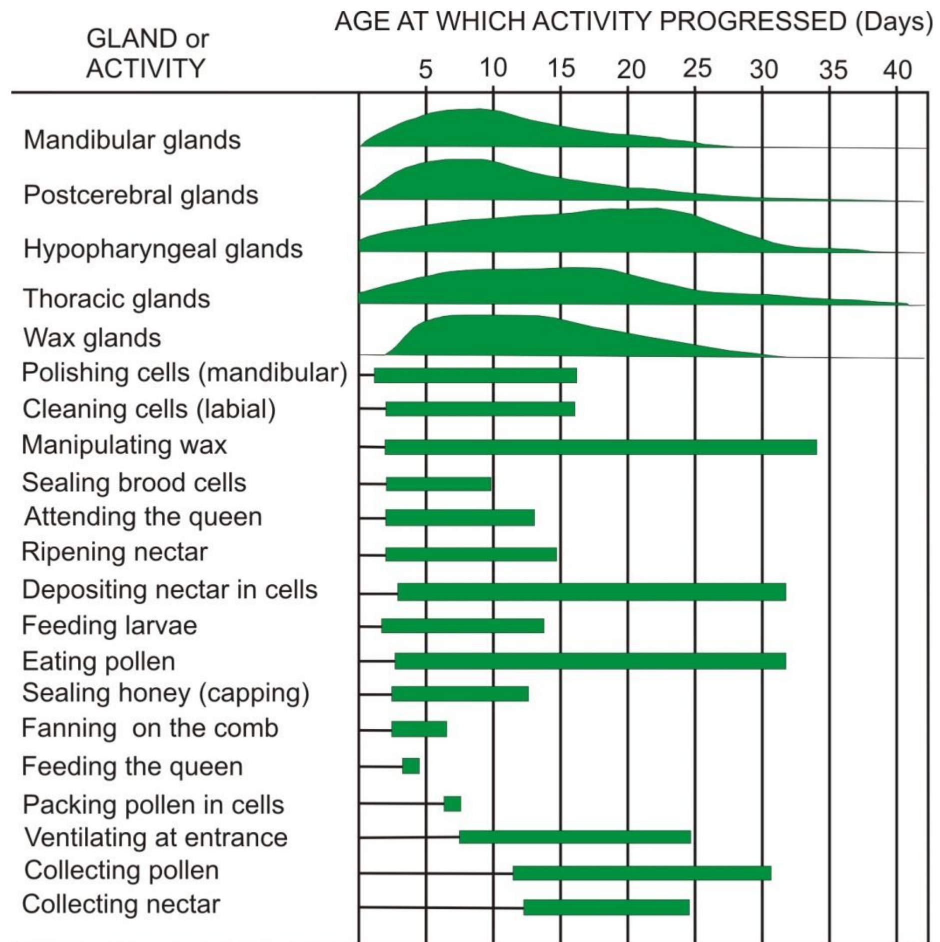

Figure 1.4: The relationship between age-related tasks and glandular development in honeybees. Redrawn and adapted from Winston (1991).

Variance in juvenile hormone levels at each developmental stage is shown to have a bearing on the growth and degradation of certain glands which in turn bears influence on worker responsibilities within the hive (Winston, 1991). 'Immature bees', therefore, would be involved in the cleaning of cells and then progress [with age] to tending the brood and queen before finally foraging until the end of their lives at around 30 to 50 days (Seeley, 1985; Winston, 1991). Collectively workers are involved in a number of tasks within the hive (Winston, 1991). Workers gather nutrition by foraging on flowers, in order to obtain quantities of nectar and pollen, which can then be stored within the hive. Foraging is regarded as a 'complex phenomenon' as it involves numerous 'coordinated

individual performances' (Colin *et al.*, 2004); including olfactory perception, memory, navigation and communication (Thompson, 2003). Whilst foraging, bees also participate in the sexual reproduction cycle of angiosperm plants via the transfer of pollen between flowers whilst collecting floral rewards (pollen, nectar, oils and resins) (Dötterl & Vereecken, 2010). At present it is believed that foragers can cover around several tens of kilometres in order to gather nectar and pollen for the hive (Rortais, Arnold, Halm, & Touffet-Briens, 2005). The location of these pollen sources are conveyed by returning foragers by using a form of communication referred to as dancing (Section 1.3.4.3, p25).

1.3.2 Beeswax

A key constituent of a highly eusocial honeybee colony is the wax that they produce (Winston, 1991), as it plays a crucial role in their survival (Michener, 2007). Wax is produced by other social bees such as bumblebees (Tribe: Bombini) and stingless bees (Tribe: Meliponini) (Michener, 2007); although the quantity of wax produced by these bee species is much smaller than that of the honeybee. Although other social bees produce wax, beeswax often refers to that produced by *A. mellifera* (Crane, 1990) and unless otherwise stated and it is in this sense that the term will be used throughout the rest of this thesis. A nesting site can be established in naturally occurring cavities (e.g. trees) or artificial hives, of which both offer a shelter for honeybees. Beeswax forms the internal structure of the nest (Berry & Delaplane, 2001), providing an area to house developing brood and to store pollen and nectar/honey (Michener, 2007). Domestication of honeybees has led to the development of artificial hives (Section 1.3.5, p29) that incorporate many of the features of a feral honeybee nest (Winston, 1991). Beekeepers

regard beeswax as an important commodity and are able to reuse or recycle old comb wax in order to help increase honey yields as well as providing a source of income.

1.3.2.1 The role of wax in the hive

Through its domestication, the honeybee is most likely to establish its nest in an artificial hive, which is designed to include many of the features of a feral honeybee nest (Winston, 1991). Beeswax is constructed into comb wax, which provides the internal structure of the nest (Berry & Delaplane, 2001). Comb is constructed vertically within the hive, with a specific spacing between each comb which allows bees to move freely throughout the hive (Crane, 1990). Each cell is also constructed at a particular angle, ensuring that food stores will not pour out of the cells (Winston, 1991). Comb wax also provides an arena on which honeybees are able to communicate (von Frisch, 1954); whilst its chemical composition plays a role in social recognition (Breed *et al.*, 1995).

1.3.2.2 Wax synthesis

Honeybees synthesise wax within wax glands which are modified epithelial cells. These glands are most developed around 5 – 15 days old (Figure 1.4) (Winston, 1991), before reducing in size when wax is no longer being synthesised (Bogdanov, 2009). The glands are located on the underside of the abdomen (Crane, 1990) and are covered by overlapping abdominal plates called wax mirrors. Liquid wax is secreted onto these wax mirrors where it then solidifies to form wax scales/flakes (Winston, 1991). Each wax scale weighs around 1 mg (Bogdanov, 2004). Around one million wax scales are needed to produce one kilogram of wax, with a worker being able to produce eight wax scales every

twelve hours (Bradbear, 2009). Upon the introduction to a hive, honeybees will immediately begin to produce beeswax in order to construct comb (Seeley, 1985; Winston, 1991).

1.3.2.3 The energetic cost and influences of wax production

The sugars obtained from honey i.e. sucrose, fructose and glucose are the main raw materials needed for wax synthesis. The ratio of sugar consumption to wax production can vary dramatically. Historical research (1788 - 1940) presented in Hepburn (1986) states experimentally determined ratios to be 1.8:1 to 104:1. The varied range seen in calculated ratios may be explained by poor experimental procedure, while others lack explanation (Hepburn, 1986). In the case of the former, some experimental colonies had a surplus of sugar solution, meaning that the calculated ratio would have perhaps been over estimated (Hepburn, 1986). Wax is known to be produced in abundance during colony establishment (Seeley, 1985). This is consistent with findings from (Whitcomb 1946, as cited by Hepburn 1986) who observed a high initial sugar to wax ratio of 104:1, before falling to an accumulative running ratio of 8.4:1 after around two months. This would again be consistent with the gradual development of the colony. A later set of experiments by (Weiss 1965, as cited by Hepburn 1986) appears to offer the most accurate experimentally determined ratios of between 3.3:1 and 16.2:1. His experiments also acknowledged a lower consumption of honey (g/per bee) in larger colonies than smaller colonies (Hepburn, 1986). The high energy cost involved in wax production means that it is only synthesised when it is deemed necessary by the colony (Butler, 1954).

There are multiple factors which stimulate the production of wax, including:

1) *Temperature*
2) *Nectar flow*
3) *Pollen availability*
4) *Queen presence*
5) *Brood rearing*

Other influential factors on wax production are reviewed elsewhere (Hepburn, 1986, 1997).

1) Temperature

The rise in ambient temperature, to around 10 °C, outside of the hive was deemed sufficient enough to stimulate comb construction; while a higher temperature of 15 °C favoured sustained comb-building (Koch 1961, as cited in Hepburn 1986). The secretion of wax and production of comb will decline towards autumn and terminate in winter months; this indirect correlation suggests that bees either choose not or will not sustain thermo-regulation of the hive and comb construction. It is difficult to place emphasis on the role that temperature plays alone as it does coincide with other factors such as nectar flow; however, the availability nectar is also closely linked to temperature (Hepburn, 1986).

2) Nectar flow

Wax production and nectar flow are directly linked (Huber, 1814). During a nectar flow there is a need for additional comb space in order to store incoming nectar, providing

that there is enough space to expand existing comb or to build new comb within the hive (Butler, 1954). Wax production is reduced when no nectar is available. Likewise, if bees are denied from foraging then comb-building is reduced. If pollen is available, yet nectar is not, then comb production stops (Hepburn, 1986).

3) Pollen availability

Not only is pollen important in the growth and development of the brood, it also plays an essential role in the production of wax (Bogdanov, 2009; Hepburn, 1986). Historical research discussed by Hepburn (1986) reveals that colonies deprived of pollen fail to produce wax, correlating with the degradation of the wax glands. Equally, an early exposure to pollen ensured rapid development of the wax gland and thus a greater potential for the production of wax.

4) Queen presence

The queen substance released by a healthy laying queen will influence the behaviour and physiology of workers (Maisonnasse *et al.*, 2010). The strong release of pheromones by the queen will suppress the construction of queen cups (Hepburn, 1986; Maisonnasse *et al.*, 2010). As a queen ages the levels of pheromones she produces will decline, which will lead to her eventual replacement and therefore the need for the construction of queen cells (Winston, 1991). However, in the absence of a queen, wax will not be produced unless brood is available from which to rear a new queen. This would result in the construction of emergency queen cells (Hepburn, 1986). It has been found that the presence of a queen can significantly increase the number of cells and thus the size of a

comb. It was also discovered that the queen controlled the size of the cells constructed; these were mainly found to be worker cells (Maisonnasse *et al.*, 2010).

5) Brood rearing

The amount of brood produced is enhanced during a nectar flow (Hepburn, 1986). As a consequent of more eggs being deposited into cells more comb space is required to house the developing brood (Bogdanov, 2009). The need to cap brood during their development is also a stimulus for wax production (Hepburn, 1997).

1.3.2.4 Comb construction

Using their mandibles (Winston, 1991), legs and antennae (Bauer & Bienefeld, 2013) worker bees manipulate secreted wax scales to produce comb wax. This occurs at a well thermo-regulated temperature of 35 °C (normal hive temperature) (Winston, 1991), although some bees will increase their body temperature in order to assist in shaping of the wax (Bauer & Bienefeld, 2013). The resulting comb is a collection of back-to-back hexagonal cells (Figure 1.5) made with exact precision. Worker cells generally have a diameter of 5.2 - 5.4 mm (Winston, 1991) and a cell depth of 15 mm; equating to a total volume of 360 mm^3 (Tremolada, Bernardinelli, Rossaro, Colombo, & Vighi, 2011). The thickness of cell walls and bases are 0.073 mm ± 0.008 mm and 0.176 mm ± 0.028 mm (Tremolada *et al.*, 2011). The angle of adjacent cells is exactly 120°, whilst the elevated angle from the base of each cell is 13° in order to avoid stores from pouring out (Winston, 1991). Drone cells are slightly larger than worker cells and are therefore mostly found clustered on comb edges, this allows for a more uniform comb construction thus

providing strength (Winston 1990). Despite their size differences, both drone and worker cells are used in the storage of food stuffs.

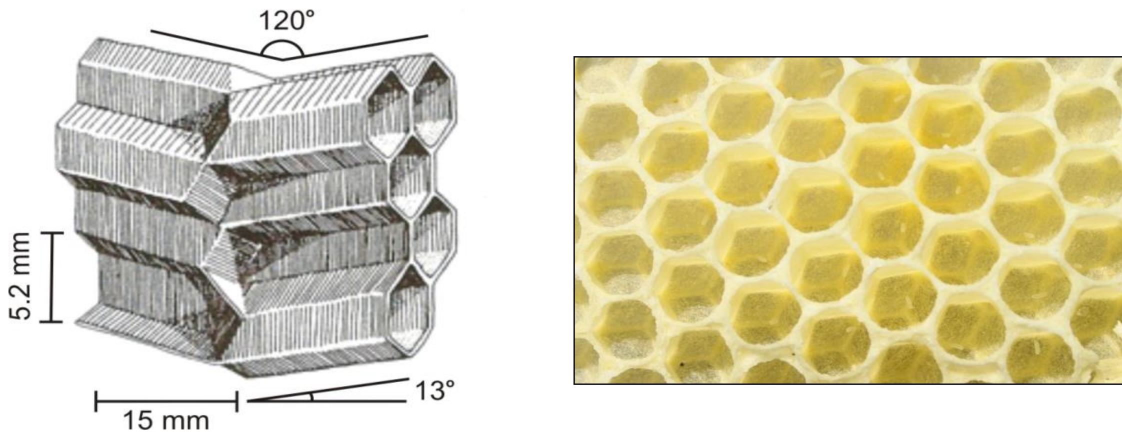

Figure 1.5: (L*eft)* A schematic display of the back-to-back and hexagonal construction of comb wax and the elevated angle of each cell. Dimensions are meant as a guide – not to scale. Adapted from Winston (1991). *(Right)* A demonstration of the repeating hexagonal shape of comb cells, cells also contain newly laid eggs. Photograph by K. D. Wisniewski (2011).

Pirk *et al.,* (2004) argue that the hexagonal shape of these cells in the comb arises as a result of the wax heating up and flowing around a closed packed arrangement of cylindrical cells and thus creating the 'illusion' of hexagonal cells. This was later disproved, as it was confirmed that the base of cells were hexagonal (Hepburn, Muerrie, & Radloff, 2007) as a result of mechanical shaping (Bauer & Bienefeld, 2013). Cells which do not possess the traditional hexagonal shape are queen cups. These are normally found hanging vertically on comb edges. Once they have had an egg deposited in them, workers will begin to extend these cups into tapered cells (queen cells), elongating them to a final length of 25 – 29 mm. These structures are especially constructed for the development of a new queen and are only found during the supercedure of a failing queen or during swarm preparation. Queen cells are deconstructed once the queen has emerged and the wax reused elsewhere in the hive (Winston, 1991).

1.3.2.5 Physical properties and chemical composition of beeswax

Newly produced comb wax shows great strength, as 1 kg of constructed comb can hold 20 – 22 kg of honey (Bradbear, 2009; Winston, 1991) and this strength is known to increase as the comb becomes adulterated/takes on other compounds over time (Hepburn & Kurstjens, 1988). The success of the comb's strength comes from the right combination of materials and geometry (Buchwald & Greenberg, 2004) as well as the temperature to which it is exposed (Buchwald, Breed, & Greenberg, 2008; Hepburn & Kurstjens, 1988). Beeswax possesses a crystalline structure at lower temperatures and becomes progressively amorphous as temperatures increase. During the construction from wax scales to comb there is also a witnessed change both mechanically and chemically. Newly formed wax also possesses a highly crystalline structure which is altered during mandible manipulation (chewing), thus making the wax easier to handle (Hepburn, 1986).

Compared to other insect waxes, beeswax has a relatively low melting point, with a range of 61 °C to 66 °C (Crane, 1990; Tulloch, 1980), while the onset melting occurs at around 37 °C (Buchwald *et al.*, 2008); therefore, beeswax is stronger at lower temperatures. At 35 °C beeswax is supple and demonstrates a level of plasticity which allows for construction of large combs - a property that is also observed at temperatures as low as 32 °C (Crane, 1990). At 40 °C wax is described as a viscous plastic, which offers reduced support to the overall comb and thus potentially leading to structural failure (Hepburn & Kurstjens, 1988). However, at 40 °C honeybees are known to die at a rapid rate compared to those living at 35 °C (Free & Spencer-Booth, 1958). Even though wax demonstrates a greater strength at lower temperatures, the energy expenditure used to construct comb at 25 °C would be at least double the amount used during construction at 35 °C (Hepburn &

Kurstjens, 1988). In addition to this, bees that are reared at colder temperatures demonstrate shorter lifespans and an increased susceptibility to pesticides (Medrzycki *et al.*, 2010). This suggests a finely tuned and balanced relationship between building material and bee, which ultimately hinges on thermoregulation (Hepburn & Kurstjens, 1988; Hepburn, 1986).

Beeswax contains over 300 substances. As given in Table 1.1, the principal components in beeswaxes are fatty acids, long-chain esters as well as hydrocarbons, in addition to other constituents at low concentrations (Tulloch, 1980). There are also a further 48 volatile components which are not presented below (Ferber & Nursten, 1977).

Table 1.1: Major non-volatile components are considered those which form more than 1 % of the fraction. For minor components (forming less than 1 %) only estimated numbers are given. Data taken from Tulloch (1980).

		Number of components in fractions	
Constituent fractions	**percentage**	**major**	**minor**
Hydrocarbons	14	10	66
Monoesters	35	10	10
Diesters	14	6	24
Triesters	3	5	20
Hydroxy monoesters	4	6	20
Hydroxy polyesters	8	5	20
Acid esters	1	7	20
Acid polyesters	2	5	20
Free acids	12	8	10
Free alcohols	1	5	?
Unidentified	6	7	?
Total		**74**	**210**

The composition of beeswax shows little variation across the world (Crane, 1990; Tulloch, 1980). Changes in the relative amounts of the component fractions, given in Table 1.1, will result in the adjustment of strength, rigidity (Kotsiomiti & McCabe, 1997) and

plasticity (Gibbs, 2002) of a comb. Protein is also found within comb wax and this is thought to be incorporated during manipulation (Buchwald *et al.*, 2008; Kurstjens, Hepburn, Schoening, & Davidson, 1985) in order to achieve the correct level of 'plasticity' suitable for comb construction (Winston, 1991). The onset of melting in beeswax can be suppressed through the incorporation of contaminants (Buchwald *et al.*, 2008).

Propolis

Collected resins, from various plant sources such as leaf buds, are often combined with beeswax to produce a substance known as propolis or bee glue (Hogendoorn, Sommeijer, & Vredenbregt, 2013). Propolis can have many roles within the hive, including: strengthening, cementing and waterproofing. It is also used to disinfect the hive and is said to play a role in the honeybees' social immunity (Bradbear, 2009). Propolis contains over 300 compounds (Bradbear, 2009) and it has been shown to be the source of flavonoids (pigments) in beeswax (Tomas-Barberan, Ferreres, & Tomas-Lorente, 1993), which can be linked to the odour of beeswax (Bogdanov, 2009).

1.3.2.6 Comb age

The age of constructed comb wax tends to loosely correlate to its use within the hive; this also has a bearing on the observed colour of the comb (Hepburn & Kurstjens, 1988). Newly constructed comb wax is described as 'white' and over time it will become discoloured through the storage of food reserves or during brood development. The discolouring of the comb can be due to an accumulation of pigments from propolis (Bogdanov, 2009) and various pollens (Owayss, Rady, & Gadallah, 2004; Varassin, 2001)

and will subsequently become 'yellow' (Free & Williams, 1974) 'orange' (Hepburn, 1986) or 'brown' (Bradbear, 2009) in colour. However, if the comb is only ever used for honey storage it will remain yellow in colour (Bradbear, 2009). Over successive generations of brood development, the comb goes through various shades of brown before becoming a final 'brownish-black' or 'black' colour (Bogdanov, 2009). The darker colours come from the inclusion of propolis, as well as increasing amounts of larval excrement and exuviae (discarded larval cocoon) (Hepburn, 1986), in addition to other contaminants which accumulate over time (Berry & Delaplane, 2001). Figure 1.6 provides a visual summary of the associated functions of comb wax and the colours witnessed with progressing age.

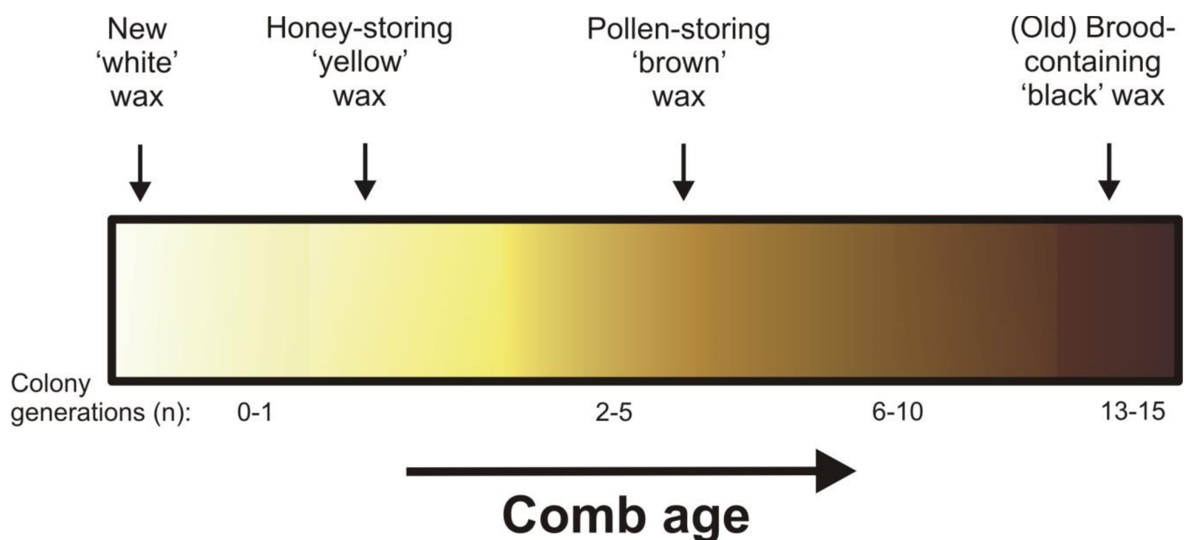

Figure 1.6: The colour of comb wax tends to correlate with the function it plays within the hive. These functions roughly correspond to comb age. The colour of the comb also becomes darker with increasing colony generations. The colours given are meant as a guide and are not accurate. Image produced from personal experience and from the description given in Bogdanov (2009).

Age is also associated with a change in the properties of the comb, such as smaller cell diameters and cell volumes; this is due to the accumulation of exuviae after each brood generation (Hepburn, 1986) as well as the recycling of wax by house bees (Winston, 1991). Smaller cells will result in a reduction in the size and weight of worker bees

(McMullan & Brown, 2006), which is also associated with a shorter life-span (Scofield & Mattila, 2015). Wild colonies would choose to shift brood rearing to newer combs during colony expansion (Free & Williams, 1974), although beekeeping practices often prevent this from happening and therefore queens are forced to lay eggs in older comb (Berry & Delaplane, 2001).

1.3.2.7 The brood nest

Not only does comb wax enable the storage of food stuffs it also provides a safe area for the developing brood (Crane, 1990; Michener, 2007; Seeley, 1985). The queen will deposit her eggs in cells which are held at a constant temperature of around 35 °C. This is normally towards the middle of each comb/frame (Winston, 1991). Food is then placed by worker bees around the resulting ‘brood nest’, with pollen immediately next to the brood, followed by honey in the outermost cells (von Frisch, 1954; Winston, 1991). This arrangement, as shown in Figure 1.7, allows the brood temperature to be easily maintained, while pollen reserves are made locally accessible to nurse bees (Hepburn, 1997; Winston, 1991). Cells reserved for drones are often found clustered on the outer edges of the brood nest, as this allows for the queen to lay batches of unfertilised and fertilised eggs (Seeley and Morse 1976 cited in Winston 1991). Likewise, queen cups and queen cells are located on the periphery of the comb (Winston, 1991).

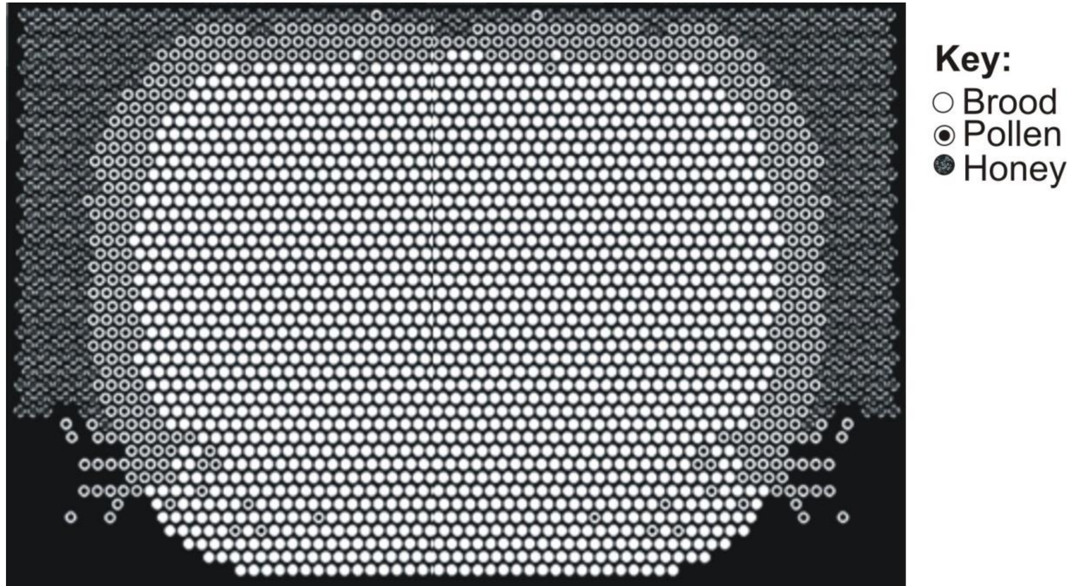

Figure 1.7: A schematic drawing of a typical brood pattern. Here the brood are deposited centrally on the frame and immediately surrounded by pollen stores while nectar/honey is found on the periphery. Adapted from Camazine (1990).

The overall shape of a brood nest (across the hive) in hotter climates is comparable to that of a rugby ball, as this allows for a greater surface area and thus a better dispersion of heat; whilst in lower temperatures the brood nest is often spherical.

1.3.4 Foraging

As we will learn in Section 1.3.4.3, p25, communication mechanisms are able to inform other foraging bees about the location of a food source; yet they fail to explain how workers are able to navigate between resources or how they recognise them (Winston, 1991). As already mentioned, foraging is a complex phenomenon and to help understand this, this section will briefly introduce some of the individual processes which will help bees to identify and locate flowers, transmit this information to other bees as well as introducing the floral rewards that are collected by foraging bees.

1.3.4.1 Bee's eye

Bees possess tri-chromatic vision which is similar to that of a human, although some key differences exist. Figure 1.8 demonstrates that bees show an increased sensitivity towards shorter wavelength light, which includes ultra-violet (UV) light (Winston, 1991). In contrast bees are unable to see red light, but they are able to see a colour referred to as 'bee-violet', which is a combination of colours found at either end of their visible spectrum. Bees are also good at spotting patterns and movement, which is a useful tool during foraging (Winston, 1991).

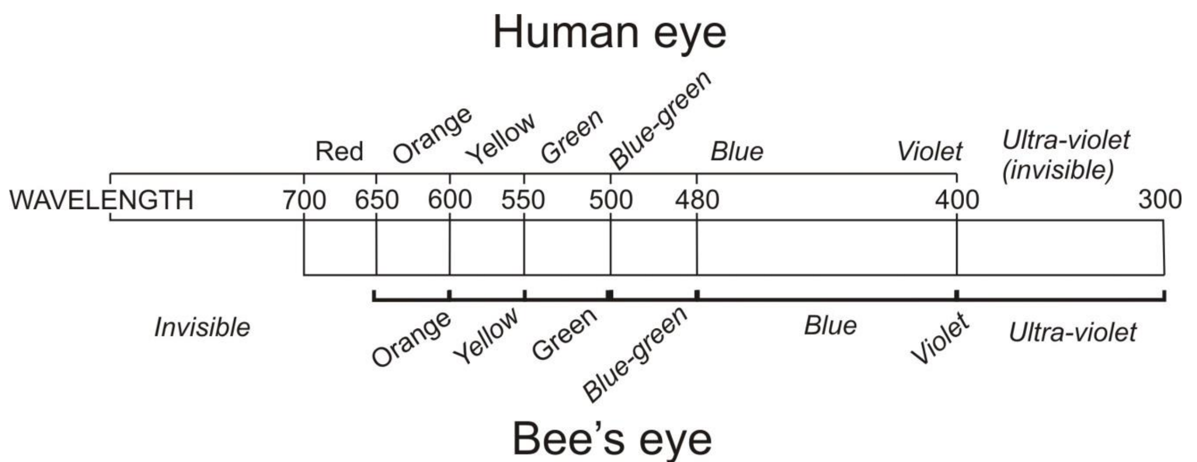

Figure 1.8: The colour spectrum as seen by the bee's eye in relation to the human eye. Redrawn and adapted from Winston (1991).

It is thought that the colours displayed by flowers are the result of an evolution process which encourages the visitation of insects (von Frisch, 1954), particularly bees (Winston, 1991). True-red flowers are thought to be more adapted to butterflies, as these are able to see at the red-end of the spectrum. Most 'red' flowers actually display a mauve or purple colour which is believed to be perceived as a blue colour to bees; yet the poppy, which is of a true-red colour, reflects UV-light and as such is regularly visited by bees (von Frisch, 1954). Flowers also produce ultra-violet patterns which include 'nectar-guides' –

these are non-reflective regions which sign-post the location of nectaries as well as other flower features i.e. stamens (Goodale, Kim, Nabors, Henrichon, & Nieh, 2014; Leonard & Papaj, 2011). Figure 1.9 shows an example of how a nectar guide would appear when a flower is viewed in UV light.

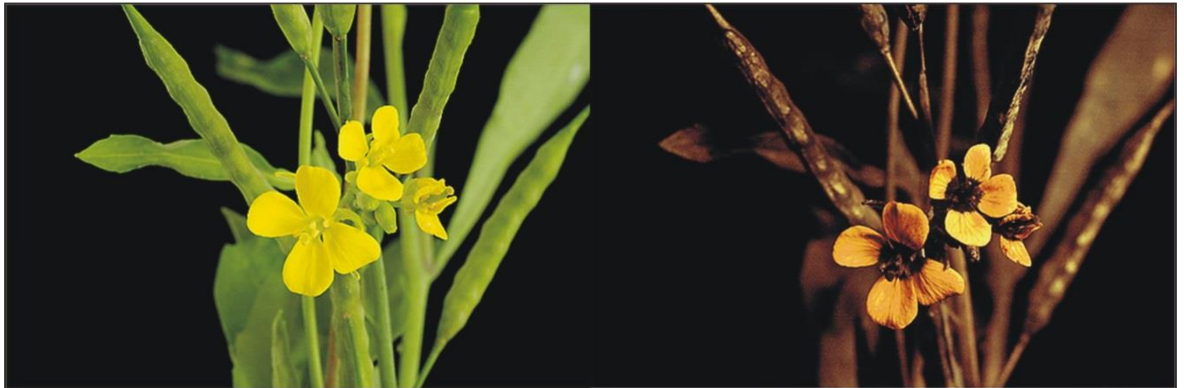

Figure 1.9: ***Brassica nigra*** **(black mustard) as seen in visible (left) and UV light (right). The darker, central 'nectar guide' which leads bees to nectar can be clearly seen in the right image. Images taken without permission from Rørslett (2005).**

A bee's ultra-violet vision allows it to locate the sun, even on overcast days, as the UV rays are able to penetrate cloud cover; this is referred to as the sun's compass (Winston, 1991). Bees will also use landmarks, such as hive location, to help with orientating themselves and in turn form a 'locale map' of an area (Crane, 1990).

1.3.4.2 A sense of smell

Bees are unable to visually distinguish between shapes, including the shapes of petals, meaning colour is only partially responsible for a bee being able to discriminate between various flower types (von Frisch, 1954). Floral odours, as well as other food resource odours, play an important role in the orientation of the honeybee (Crane, 1990; Winston, 1991). Odours are detected by a bee's antennae found at the front of the head and

odours can be remembered for up to five days (Crane, 1990); once an odour has been learnt by an individual bee they will continue to visit flowers of the same scent, as they have now become accustomed to the foraging conditions of that particular flower type (von Frisch, 1954) i.e. knowing which nectary will give the most nectar. Odours are also used during a return flight to the hive as the bee can easily discriminate between a foreign colony and their own (Winston, 1991).

1.3.4.3 Communication

The sum of a colony's total workload and efficiency is greater than that which can be achieved by the same number of bees working independently. Therefore in order for a colony to successfully function as a unit it is important that individual bees are able to communicate to other nest mates; this then ensures, for example, that essential levels of pollen, nectar, water and resins are collected for the benefit of the whole colony. Following a foraging trip for pollen or nectar and in some cases water, a bee returning to the hive will/can perform a dance, which is aimed to recruit or inform workers of a food source's locality to the hive. Two forms of dance exist: the waggle dance and the round dance.

The waggle dance is carried out by the honeybee in the form of abdominal shakes and a figure-of-eight movement (Figure 1.10); it is used to inform nest mates of the precise location (based on the amount of energy expelled to get to location) and the quality of a food source which can be found within a radius greater than a hundred metres from the hive.

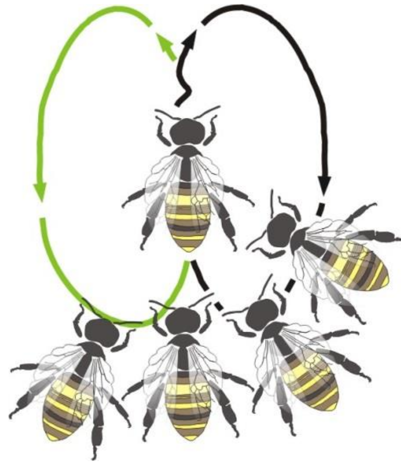

Figure 1.10: A forager conducting the 'waggle dance' in order to recruit other foragers. Redrawn and adapted from Winston (1991).

The dance also specifies the direction of the food source in relation of the sun (Figure 1.11), which can be compensated for and accurately navigated to within one degree by other foragers (Winston 1991).

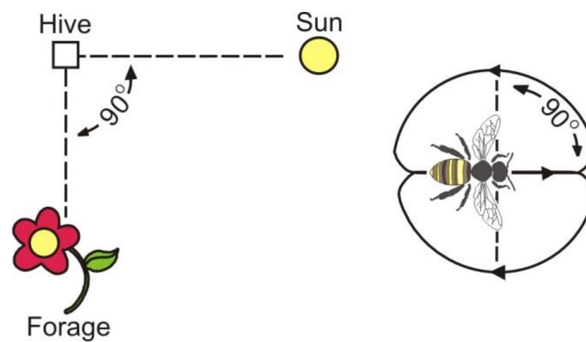

Figure 1.11: The angle of the waggle dance from the vertical (dashed line) informs foragers of the location of a floral source in relation to the sun position. Redrawn and adapted from Winston (1991).

In comparison, the round dance (Figure 1.12) is rather more simplistic in both its process and the message it delivers. The dance does not transmit any precise information on the distance or direction of forage but merely notifies that a floral reward is within a proximity of 15 metres to the hive (Winston, 1991).

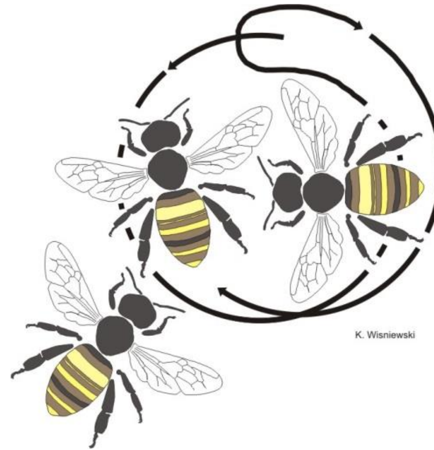

Figure 1.12: The round dance informs recruits that a food source is close to the hive. Redrawn and adapted from Crane (1990).

The dance is performed by the 'dancer', who continually walks in small circles and then reverses direction on every first or second revolution; up to twenty reversals can be performed in a dance that can last from a few seconds to no longer than a few minutes. Nectar or pollen samples are distributed to surrounding bees prior to or during the dance. Once the dance is completed the recruited bees then circle the hive, gradually increasing their radius until the food source is found. It is believed that the food source is identified using the odours exchanged during the round dance. An amalgamation of both the waggle and round dances is used for floral sources that exist between 15 and 100 metres (Winston, 1991).

During both the waggle and round dance, other recruits will read the vibrations of the dancer using their antennae; as well as taking note of any odours which may help to identify the food source (Winston 1991).

1.3.4.4 Pollen and nectar

Pollen and nectar are floral rewards that are produced by flowers and collected by bees as a food source. The main composition of nectar is between 5 - 80 % sugars and this high composition of carbohydrates acts as a bee's main source of energy; other compounds such as minerals, vitamins and organic acids are also found (Rortais *et al.*, 2005). The rate of nectar and/or honey consumption can vary according to the stage within the life cycle that the bee is at i.e. larvae or adult bee, plus the tasks undertaken within the hive; for example, it was shown that wax-producing bees, foragers and "winter" bees would use larger amounts of nectar to cover the high amounts of energy required for their respective duties (Rortais *et al.*, 2005).

Pollen can contain between 6 – 28 % protein, 1 – 20 % lipids (mainly polar and neutral), carbohydrates, vitamins, minerals and ~0.5 % sterols. The latter is required for bee metabolism, as without this bees are not able to synthesise cholesterol (Campos *et al.*, 2008; Winston, 1991). The collected pollen grains are packed with a combination of nectar and/or honey in addition to salivary enzymatic secretions such as amylase and catalyse, which helps aid pollen transportation to the hive for storage. This is often referred to as bee bread (Campos *et al.*, 2008; Isidorov, Isidorova, Sczczepaniak, & Czyżewska, 2009). As with nectar, pollen consumption varies according to the stage within a bee's life cycle. Nurse bees (8 - 10 days old) will consume high levels of pollen in order to develop the hypopharyngeal and mandibular glands needed for larval feeding (Rortais *et al.*, 2005).

1.3.4.5 Honey production

Bees will forage and collect nectar so that honey can then be later produced and stored. The nectar collected by foragers is stored within the 'honey stomach' where it undergoes enzymatic manipulation. The enzyme (invertase) breaks down sucrose into two simpler sugars: glucose and fructose (Butler, 1954). Once at the hive, workers then accept regurgitated nectar from a returning forager's tongue and mouth parts, before retiring to a quieter region of the hive where she will then reduce the water content of the nectar; this is achieved by exposing the nectar to air by folding her mouth parts repeatedly, before it is then stored in a wax cell (Crane, 1990). Water evaporation is further achieved by bees fanning their wings near the cell in order for air to circulate over the nectar. The average water content of nectar from the UK is 60 – 65 %, whilst the water content of honey is around 18 – 20 % (Butler, 1954; Winston, 1991). The cell containing the mature nectar, which has a sugar content of around 80 % (Rortais *et al.*, 2005), is then capped with wax until it is needed (Crane, 1990).

1.3.5 Beekeeping (honeybee)

Beekeeping refers to the management of bees in artificial hives by a beekeeper (apiarist) (Crane, 1990). Owing to the large colony numbers and proving to be the easiest bee species to manage, honeybees are typically the choice species when it comes to beekeeping (McGregor, 1976). It is thought that honeybees have been kept within hives of various forms for over 4,500 years (Crane, 1990). Early primitive examples of hives include mud or clay cylinders; these, along with the much later wicker skep, had fixed combs and had to be destroyed to remove honey stores. It is only since 1851 when the

first 'removable frame' hive (Langstroth hive) was created and that modern beekeeping was born (Crane, 1990; Winston, 1991). The introduction of the removable frame hive also became important for modern agriculture, as this allowed for hives to be transported to blooming crops (Winston, 1991). A typical removable frame hive used in England is the British National hive, seen in Figure 1.13, whilst the 'Smith' hive is often used in Scotland; although very little differences exist between these two hives.

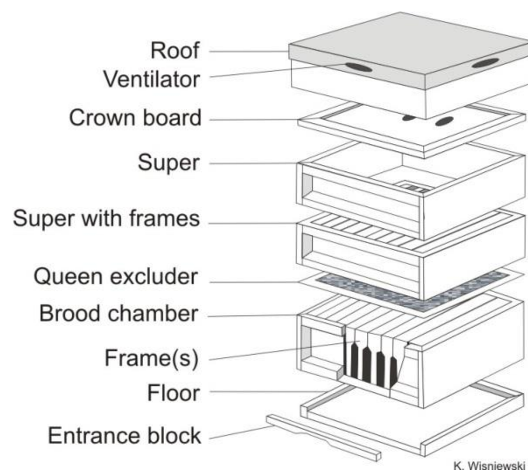

Figure 1.13: A schematic diagram of a typical British National hive. The exploded view provides details of individual components. Redrawn and adapted from Crane (1990).

A hive is traditionally wood in construction and consists of a brood chamber and super, the latter is normally exclusively for honey storage. In order to ensure that only honey is collected by the beekeeper, he or she will use a queen excluder between the brood chamber and honey supers. The queen excluder is typically a mesh with spacing too small for the queen to pass through, and hence she will not be able to deposit eggs in the super.

A beekeeper is able to manipulate a colony to produce more honey than is actually required so that he/she is able to harvest the surplus (Crane, 1990); a higher yield can be achieved through the use of removable frames, as the beekeeper is able to extract honey from a frame centrifugally, thus being able to reuse the frame over several years (Crane, 1990; Winston, 1991). Additional brood boxes as well as supers can be added allowing for colony growth and increased honey stores (Winston, 1991). The surplus honey collected by the beekeeper can be used as a source of income.

1.3.5.1 Migratory beekeeping

Migratory beekeeping allows for bigger honey yields by taking advantage of different flowering times of each plant species around a particular region (Crane, 1990). This occurs on a small scale within the UK as bee keepers move a few hives to a later flowering plant species, such as heather, in order to increase their honey yields. In contrast, American beekeepers may collectively relocate up to a million colonies in order to meet the pollination demands of the Californian almond trade; an industry valued at $2 billion alone (Ratnieks & Carreck, 2010). Figure 1.14 shows an example of how honeybee colonies may be relocated to major crop producing states across the USA throughout a single season. In order for a crop to be pollinated to a satisfactory level, colonies may remain at the crop for 3 – 5 weeks before being relocated (Bond, Plattner, & Hunt, 2014).

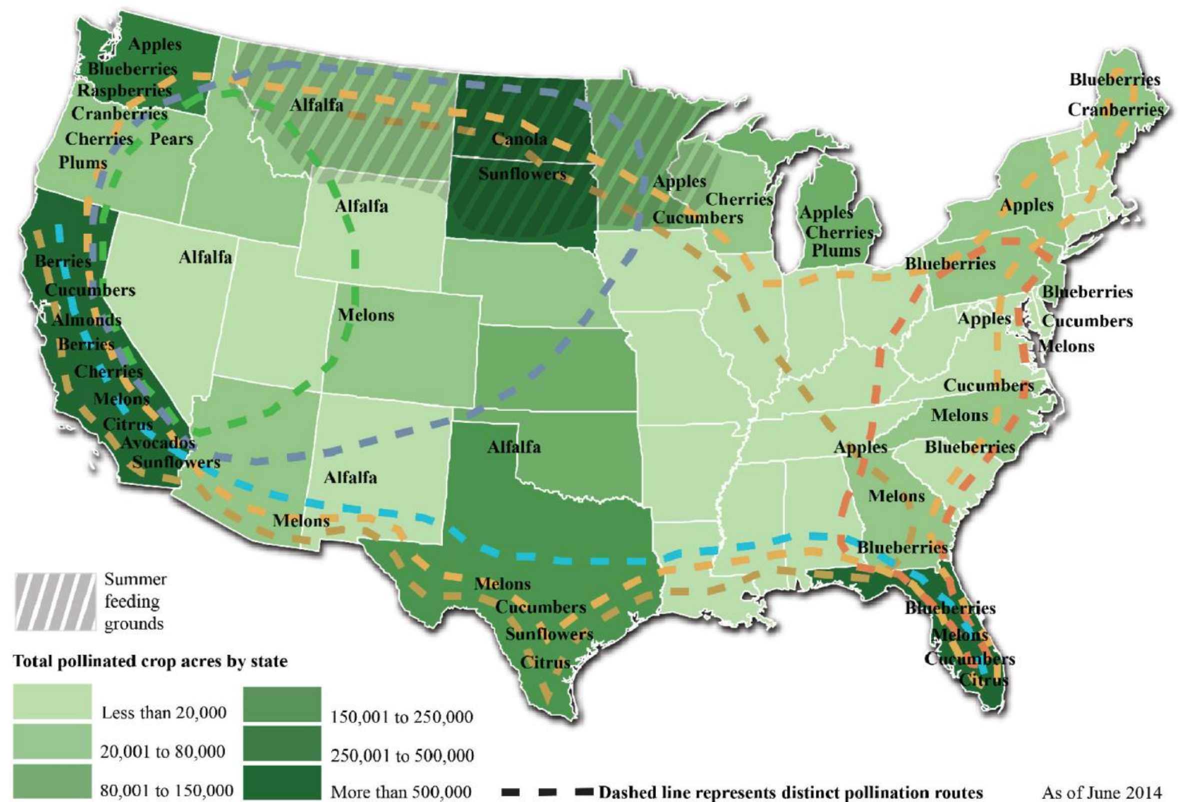

Figure 1.14: The various potential movements of honeybee colonies and the crops that they may visit within the USA. Taken without permission from (Bond *et al*., 2014).

There are two subfamilies of the order *Apidae* which produce honey in high enough quantities for its harvest to be worthwhile: *Apinae* (honeybees) and *Meliponinae* (stingless bees). However it is the former whose only species *Apis* carries more economic importance than any other (Crane, 1990). The European honeybee, *Apis mellifera*, is a highly adaptable species of bee and through deliberate human transport it now encompasses a vast native range that has expanded to nearly all habitable corners of the world (vanEngelsdorp & Meixner, 2010). There are thought to be no or very little feral (wild) honeybee colonies found within Great Britain, leaving only those managed by beekeepers (Potts, Roberts, *et al.,* 2010).

1.3.5.2 The recycling of beeswax

As previously discussed in Section 1.3.2.3, the overall energy required to produce beeswax is high and as such the production of wax only occurs when necessary (Hepburn, 1986). Honeybees will also recycle old wax in order to save energy; this includes wax cappings (Seeley, 1985), as well as queen cups and queen cells (Winston, 1991). Any unused wax tends to be placed at a cell's edge by nurse bees until required (Seeley, 1985). A common beekeeping practice is to also save the energy needed for wax production and to revert it to other duties i.e. colony growth and nectar collection; this helps to maximise honey production (Bradbear, 2009). Such a result can be achieved through the use of removable frame hives and readymade wax foundation sheets. Foundation wax sheets are produced by running a sheet of wax through embossed rollers – resulting in a hexagonal cell pattern on which bees will draw out the rest of the comb. The reuse of drawn comb can ensure a larger honey crop, as the amount of honey needed for further wax production (i.e. cappings) will reduce to around 1.5 – 2 % (Crane, 1990).

It is suggested that comb wax should be replaced every two to three years, with the old wax undergoing a recycling process (Bogdanov, 2004). Beekeepers can trade in their old comb wax for new foundation sheets as a way of offsetting costs and, once processed, any old wax will most likely be used to form new foundation sheets (Bradbear, 2009).

The recycling of beeswax can vary in both price and sophistication: a few common examples, which are also adopted by beekeepers and involved with this study, are:

1) *Solar wax extraction*
2) *Steam wax extraction*

Both solar and steam extraction will return wax of suitable quality for exchange with manufacturers for new foundation; however, if the wax was to be formed into candles and sold to the general public then further refinement will be necessary – these techniques are outside the scope of this thesis and shall therefore not be considered. The return yield from either extraction can vary, depending on the quality of comb that is being recycled; older, darker combs will usually yield between 30 – 50 %, however newly drawn comb can see returns of up to 100 % (Bogdanov, 2004). Some beekeepers may choose to recycle their own wax; this may be done as a cost saving activity, but in addition, some beekeepers prefer to have a history of the wax they use for fear of bringing in contaminants or low grade wax into their colonies (personal communication with beekeepers).

1) Solar wax extraction

Solar wax extraction is a simple and effective process which uses the heat of the sun to melt and purify beeswax. A solar wax extractor can either be shop-bought or can easily be made at home at little cost to the beekeeper. A schematic diagram of a solar extractor is given in Figure 1.15; it consists of a box with a transparent lid; inside is a metal sheet on to which old beeswax is placed that slopes towards a container. In order for the wax to melt the internal temperature of the extractor needs to be around 68 °C; thermal insulation as well as two sheets of glass or Perspex help to retain heat within the extractor. During extraction a mesh screen catches any debris contained within the old comb wax, whilst clean wax is free to run into the container. A single extraction is often adequate enough to produce a high quality wax block; however, this may vary according to the initial cleanliness and age of the comb wax used (Bradbear, 2009).

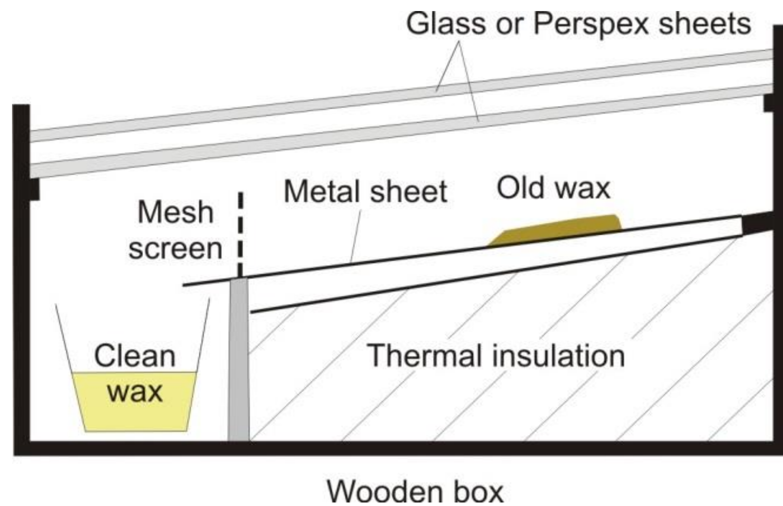

Figure 1.15: A schematic drawing of a solar extractor.

In order for the extractor to work at maximum efficiency it must be continuously positioned in direct sunlight and so is perhaps best suited for summer months, during which the smell of hot wax and honey can attract bees and may prove to be an inconvenience to the beekeeper (Bradbear, 2009).

2) Steam wax extraction

Steam wax extractors involve placing beeswax in a sealed cylinder, which houses a basket to hold the old wax, and then introducing hot steam to melt the wax. The steam melts and cleans the wax, where by the melted wax then drains through an opening at the bottom of the container, where it is then collected in a suitable receptacle (Bradbear, 2009). Steam extractors, although perhaps not as cheap as solar extractors, can again be made at a relatively small cost. This method of extraction offers a benefit over solar extraction as it can be conducted anytime of the year.

1.4 Honeybee decline

Throughout their lifetime, honeybees are at risk of being attacked by parasitic mites (*Varroa destructor*) (Genersch, Evans, & Fries, 2010), bacteria (e.g. European and American foulbrood: *Melissococcus pluton* and *Paenibacillus larvae*, respectively) (Hornitzky & Anderson, 2003), viruses (sacbrood: *Morator aetotulas*) (FAO 2006) and fungi (e.g. *Nosema apis*) (Genersch *et al.*, 2010) to name but a few. The aforementioned factors can all have a negative effect on the health of the honeybee (Genersch *et al.*, 2010), in addition to other factors such as climate change, loss of habitat and the use of pesticides in agriculture (Potts, Biesmeijer, *et al.*, 2010).

Honeybee numbers are currently in decline and this has sparked massive media interest and research operations investigating reasons as to why these losses are occurring (Neumann & Carreck, 2010). A huge loss of honeybees could massively threaten the supply of around one hundred pollinated crop types, in addition to habitat diversity within the wild (Dötterl and Vereecken 2010). Nevertheless, threats to honeybee numbers are not a new phenomenon and incidents have been noted throughout the last 150 years or so (Underwood & vanEngelsdorp, 2007). The first occurrence of major honeybee loss was recorded in Kentucky 1868, where it was said that bee numbers had declined whilst still leaving plenty of honey within the hive. Since then, no less than eighteen major losses have been recorded up to 2009 (Underwood & vanEngelsdorp, 2007; vanEngelsdorp *et al.*, 2009). For example, in 1905 and 1919 the Isle of Wight was struck by 90 % colony losses. Honeybees suffering from the 'Isle of Wight' disease were unable to fly and were often seen crawling out of their hives (Neumann & Carreck, 2010; Underwood & vanEngelsdorp, 2007). The cause of these losses was thought to be down

to the tracheal mite *Acarapis woodi* (Wilson-Rich, Allin, Carreck, & Quigley, 2014), but it was later found to be a combination of chronic bee paralysis virus, poor weather conditions and excessive colony numbers in a limited available foraging space (Neumann & Carreck, 2010; Wilson-Rich *et al.*, 2014).

France also played victim to heavy honeybee losses in the winters of 1998 - 1999 and 1999 - 2000; research showed one or multiple diseases e.g. tracheal mites being present in 76 % of the affected colonies. Yet, despite this, the cause still remains unknown (Underwood & vanEngelsdorp, 2007). During the same time (late 1990s/early 2000s) both French and Italian beekeepers reported honeybee losses in hives placed near fields of sunflowers (France) and maize (Italy). Both of these crops were treated with imidacloprid (insecticide), which was introduced around this time (Bortolotti *et al.*, 2003), thus suggesting that pesticides are a possible driver behind bee decline (Potts, Biesmeijer, *et al.*, 2010).

American honeybee losses are well documented; however, the evidence for honeybee decline in Europe has been described as patchy and poorly documented (Potts, Roberts, *et al.*, 2010). The most recent and current incident of large scale bee losses was first reported along the East Coast of the USA in the latter months of 2006 (Underwood & vanEngelsdorp, 2007); yet beekeepers had noticed dwindling numbers with consistent symptoms two years earlier (Cox-Foster *et al.*, 2007). Since 2006 this condition has taken on many aliases, for example: 'honeybee vanishing' (vanEngelsdorp & Meixner, 2010), 'fall dwindle disease' (Underwood & vanEngelsdorp, 2007; vanEngelsdorp & Meixner, 2010) and the finally adopted name of 'colony collapse disorder' (CCD) (Cox-Foster *et al.*, 2007). The characteristic symptoms of CCD are defined as the nonexistence or absence of

the extreme majority of adult honeybees within a colony without signs of mortality within or near the hive (Cox-Foster *et al.*, 2007; Kamel, 2010). The condition only appears to effect worker bees, as both the queen and brood remain present in the hive (Kamel, 2010). In addition to this, a good amount of food stores remain untouched by robbing bees, wax moths and hive beetles, which remains true weeks after the collapse (Underwood & vanEngelsdorp, 2007). For deaths to qualify as CCD, the previously described symptoms must be met, as bees can die in many particular ways (Neumann & Carreck, 2010). However, within the literature there are currently seventeen definitions used to describe CCD, therefore making it difficult to determine whether the same phenomena is always being referred to (Garwood, 2010). Many theories and speculations exist to date that look to identify and point out the current cause of honeybee decline, with the more unlikely suggestions holding mobile telephones, genetically modified crops and even nanotechnology responsible (Neumann & Carreck, 2010). A combination of diseases, mite stresses and pesticides, amongst other stressors contribute to the hypothesis that no one factor is solely responsible for honeybee decline or CCD (Mullin *et al.*, 2010).

It must be noted that the phenomena of CCD does not occur in the UK and Europe. It is suggested that UK losses are perhaps down to the occurrence of *Varroa* mites, habitat loss and shifts in weather patterns (Wilson-Rich *et al.*, 2014), while pesticide applications are still regarded as an important driver behind the declines (Potts, Biesmeijer, *et al.,* 2010). In any case, the UK has lost over 75 % of its colonies over the last century. Britain has also seen a 78 % reduction in the number of beekeepers since 1953, leaving the country with only around 25 % of its total honeybee requirement (Potts, Biesmeijer, *et*

al., 2010). Other UK bee species have also been in decline during the last 50 years. However, as these are often wild bees their disappearance is not immediately as obvious as domesticated honeybees (Wilson-Rich *et al.*, 2014). Solitary bee species were also found to have reduced by 52 % across surveyed areas (Biesmeijer *et al.*, 2006). Three species of bumblebee have also become nationally extinct, whilst a further eight species have experienced major declines (Goulson, 2010); and despite a lack of monitoring schemes (Breeze *et al.*, 2011) there are still concerns into how various stressors affect bumblebees (see Chapter 6).

Weather and climate

Climate change has been identified as a potential contributor behind bee losses, including CCD. Winter weather has increasingly become more variable and this can be linked to bee losses, not only in the UK, but also across Europe and the USA (Wilson-Rich *et al.*, 2014). Although honeybees are capable of tolerating a varied range of climates, they remain sensitive to prolonged cold and wet spells which prevent them from foraging (Grunewald, 2010). The flowering times of many temperate plant species appear to have been affected by climate change, resulting in a potential mismatch between pollinator visitation and the onset of flowering (Rafferty & Ives, 2012); prompting concerns as this may lead to starvation of early emerging bees or that flowers may not fully receive the pollination they require (Wilson-Rich *et al.*, 2014). There is a possibility that bees and flowers may adjust to such a mismatch over time (Wilson-Rich *et al.*, 2014); however, this is not confirmed and the mismatch may become further exacerbated (Rafferty & Ives, 2012). Once the queen starts to lay eggs, a constant supply of pollen is needed for the

colony and disruption to the supply of this important resource may affect brood development (Grunewald, 2010) and consequently colony growth.

Recent official 'overwintering statistics' (Figure 1.16), produced by the British Beekeepers Association (BBKA), show a promising improvement in the overwintering success of UK honeybees, with the exception of the winter 2012/13 which was considered a severe winter following a poor summer (BBKA, 2014).

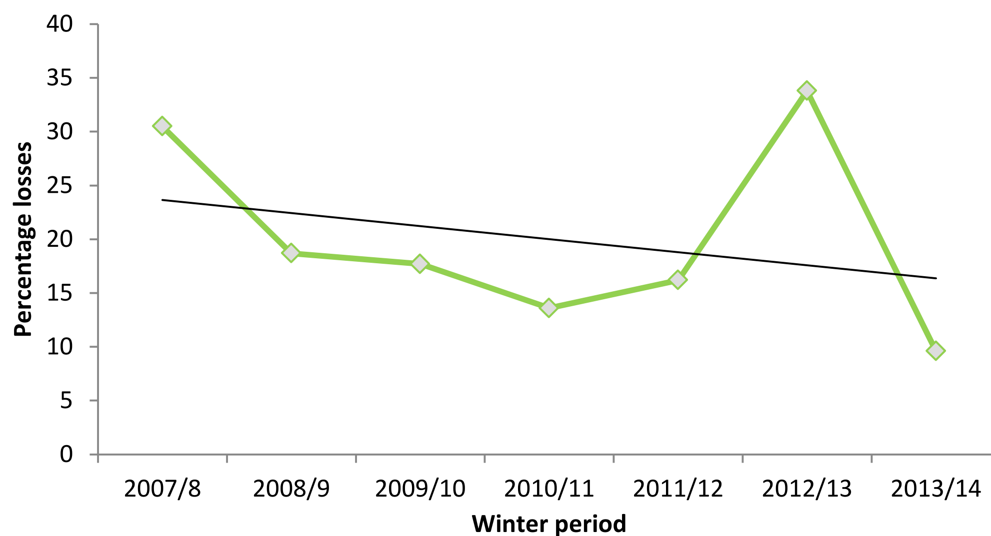

Figure 1.16: Percentage honeybee losses in the UK over the winter periods 2007/08 to 2013/14. Redrawn from data presented in BBKA, (2014).

Despite improvements, the BBKA considers the current losses (9.6 %) to be too high and hope to see an improvement in the long term, during which time beekeepers are encouraged to keep a high-standard of husbandry in order to minimise the levels of *Varroa* (BBKA, 2014).

Habitat loss

Agricultural intensification, as a result in the reclamation of land in order to supply the growing human demand for various produce (Wilson-Rich *et al.*, 2014), has resulted in a loss of nesting and forage sites for a number of bee species. Habitat loss is still considered the primary driver behind declines in the UK and across Europe (Brown & Paxton, 2009; Kuldna, Peterson, Poltimäe, & Luig, 2009). Species-rich hay meadows were commonly used as livestock feed; however, since the 1930s silage production has become more preferred, resulting in a 97 % reduction in hay meadows (Breeze *et al.*, 2011). Despite their negative effects on available nesting sites, crop monocultures i.e. oilseed rape, can provide a rich source of pollen and nectar, albeit for a short period of time (Grunewald, 2010). Hedgerows provide safe corridors for bees to move between nest sites and food sources; yet a continued reduction of hedgerows threatens to isolate certain bee species, promoting inbreeding and suppressing gene diversity (Williams *et al.*, 2010). A similar effect is witnessed through urbanisation. However, it should be noted that honeybees are able to forage over a much larger range than most bees and so are able to thrive in a urban environment (Wilson-Rich *et al.*, 2014).

1.5 Pesticides

Since the middle of the 20th century pesticides have been used worldwide as a result of agricultural intensification (Wilson-Rich *et al.*, 2014). The term pesticide is used as a title that incorporates a wide range of chemicals (natural, semi-synthetic or synthetic) that aim to manage/eradicate insects (insecticides), rodents (rodenticides), fungi (fungicides) and weeds/plants (herbicides) as well as other pests (Johnson, Pollock, & Berenbaum,

2009). Pesticide formulations consist of an active ingredient(s) and 'inert' chemicals, although the latter can be misleading. These can often assist in the successful delivery of the active ingredient to a specific target, in addition to possibly increasing product stability (Curtis, 2006). Both the chemical and physical properties of pesticides can vary substantially (Alder, Greulich, Kempe, & Vieth, 2006) and this of course has an effect on the intended target species and target area as well as method of application (Curtis, 2006). As of 2009 the British Crop Protection Council identified 908 active substances from over 100 different classes i.e. neonicotinoids and organochlorides (Tomlin, 2009).

1.5.1 The need for pesticides

The world's population has been forecast to increase by 50 % over the next 50 years, approaching around 9 billion people - which is expected to double food demands by 2050 (Sexton, Lei, & Zilberman, 2007). In order to meet these demands chemical treatments are used to reduce or prevent damage to the crops or fruit, which would otherwise be rendered unmarketable or sold at a lower quality grade for a reduced price (Sexton *et al.*, 2007). As a consequence of being able to maintain high crop yields, farmers are also able to produce more food on less land (Fishel, 2015). Modern farming techniques often see crops grown as part of a large monoculture, which are particularly threatened from pest destruction (Oerke & Dehne, 2004). This therefore gives incentive for growers to adopt the use of pesticides (Sexton *et al.*, 2007). A farmer will show a tolerance towards pests within the field; with pesticide applications only been made once it is deemed economically viable to do so. This takes into account whether the cost of application is equal to the amount of reducing the damage to the crop (Sexton *et al.*, 2007).

Pesticides are not exclusive to agriculture, as their use also contributes to the control of various human pests which, if left uncontrolled, can have serious health consequences i.e. mosquito vector diseases. Other uses can include the control of non-native plant species which would otherwise threaten native environments. From a recreational standpoint herbicides are used to control weeds found in gardens or parks. Damage to wooden structures can also be reduced through the use of appropriate chemical applications (Fishel, 2015). As will be covered in Section 1.5.3.1.2, p54, chemical treatments can also be used for veterinary practices including the control of *Varroa destructor*.

1.5.2 A brief history of pesticides

The use of chemicals to control pests is not a new phenomenon. During pre-Roman civilisations sulphur (brimstone) was used in as a bleaching agent and fumigant (Fishel, 2013). Similarly, the Romans also used sulphur fumes as an insecticide as well as salts to control weeds (Delaplane, 1996) and by 800 AD arsenic was used as an insecticide in China. It is thought that the development of pesticides was hindered during a time of superstition and ignorance towards pests; it was not until pests were viewed as part of the natural world, rather than a punishment from God, could they then be controlled (Taylor, Holley, & Kirk, 2007). Plant-based insecticides consequently emerged throughout the years, which included nicotine; originally extracted from tobacco (1690) (Fishel, 2013). From about 1750 – 1880 an ‘agricultural revolution’ occurred in Europe, which saw an increase in the use and availability of crop protectants. By the nineteenth century chemicals including copper acetoarsenite (Paris green - 1867) (Taylor *et al.*, 2007), hydrogen cyanide (1886) and lead arsenate (1892) were being applied as insecticides

(Fishel, 2013). Organic mercury compounds were later introduced in 1913 in order to protect seeds from various diseases (Taylor *et al.*, 2007). Basic chemistry and crude application methods of early chemicals often meant that treatments proved ineffective (Delaplane, 1996). The development of sprayers in 1880 allowed chemicals to be applied as sprays (Figure 1.17a), whilst the first aerial application of lean-arsenate was made in 1921 (Figure 1.17b).

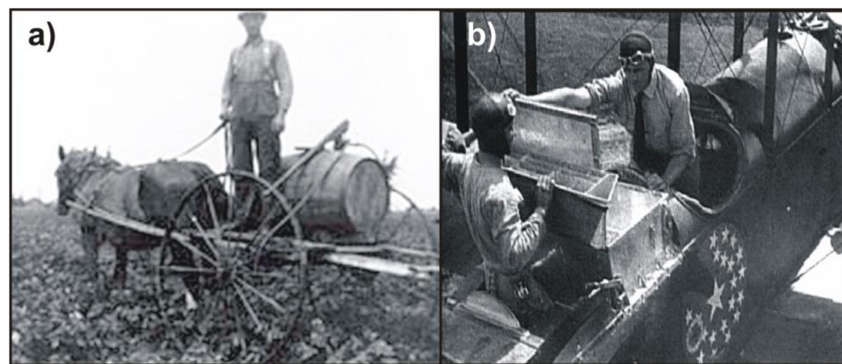

Figure 1.17: a) An example of a late 19th Century horse-drawn spraying application of Paris green on a potato crop; b) The first aircraft altered to make aerial applications of lead-arsenate. Adapted from (Taylor, Holley and Kirk, 2007).

Interest in chlorinated insecticides, between 1920 and 1940, was quite considerable. The insecticidal properties of DDT (4,4'-dichlorodiphenyl trichloroethane) were tested in the 1930s, before being officially sold as an insecticide in 1942 (Jarman & Ballschmiter, 2012). The role played by DDT in agricultural practices during World War II lead to an eventual reliance on chemical treatments (Fishel, 2013). In the years to follow a large number of these effective and inexpensive chlorinated compounds began to emerge. The 1950s and 1960s saw the introduction of many organophosphate compounds (Taylor *et al.*, 2007). However, the liberal applications of pesticides eventually lead to pest developing a resistance to chemicals, as well as harm to non-target organisms (Delaplane, 1996). Since

the 1940s resistance towards chemical treatments has increased at an exponential rate. Consequently, it is thought that there are over 500 pest species which exhibit some degree of resistance to insecticides (Taylor *et al.*, 2007).

Awareness into the environmental risks of pesticides came to light during the early 1960s, most famously following the publication of "Silent Spring" by Carson (1962). This highlighted poisonings and bioaccumulation of pesticides in the environment. The preceding years saw the development of more pest-specific pesticides (Delaplane, 1996) as well as 'integrated pest management' (IPM). The concept of IPM aims to manage pests using crop production techniques as oppose attempting eradicating them. This saw the development of pesticides which were more effective at lower doses and less harmful to both the environment and beneficial insects (Taylor *et al.*, 2007). This included pyrethroids, which were developed from 1975 to 1983 and included fluvalinate. However, their continuous use led to widespread resistance (Davies, Field, Usherwood, & Williamson, 2007). Interestingly, the UK ban of DDT was not until 1984, whilst other organochlorines agrochemicals such as aldrin (insecticide) and dieldrin (insecticide) were restricted in 1986 (Thompson, 2003). A strong market demand for new broad-spectrum insecticides was met following the introduction of neonicotinoids at the beginning of the 1990s (Wollweber & Tieyen, 1999). Neonicotinoids are covered in more detail in Section 1.5.4, pp 60 - 65.

1.5.2.1 The use of halogens in pesticides

Over the last thirty years there has been a large increase in the number of halogens being incorporated into agrochemicals. A statistical pattern relating to the number of fluorine

(F) atoms incorporated into each type of agrochemical has been observed for fungicides (2+ F atoms), herbicides (3+ F atoms), insecticides and acaricides (4+ F atoms). Mixed halogens tend to be preferred in both insecticide and acaricide compositions; i.e. both chlorine (Cl) and fluorine; whilst only chlorine or fluorine atoms are used in fungicides. Halogens are often incorporated to modify the physiochemical and pharmacokinetic properties of a compound; such as metabolic stability, increased insecticidal activity and increased lipophilicity - the latter can determine a chemical's ability to cross membranes (Jeschke, 2010). Other influences concerning halogen effects have been considered and discussed elsewhere (Jeschke, 2010).

Lipophilic properties of pesticides

The lipophilicity of a pesticide can be determined by its distribution (at equilibrium) between two immiscible phases, this is known as a partition coefficient (K). The organic solvent *n*-octanol has traditionally been used to represent biological systems (Sangster, 1988). The octanol-water partition coefficient (K_{ow}) is defined in Equation 1.1:

$$K_{OW} = \frac{[\text{Concentration in octanol phase}]}{[\text{Concentration in water phase}]}$$

Equation 1.1: Octanol-water partition coefficient (K_{ow}).

K_{ow} is a unitless parameter which can be used to predict the physical properties of most pesticides (mw <500 Da). The resulting K_{ow} values tend to be quite large (10^{-2} to 10^{6}) (Sangster, 1988); consequently, these are often expressed as Log (K_{ow}), with the resulting values ranging from -3 to 7 (Zacharia, 2011). Polar pesticides (Log K_{ow} <1) (Nicholls, 1988) tend to demonstrate greater solubility in water and so will therefore have a low Log K_{ow}

value (Zacharia, 2011). The lipophilic properties of pesticides can vary: polar pesticides (Log K_{ow} <1), herbicides (Log K_{ow} 0.5 - 3), fungicides (Log K_{ow} 2 – 5), and lipophilic insecticides (Log K_{ow} >4). Those which demonstrate a high Log K_{ow} value will show little absorption into water-based matrices (Sangster, 1988).

1.5.3 History of bees and pesticides

As discussed previously, the agricultural revolution (mentioned in Section 1.5.2) brought about the use of many chemical treatments to control pests. It is at this point that the story of bees and agricultural pesticides became entwined. Reports of accidental honeybee poisoning first appeared during the early 1870s, these were attributed to the use of Paris green on flowers during bloom (Todd & Mcgregor, 1952). In an historical review paper by (Shaw, 1941) it was evidenced that researchers found many copper-, sulphur- and arsenic-based compounds to have lethal effects on bees. However, modern pesticides and application procedures have been developed to be safer for beneficial pollinators. Despite this there are still instances of poisoning, including from neonicotinoids. The remainder of this section will consider the routes of pesticide exposure, toxicity and the chemistry of neonicotinoids - which have become of particular interest in recent years.

1.5.3.1 Typical methods of exposure and accumulation of pesticides

The two main methods of pesticide exposure to which honeybees are subject to are contact (dermal) and oral exposures. Dermal exposure to pesticides can result from an insect receiving a direct application of pesticide or they might fly through any resulting

dust particles which can occur from spray application (Jeschke, 2010). Oral exposure is the result of direct consumption of contaminated foodstuffs.

Honeybee exposure to pesticides can arise from environmental sources (i.e. agriculture) or apicultural practices (Rortais *et al.*, 2005), although little work has been conducted in to the contamination of beeswax from agricultural treatments (Bogdanov, 2006; Chauzat & Faucon, 2007). A search of the published literature reveals that there are no current studies within UK that have investigated pesticide residue levels in beeswax. However, an investigation into the levels of miticides and agrochemicals contained within North American apiaries discovered that of the 259 wax samples analysed, 87 pesticides and metabolites were found (with an average of 6 pesticides per sample); in one instance 39 different pesticides were found in a single hive (Mullin *et al.*, 2010).

1.5.3.1.1 Agricultural exposure

In agriculture, the main aim of pesticide application is to control moulds, weeds and to act as an insect deterrent from crops (Mullin *et al.*, 2010); an implication of this is that multiple categories of pesticide may be present/in contact with fruit and vegetables throughout their entire period of growth (Selim, EL-Saeid, & Al-Dossari, 2011) and in some cases post-harvest (Shuling, Xiaodong, & Chongjiu, 2007). It is worthwhile noting that a combination of various pesticides can have toxic implications through synergistic interactions (Selim *et al.*, 2011).

Agricultural pesticides are applied in many ways, typically in one of the following: *(1)* spraying a pesticide mixed with a dilutant (such as water), *(2)* spreading a pesticide that has been impregnated into an inert solid i.e. granules, *(3)* burning the compound to

create pesticidal smoke (often reserved for confined spaces) and *(4)* coating a seed with an active ingredient before sowing (Johnson *et al.*, 2009). The poor application of pesticides during the treatment of crops can cause the potential contamination of surrounding media i.e. water and soil (van Emden, 1989) and this can lead to the unintentional exposure of pesticides to non-target organisms (García-Chao *et al.*, 2010). A major downfall with most pesticides is that they are often nonspecific and so may exhibit toxic effects to these non-target organisms such as bees for example (Desneux, Decourtye, & Delpuech, 2007).

Honeybees that fly through dust clouds during spraying treatments can be exposed to toxic levels of pesticides (Daintith, 2004), although the short-residual life of sprayed-on pesticides on a plant surface means that they usually have a short lasting action from a few hours to a few days (Rortais *et al.*, 2005). Systematic pesticides, such as imidacloprid (Figure 1.18), can penetrate plants at the point of their original application and subsequently travel throughout the plant; leading to the unintended contamination of both pollen and nectar (van Emden, 1989). Pollen has also been found to contain pesticide residues (Mullin *et al.*, 2010), not only from agricultural sources but also from garden-use pesticides (Smodis Skerl, Velikonja Bolta, Basa Cesnik, & Gregorc, 2009; Yang, Chuang, Chen, & Chang, 2008). Returning contaminated pollen to the hive therefore has the potential to intoxicate the whole of the colony.

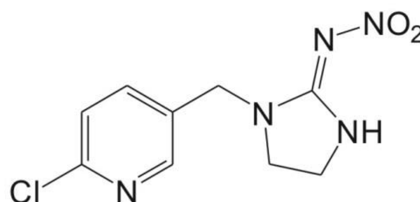

Figure 1.18: Imidacloprid - a neonicotinoid insecticide.

As the majority of pesticides fail to degrade naturally or degrade slowly over time (Rortais *et al.*, 2005) it can often witness a bioaccumulation of pesticides within animals and plant materials (Chu, Hu, & Yao, 2005). Fat-soluble/lipophilic compounds, typically pyrethroids and organophosphates (Halm, Rortais, Arnold, Taséi, & Rault, 2006) often have good stability in wax/fatty mediums and are capable of accumulating within the hive (Johnson, Ellis, Mullin, & Frazier, 2010).

According to an online pesticide usage survey, the total amount of agricultural pesticides used in the UK for 2013 stands at 17,025,465 kg; which were applied over 77,980,580 hectares (ha) in the UK (Figure 1.19) equating to an average of 0.218 kg/ha; this is a decrease on the previous year and a continuing trend since 1990 (Figure 1.20) (Food and Environmental Research Agency (FERA), n.d.).

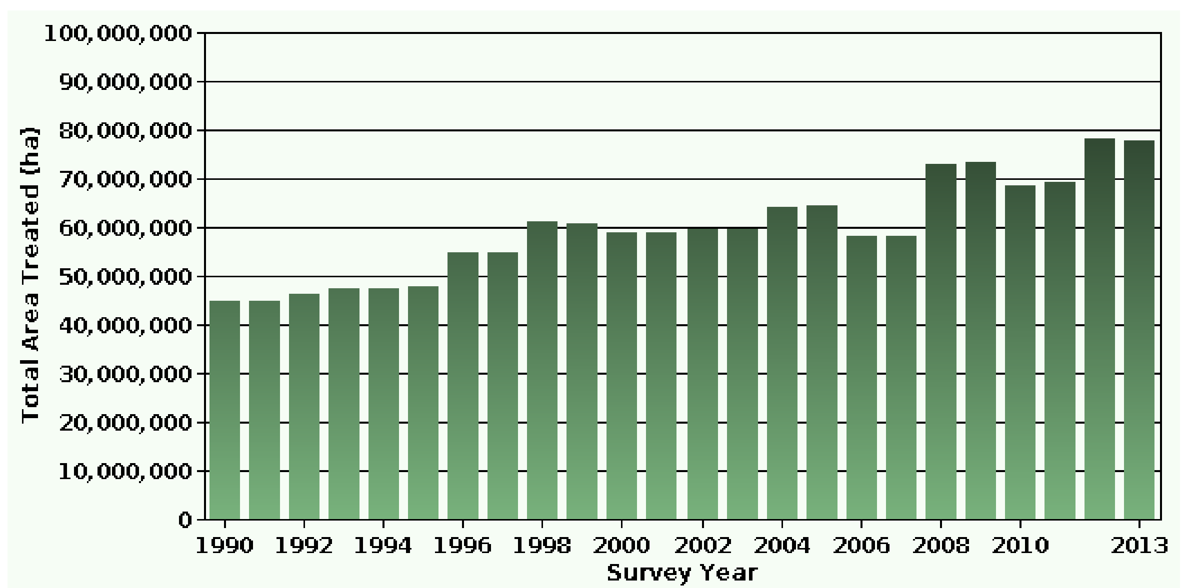

Figure 1.19: Total area treated (ha) of all crops types in Great Britain. Taken from Food and Environmental Research Agency (FERA), (n.d.)

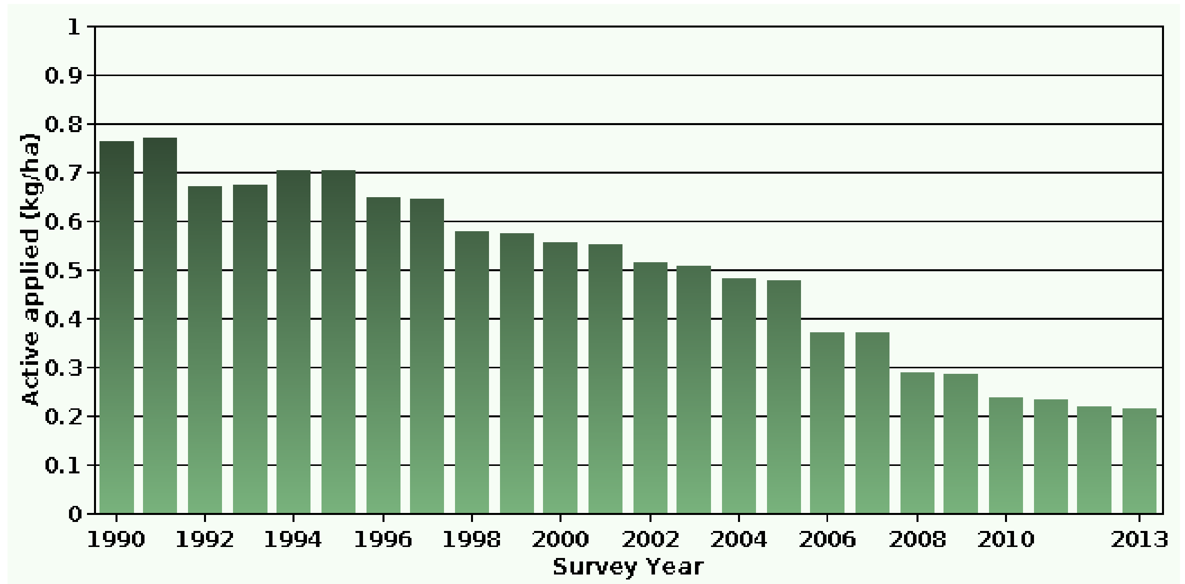

Figure 1.20: Amount of active applied (kg/ha) for all pesticides applied to all crops types in Great Britain; figures represent total amount of active ingredient per hectare including any repeat applications. Taken from Food and Environmental Research Agency (FERA), (n.d).

The continued falling trend in the weight of pesticide applied per hectare is a consequence of a greater efficiency of newer chemical treatments (Garthwaite *et al.*, 2008). For example, neonicotinoid seed treatments can be applied directly to the site of action, less active ingredient (a.i.) applied per hectare than sprays or granular applications (Jeschke, Nauen, Schindler, & Elbert, 2011), as shown in Figure 1.21.

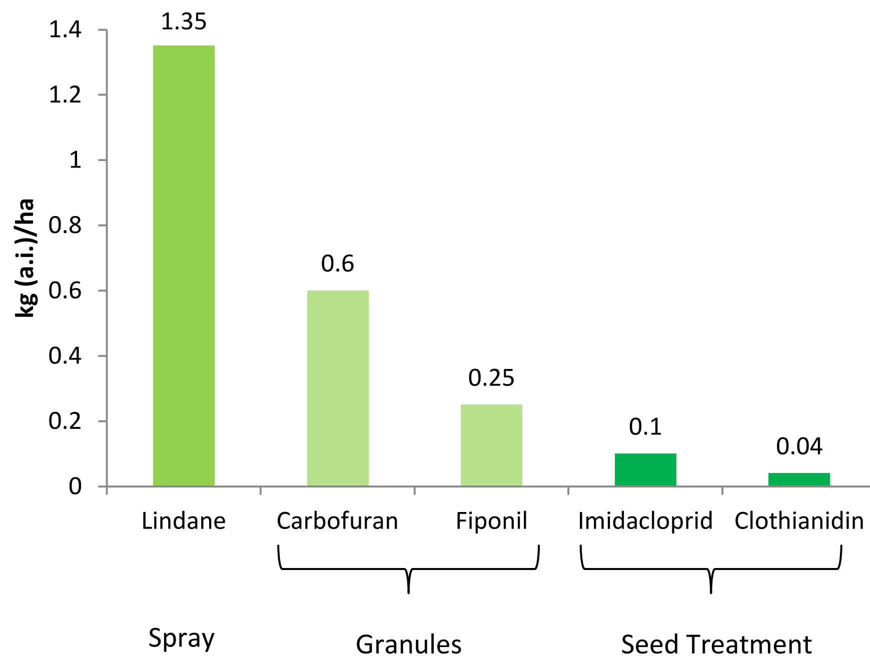

Figure 1.21: The development of pesticide application methods in maize; applied grams of active ingredient (a.i.) per hectare. Information edited from Jeschke, Nauen, Schindler, & Elbert, (2011).

Entombed pollen

The phenomenon of 'entombed pollen' was first identified by vanEngelsdorp *et al.,* (2008); here they describe bee bread that is brick red in colour and sealed under a sunken wax capping (Figure 1.22). It was noted that colonies containing entombed pollen had a higher mortality rate than those that only contained normal pollen. The increase in honeybee mortality was directly linked to entombed pollen, as there was no significant reduction in the longevity of larvae or adult bees; however, an accumulation of pesticides was thought to be a factor. Tests on the entombed pollen revealed thirty pesticides, with the most occurring pesticides being fluvalinate in 96 % of samples in addition to coumaphos and chlorothalonil both being found in 100 % of samples. In comparison, chlorothalonil only occurred within 45.5 % of 'normal' hives.

Figure 1.22: The arrows indicate 'Entombed' pollen which is identified by its sunken, wax-covered cells. Other Surrounding cells (mostly yellow in colour) are 'normal', uncapped cells containing bee bread. Adapted from vanEngelsdorp *et al.,* (2008).

As discussed previously, various numbers of bees within a colony perform different age related tasks. Therefore exposure to pesticides would vary throughout the colony. Nectar foragers are found to have the greatest need for sugar (224 - 898.8 mg/week) and thus the highest exposure to pesticides. In the example of the imidacloprid, this would equate to 1.1 - 4.3 ng [assuming 4.75 pg of imidacloprid in 1 g of sugar] (Rortais *et al.*, 2005). Table 1.2 provides further estimations of the amount of imidacloprid contaminated nectar/pollen consumed by the various categories of honeybee within the hive.

Table 1.2: Estimated amounts of sugar (contained in nectar or honey), pollen and imidacloprid consumed by larvae during their development over N days (N = 5 days for workers and N = 6.5 days for drones) and by adults over a period of N days of activity (N = 10 days for nurses, N = 6 days for wax producing bees, N = 8 days for brood attending bees, N = 90 days for winter bees and N = 7 for foraging bees). The amount of imidacloprid consumed by honeybees is determined by the following equivalence: 1 mg of sugar contained in nectar or honey = 4.75 pg of imidacloprid and 1 mg of pollen = 3.4 pg of imidacloprid in nectar and pollen coming from Gaucho seed-dressed plants. N/A = no data available. Redrawn and adapted from Rortais *et al.,* (2005).

Category of bee	Caste	Estimated amounts of food (sugar and pollen) and imidacloprid consumed per bee over N days		
		Sugar (mg)	Pollen (mg)	Imidacloprid (ng)
Larvae	Workers	59.4	5.4	0.3
	Drones	98.2	N/A	0.5
	Nurses	-	65	0.2
Hive bees	Wax-producing bees	108	-	0.5
	Brood-attending bees	272-400	-	1.3-1.9
	Winter bees	792	-	3.8
Foraging bees	Nectar foragers	224-898.8	-	1.1-4.3
	Pollen foragers	72.0-109.2	-	0.3-0.5

Interestingly, a honeybee will increase its food intake when exposed to a pesticide, in order to reduce the pesticide’s overall concentration within the honey sac and thus reducing the poisonous effect. However, the eventual regurgitation of a proportion of the contaminated foodstuff into a cell exposes the immediate area to the pesticide (Wallner, 1999).

1.5.3.1.2 Apicultural exposure

Apicultural exposure refers to any contamination which results from chemical applications made by a beekeeper; this shall be considered in regards to the control of *Varroa destructor.* A colony of *A. mellifera* is likely that will succumb within a few years if an infestation of *V. destructor* is not controlled. Since *A. mellifera* lacks any natural defences against the parasite chemical treatments are often used to control mite

populations (Rademacher & Harz, 2006). However, before chemical intervention the severity of mite infestation must first be determined. Mites regularly fall from their host and consequently beekeepers will commonly monitor the amount of varroa found at the base of the hive (Ellis & Nalen, 2013). Such monitoring procedure has been found to have a linear relationship with the population size of *V. destructor* within the colony (Delaplane & Hood, 1997). Such concepts are employed to ensure that treatment is justifiable and effective (Boecking & Genersch, 2008). Many varroa treatments are available to beekeepers. The most commonly-used treatments are organic acids, thymol, synthetic pyrethroids and organophospates; depending on each country's own approval. Different treatments are required throughout the year due to the biology of both the honeybee and the mite (Rademacher & Harz, 2006). A comprehensive list of in-hive treatments and their respective UK approval status can be found in Section 3.3, p125. Synthetic treatments include tau-fluvalinate, which acts sodium channels of the central nervous system, causing paralysis (Davies *et al.*, 2007).

Natural products have now become more widespread since the effectiveness of synthetic chemicals has declined, due to mite resistance (Johnson *et al.*, 2010). Organic acid treatments include formic, oxalic and lactic acids and can be naturally found within honey. During the summer months, formic acid is applied to the hive as a solution and is allowed to evaporate throughout the hive for a number of weeks. Varroa contained within the cells of emerging brood will be killed, along with those mites found on bees walking around the hive (Ritter & Akratanakul, 2006). The use of formic acid eventually interferes with both the metabolic and respiratory processes of the mite (Rosenkranz, Aumeier, & Ziegelmann, 2010). Oxalic acid can be applied in a number of ways and works

by direct contact. Lactic acid is also by direct contact, but is disadvantaged by a slow application process, as it must be sprayed to individual frames (Ritter & Akratanakul, 2006). Both acids are required to be applied to broodless colonies (Rosenkranz *et al.*, 2010) and since they do not rely on evaporation, these chemicals can be applied in colder months (Rademacher & Harz, 2006). Thymol is a volatile compound which can be applied to the hive in the form of a gel or as part of a formulation containing essential oils impregnated into a strip. It is believed that thymol inhibits the mite's growth, feeding and reproduction (Rosenkranz *et al.*, 2010).

1.5.3.2 Toxic effects of pesticides

The toxicity of a pesticide, following oral or dermal applications, is determined by the acute LD_{50} value of a substance (Curtis, 2006). The subscripted number 50 of LD (lethal dose) indicates that at a said dosage the administered chemical is toxic to 50 % of the sampled population (Nesheim, Fishel, & Mossler, 2008). Therefore an LD_{50} value of 50 µg/kg would be less toxic than a LD_{50} value of 5 µg/kg. Expression of the LD_{50} value can also be represented by µg/bee (Desneux *et al.*, 2007). The Washington State Department of Agriculture considers four levels of pesticide toxicity to bees: highly toxic (acute LD_{50} <2 µg/bee), moderately toxic (acute LD_{50} 2 µg/bee – 10.99 µg/bee), slightly toxic (acute LD_{50} 11 µg/bee – 100 µg/bee) or practically non-toxic (acute LD_{50} >100 µg/bee) (WSDA, 2010).

In order to understand the potential exposure dose, the LD_{50} must be converted to an equivalent food concentration (LC_{50}). The units of LC_{50} are in parts per billion by volume (ppbv), thus allowing for a direct comparison with experimentally measured doses. Equation 1.2 shows this conversion (Fischer & Chalmers, 2007).

$$LC_{50}(ppbv) = \frac{LD_{50}\ (\mu g\ /\ bee)}{Amount\ of\ sucrose\ solution\ ingested\ (mg)}\ .10^{6}$$

Equation 1.2: Conversion from LD_{50} (ug/bee) to LC_{50} (ppbv). The LC50 value can then be used to make direct comparisons with environmental values in pollen or nectar, for example (Fischer & Chalmers, 2007).

Most pesticides are often nonspecific and therefore may have toxic effects on non-target organisms such as bees (Nesheim *et al.*, 2008). Different levels of intoxication can be witnessed in honey bees and other insects according to the quantity of pesticides to which they have been exposed as well as the age and condition of an individual amongst other factors (Curtis, 2006). A dose is said to be sub-lethal if no evident mortality is witnessed in the experimental population (Desneux *et al.*, 2007). Desneux *et al.,* (2007) define sub-lethal effects as either a physiological or behavioural effect(s) on an individual that survives exposure to a pesticide at a concentration deemed to be either sub-lethal or lethal.

The genome of *A*. *mellifera* has been shown to contain fewer genes (≈11,000 genes) than, for example, that of the fruit fly Drosophilia *melanogaster* (≈13,500 genes). It is thought that a lack of detoxifying genes, which are involved in pesticide metabolism, may explain the honeybees' increased sensitivity to pesticides (Johnson *et al.*, 2010). It is even suggested that synergistic effects of pesticides may result from the competition between multiple pesticides trying to access the limited numbers of P450 enzymes (Claudianos *et al.*, 2006) – resulting in a greater level of toxicity. A reduced level of body fat can cause a greater susceptibility to pesticides; this reduction in body fat may be an effect of parasitic mites (Ritter & Akratanakul, 2006) or from poor nutrition caused, for example, by agricultural monocultures (Johnson *et al.*, 2009).

Royal jelly is an important foodstuff for development and is fed to both worker larvae and queen; although the queen will receive greater amounts of this substance. A reduction in the hypopharyngeal gland in nurse bees, through pesticide exposure (Hatjina *et al.*, 2013), can result in malnutrition of the developing larvae, thus reducing the number of emerging adults and threaten queen renewal (Daintith, 2004). Investigations into the effect of contaminated wax on colony health found that exposure to sub-lethal levels of pesticides in the brood comb led to an increased level of brood mortality, as well as reduced adult bee longevity (Desneux *et al.*, 2007). Larval exposure to pesticides can come from the consumption of contaminated food, fed to them by nurse bees, or from direct contact with comb wax whilst in the cell (Wu, Anelli, & Sheppard, 2011). Although the quantity of pesticides found within pollen and nectar are considered to be low, the high toxicities that these residues exhibit may induce sub-lethal effects within honeybees at low doses (Rortais *et al.*, 2005).

Studies into the effects on a honeybee's behaviour are typically performed under a watchful eye in the laboratory and therefore some of the more subtle symptoms of pesticide poisoning e.g. a reduction in foraging by an individual worker, may perhaps be overlooked by most beekeepers. The more obvious indications to the layman are an increase in 'aggression', paralysis, erratic body and/or spinning movements (Johansen, 1979). An accumulation of dying or dead worker bees at the hive entrance is also a probable sign that an exposure to pesticides has occurred – most likely during foraging (agricultural exposure) (Tremolada, Bernardinelli, Colombo, Spreafico, & Vighi, 2004). Poisonings from a lethal dose can often go unnoticed, as most honeybees will die in the field and therefore fail to return to the hive (Johansen, 1979). The effects of sublethal

doses can also have a dramatic effect on colony numbers; for example, deltamethrin (insecticide) was found to alter the homing-flight ability of bees (Vandame, Meled, Colin, & Belzunces, 1995); it was suggested that bees failed to acknowledge the visual pattern of landmarks in relation to the sun and so were unable to return to the hive. Eventually the inability for foragers to return to the hive can threaten colony survival, as nurse bees will often be recruited for foraging duty (precocious foraging) and this can lower brood production (Thompson, 2003); since nurse bees are needed to feed the brood. A reduction in brood surface area/numbers ultimately poses the biggest threat to colony survival, as mature bees will not be replaced at a sufficient rate (Desneux *et al.*, 2007). In either instance the beekeeper is unlikely to realise the magnitude of the situation until the colony has succumbed (Thompson, 2003; Wu *et al.*, 2011; Yang *et al.*, 2008).

However, efforts are made by the farmer to minimise pesticide exposure to bees and other beneficial insects. This can be achieved by making applications during the early morning or late in the evening. However, early morning applications were found to result in a greater mortality by as much as two to four times that of late night applications. This may be as a result of a rising temperature during the morning; meaning that bees will begin to forage, as this is when a flower will begin to shed pollen and release nectar (Johansen, 1979).

A honeybee's ability to communicate via the waggle dance has also been found to be adversely affected by exposure to organophosphorus, carbamate (Johansen, 1979) and neonicotinoid insecticides (Thompson, 2003). During the dance honeybees were found to underestimate the appropriate angle of the food source in relation to the sun; this would have a greater affect in misguiding novice foragers (Maini, Medrzycki, & Porrini, 2010).

Sub-lethal levels of imidacloprid are also known to affect the ability to communicate the distance to a food source (Thompson, 2003). Exposure to pyrethroids, whilst foraging, was found to affect the ability of a bee to return to its hive, while permethrin was shown to induce severe disorientation in foragers in addition to a large disturbance in behaviour i.e. less foraging and more self-cleaning (Thompson, 2003).

1.5.4 An introduction to neonicotinoids

Originally developed in the 1980s and first available on the commercial market in the early 1990s, neonicotinoids offered an attractive solution and have quickly become the most popular insecticide class in the world (Goulson, 2013); as such neonicotinoids are currently registered for use in more than 120 countries (Jeschke *et al.*, 2011).

Owing to their systemic properties, neonicotinoids can be used as: seed treatments, soil applications and as foliar sprays (Jeschke *et al.*, 2011; Jeschke & Nauen, 2008; Wollweber & Tieyen, 1999). In developed countries the single and most popular use of neonicotinoids compounds is for seed treatments (Dively & Kamel, 2012; Goulson, 2013). During 2011, 91 % of neonicotinoids used in the UK were applied as seed dressings (Goulson, 2013), whilst the global value was around 60 % (Jeschke *et al.*, 2011). Prior to 1st December 2013, five neonicotinoids were registered for use within the UK: acetamiprid, clothianidin, imidacloprid, thiacloprid and thiamethoxam. The seed treatments used on UK oilseed rape are: Cruiser® OSR (thiamethoxam) by Syngenta; Modesto® (clothianidin), Chinook® and Chinook Blue® (imidacloprid) by Bayer CropScience.

1.5.4.1 Chemistry of neonicotinoids

Neonicotinoids are a group of nicotine-related insecticides which selectively bind to insect nicotinic acetylcholine receptors (*n*AChRs). These receptors are increasingly becoming an important biochemical target site for insecticides (Jeschke *et al.*, 2011). In total, there are seven market-registered compounds belonging to the neonicotinoid class. These can be classified according to their pharmacophore moieties (Figure 1.23); for example: *N*-nitro-guanidine (imidacloprid, thiamethoxam, clothianidin and dinotefuran), nitromethylene (nitenpyram) and *N*-cyano-amidines (acetamiprid and thiacloprid) (Elbert, Haas, Springer, Thielert, & Nauen, 2008; Jeschke *et al.*, 2011).

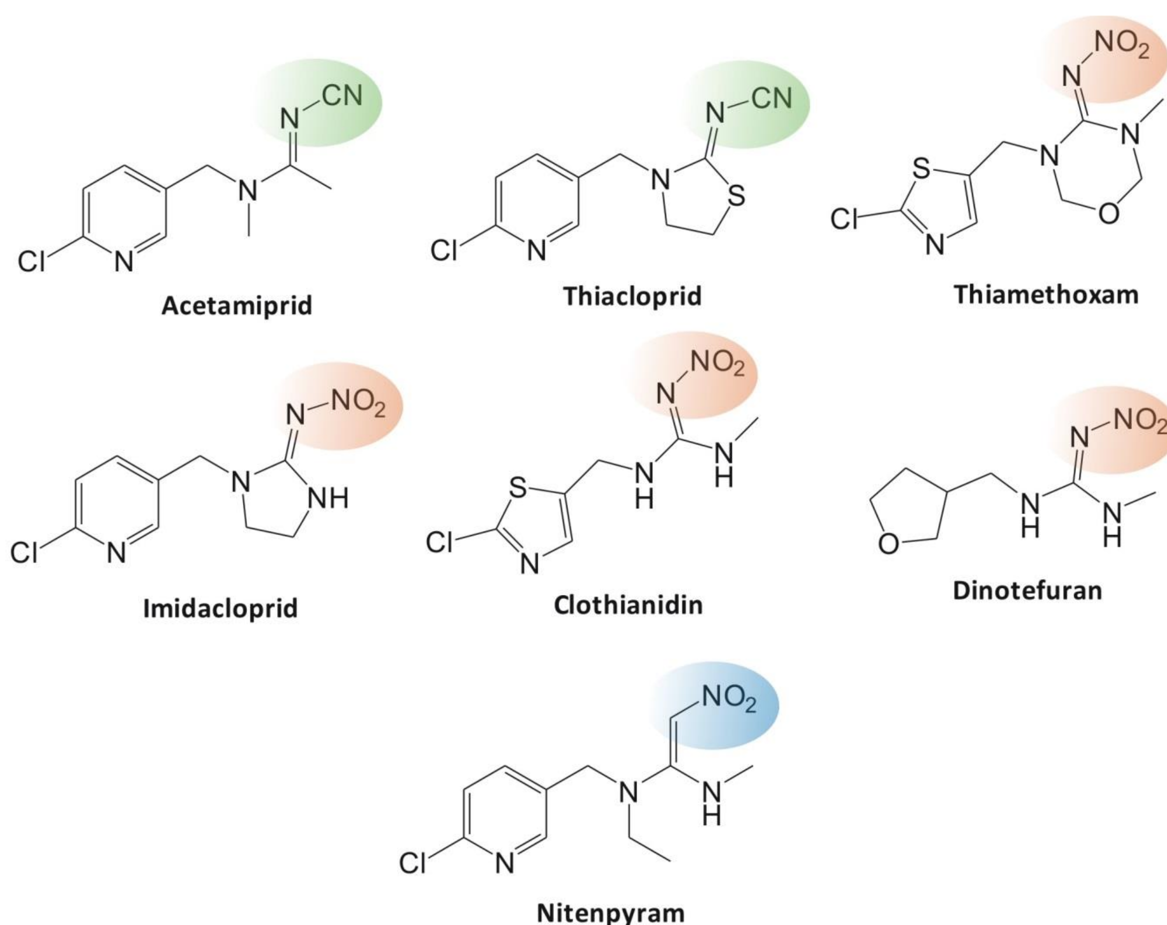

Figure 1.23: Neonicotinoid structures. Each pharmacophore has been circled: *N*-cyano-amidine (green), *N*-nitro-guanidine (orange) and nitromethylene (blue).

Neonicotinoids may also be regarded as cyclic (imidacloprid, thiacloprid and thiamethoxam) and non-cyclic (nitenpyram, acetamiprid, clothianidin and dinotefuran) (Jeschke & Nauen, 2008). The polar, non-volatile nature of neonicotinoids means that they demonstrate relatively poor lipophilicity compared to other non-polar pesticide classes. Non-cyclic compounds are generally less lipophilic than the cyclic ring systems (Jeschke & Nauen, 2008); this is reflected by their octanol-water partition coefficient (log K_{ow}) values (Table 1.3).

Table 1.3: Log K_{ow} and solubility values for the seven commercially available neonicotinoids. Adapted from Jeschke & Nauen (2008).

	Neonicotinoid	Log K_{ow} (at 25 °C)	Solubility in water (g L^{-1} at 20 °C)
Cyclic compounds	Imidacloprid	0.57[a]	0.61
	Thiacloprid	1.26[b]	0.185
	Thiamethoxam	-0.13	4.1[c]
Non-cyclic compounds	Nitenpyram	0.64	840
	Acetamiprid	0.8	4.2[c]
	Clothianidin	0.7	0.327
	Dinotefuran	0.644	54.3

[a] At 22 °C; [b] At 20 °C; [c] At 25 °C.

The uptake of an agrochemical by the roots of a plant is dependent on the lipophilicity of the compound (Briggs, Bromilow, & Evans, 1982). Neonicotinoids with a comparatively higher log K_{ow} are slightly more lipophilic than those with a lower value, therefore making the latter more effective as a seed treatment. Neonicotinoids with lower log K_{ow} will, however, show a better mobility in the xylem of the plant (Jeschke & Nauen, 2008). Once within the xylem, all neonicotinoids will be transported via the vascular system throughout all parts of the plant. Figure 1.24 shows how a neonicotinoid would be distributed throughout the plant when applied as a seed treatment.

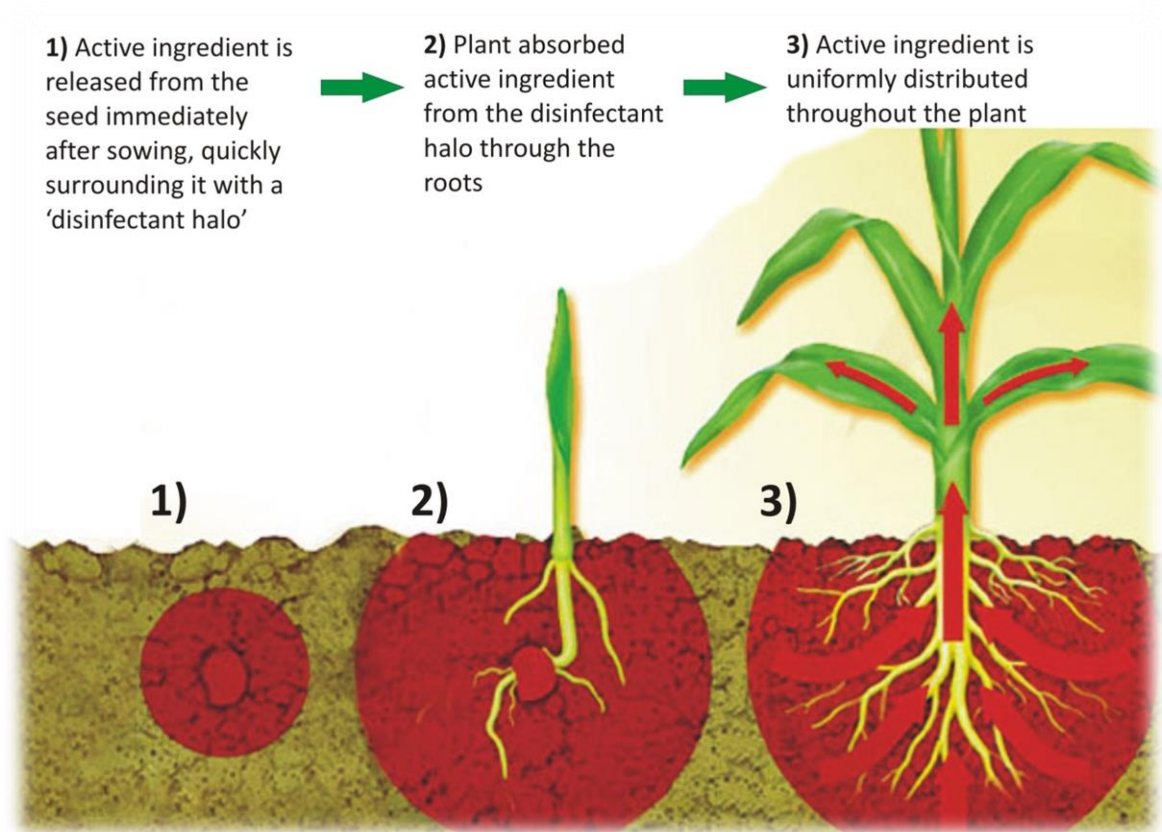

Figure 1.24: The distribution of a systemic pesticide, originally applied as a seed treatment, throughout a plant. The red 'disinfectant halo' offers protection from soil-borne pests and disease. Adapted from Bayer CropScience, (2012).

Neonicotinoids with an *N*-cyano-amidine moiety (acetamiprid and thiacloprid) are almost non-toxic to bees (Jeschke & Nauen, 2008). However, *N*-nitroguanidine containing neonicotinoids (thiamethoxam, Imidacloprid and clothianidin) are the most prominent neonicotinoid subclass (Jeschke *et al.*, 2011), that show high selectivity for binding to insect *n*AChRs (Kanne, Dick, Tomizawa, & Casida, 2005) and are therefore considered toxic to bees (Jeschke & Nauen, 2008). Based on LD_{50} values, thiamethoxam (29.9 ng/bee) and clothianidin (21.8 ng/bee) are found to be less toxic than imidacloprid (17.9 ng/bee) (Iwasa, Motoyama, Ambrose, & Roe, 2004); this had resulted in a shift from the use of imidacloprid to thiamethoxam (García-Chao *et al.*, 2010).

Due to their use as an oilseed rape seed treatment (Chapter 5), imidacloprid, thiamethoxam and clothianidin will be the main focus of this thesis and so, henceforth, only these are discussed.

1.5.4.3 The effects of neonicotinoids on honeybee health

A vast array of studies has investigated the effects of neonicotinoids on honeybee behaviour and immunity; although not an exhaustive list, a few examples of this shall be given here. Reported doses of thiamethoxam, at concentrations of 1.34 ng/20 µl (67 ppbv), were found to impair the homing ability of honeybees (Henry *et al.*, 2012). Clothianidin was shown to reduce immunity, thus making honeybees more susceptible to pathogens (Di Prisco *et al.*, 2013). Imidacloprid was found to affect cognitive behaviours (Decourtye *et al.*, 2005). Other behavioural abnormalities have also been observed, such as arching of the abdomen and uncoordinated movements along with food regurgitation (Girolami *et al.*, 2009). The ability to communicate can also be affected, which is suggested to impair social behaviour (Medrzycki *et al.*, 2003), whilst the success of foragers returning to the hive can be severely reduced (Bortolotti *et al.*, 2003). A greater in-depth review on the side-effects of neonicotinoids to honeybees is presented elsewhere (Blacquière, Smagghe, van Gestel, & Mommaerts, 2012).

Interestingly, other studies report that imidacloprid-treated sunflowers pose “no risk to honeybees” (Schmuck, Schöning, Stork, & Schramel, 2001). A study into the exposure of seed-treated canola on honeybees found clothianidin to have no adverse long-term effect on longevity or brood production in honeybees (Cutler & Scott-Dupree, 2007). This was found to be true for other bee species, including: the common eastern bumblebee

(*Bombus impatiens*) (Franklin, Winston, & Morandin, 2004) and the buff-tailed bumblebee (*Bombus terrestris*) (Thompson *et al.*, 2013). The effect of neonicotinoids on bumblebees is discussed further in Chapter 6.

Nevertheless, in light of a report from the European Food Safety Authority (EFSA) (EFSA, 2013), in addition to scientific information into the sublethal effects of neonicotinoids (Henry *et al.,* 2012; Whitehorn *et al.,* 2012), the European Commission imposed a two-year restriction on the use of imidacloprid, thiamethoxam and clothianidin for seed-treatments, soil applications and foliar treatment on cereals and plants deemed attractive to bees (European Commission, 2013). The restrictions were opposed by many, including the UK government (Gross, 2013) who were said to be unaware of any issues regarding the use of neonicotinoid seed treatment (PAN UK, 2012).

The EFSA identified "high acute risks for bees from exposure via dust…consumption of residues in contaminated pollen and nectar…and guttation fluid [in maize]". The regulation specifies that a "sufficient period of transition" is to be placed on the sale of treated seeds; subsequently, as of the 1st December 2013, crops including soft fruit and cereals can no longer be treated with any of the three neonicotinoids[1] (European Commission, 2013).

1.6 Laboratory verse field studies

It can be argued that a debate which divides the scientific community evolves around the validity/conclusions of laboratory verse field studies. Laboratory studies are conducted

[1] * Treated seeds sown before 1st December 2013 are therefore still legally allowed to flower in 2014.

under controlled conditions, thus allowing for replications to be achieved by other researchers. Independent variables are manipulated and the measured outcome is known as the dependant variable. Therefore, laboratory studies are able to show a cause and effect. Policy makers are able to use important information gained from laboratory studies, but they need to use doses which represent those found in the field (Carreck & Ratnieks, 2014). In contrast, field experiments are conducted away from the laboratory under a natural setting i.e. outdoors. Studies are able to establish the true impact of pesticide on bee behaviour and colony performance, or to observe the true levels of pesticide exposure under normal use (Godfray *et al.*, 2014). Field studies can easily be criticised if poorly designed or executed. However, the costs to carry out an ideal study would be too expensive, whilst an idyllic experimental plot would be hard to come by (Carreck & Ratnieks, 2014). The lack of replication in field studies also makes it difficult to extrapolate cause and effect (Godfray *et al.*, 2014).

A review paper by Carreck and Ratnieks (2014) investigates the origins of a "field-realistic" dose, which is often used and quoted within the literature. They highlight that laboratory studies have somewhat overestimated three key factors which affect field exposures: concentration, duration and choice. Henry *et al.,* (2012) offered 20 µl of sugar solution, containing 67 ppbv of thiamethoxam, to individual honeybees. When tracing this *concentration* back to its origin, it was found that the data was based on a cited Rortais *et al.,* (2005) which contained estimates of exposure to imidacloprid during foraging. It was actually estimated that the dose received would be the result of 7 days foraging. This demonstrates that the *concentration* and *duration* of pesticide exposure conducted in the laboratory is not always representable of long field exposures (Carreck

& Ratnieks, 2014). Laboratory feeding trials often make the assumption that a colony will feed on a single crop and so exclusively expose bees to a single pesticide; thus representing the treatment of a single crop. However, this is found not to be the case as bees have a *choice* of forage in the field (Carreck & Ratnieks, 2014; Osborne, Carreck, & Williams, 2001).

Field studies have evidenced lower levels of neonicotinoids in the field (Cutler, Scott-Dupree, Sultan, Mcfarlane, & Brewer, 2014; Cutler & Scott-Dupree, 2007; Pilling, Campbell, Coulson, Ruddle, & Tornier, 2013) than those was used within laboratory studies (Bryden, Gill, Mitton, Raine, & Jansen, 2013; Gill, Ramos-Rodriguez, & Raine, 2012). Therefore, proposing that laboratory studies are more representative of "worse-case" scenarios (Carreck & Ratnieks, 2014). However, field studies have demonstrated neonicotinoids to have little or no adverse effects on the overwintering of honeybees (Cutler *et al.*, 2014; Cutler & Scott-Dupree, 2007, 2014; Pilling *et al.*, 2013).

1.7 Rationale of research

Pesticides have been identified as one of the key drivers behind pollinator declines. The routes of pesticide exposure can originate from the treated crops on which bees forage, as well as from the beekeeper during the treatment of various honeybee pests. This can ultimately lead to the contamination of comb wax, food sources and developing larvae. The pesticides found within wax are often stable and persistent for many years, resulting in chronic exposure to sublethal levels of a number of pesticides. Consequently, this can have negative effects on the growth and survival of a honeybee colony. A vast majority of the data on residual pesticide levels in beeswax comes from the US or from across

Europe; this excludes the UK, where no information has been published within this area. It is generally accepted that levels found in the US are applicable to the UK. However, differences in beekeeping and agricultural practices as well as climate, habitat and the frequency of honeybee diseases, mean that each country should be considered individually; as each of the aforementioned stressors may play a more or less significant role across the world. For example, CCD has been described within the USA; however, it is not recognised within Europe.

The purpose of this study is to establish an understanding of the presence of residual pesticides contained within UK honeybees comb wax samples; as well as aiming to understand their accumulation in comb wax over time. Oilseed rape is one of the top economically important crops grown in the UK and so samples of nectar and pollen from this crop will also be analysed in order to appreciate what levels of pesticides honeybees are exposed to during foraging. Such knowledge may help to structure efforts to help mitigate further honeybee losses.

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CHAPTER 2

Methodology - sample preparation and analysis

2.1 Overview

This chapter will cover the theory and principles of sample preparation and the analytical instruments used in this study. Before discussing the various aspects of analytical instrumentation, it is important to consider the preparative steps which must be taken to ensure that samples are ready for analysis. This includes 'QuEChERS'; the primary sample preparation technique used within this study. The analytical techniques used within this study include both gas chromatography – mass spectrometry (GC-MS) and liquid chromatography – mass spectrometry (LC-MS). These systems shall be primarily considered as isolated techniques (GC, LC and MS) and the principle component which make up the instruments.

2.2 Introduction

Determining the levels of pesticides to which honeybees are exposed to can be achieved using various sensitive analytical techniques. Traditionally this analysis was carried out using gas chromatography (GC) coupled to a suitable detector i.e. electron capture detector; however further analysis was needed to obtain a conformational result (Alder, Greulich, Kempe, & Vieth, 2006). Although liquid chromatography (LC) based methods in combination with UV and diode array detectors do exist, these were often less sensitive than GC instruments and therefore rarely adopted (Alder *et al.*, 2006). However, the sensitivity of analytical equipment used in modern analytical laboratories is ever improving, most notably GC and LC instrumentation coupled to a mass spectrometer (MS) and is now enabling researchers to detect residual pesticide levels typically around one part per billion (ppbv). The amount of pesticides used in today's agricultural practices has led to the development of multi-residue extraction techniques i.e. "QuEChERS" (Anastassiades, Lehotay, Stajnbaher, & Schenck, 2003) as well as suitable (multi-residue) analytical methods (Wiest *et al.*, 2011). These will all be covered in more detail below.

2.3 Sample preparation: Extraction and clean-up techniques

It is often necessary to prepare a sample before it can undergo instrumental analysis; this may involve the exchange of solvent, concentrating the sample or applying an extraction technique to remove analytes from a dirty or complex matrix. Unwanted compounds may cause background ions or ion suppression, which result in a loss of sensitivity during mass spectral analysis. The samples analysed within this thesis: wax, nectar, pollen and bees

are considered complicated and 'dirty' matrices, containing many different compounds; thus a suitable extraction and clean up procedure is required.

2.3.1 Solid phase extraction

A technique used by most chromatographers in sample preparation is solid phase extraction (SPE). SPE is often used to separate and increase the sensitivity of detection of target analytes from interfering matrix components, which would otherwise interfere in sample analysis. A hydrophobic organic material, that can be polar, moderately polar or nonpolar in nature is adhered to powdered silica in order to form the solid phase (adsorbent), often contained within a disposable cartridge.

In the case of removing interfering components from a solution the adsorbent will have a selected polarity similar to that of the unwanted compounds. The adsorbent will then retain these within the cartridge whilst the analyte of interest, e.g. pesticides, are free to elute ready for analysis (Figure 2.1).

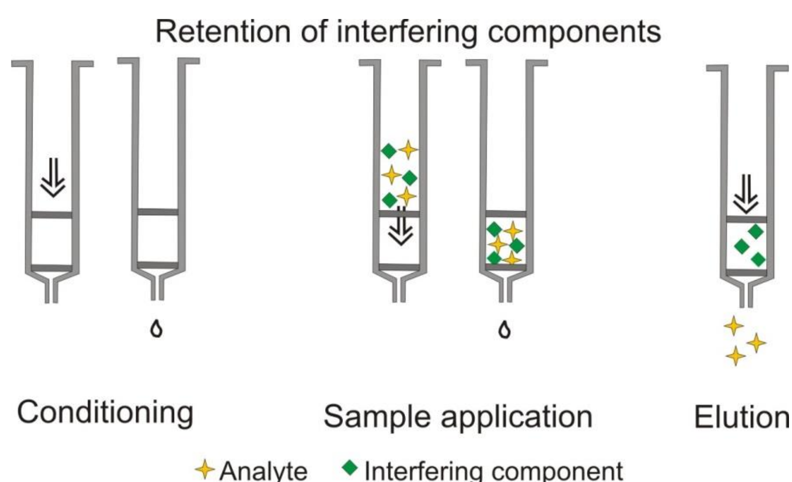


Figure 2.1: A schematic diagram of a typical SPE procedure whereby the analyte is eluted whilst the interfering component is retained by the adsorbent. Redrawn and adapted from Macherey-Nagel (n.d.).

Another variation of SPE is whereby the adsorbent will retain the compounds of interest, leaving the interfering components to elute to waste. The target analytes can then be eluted with an appropriate solvent and readied for analysis.

2.3.2 QuEChERS

The extraction and partitioning method, most commonly referred to by its acronym "QuEChERS" (**Qu**ick, **E**asy, **Ch**eap, **E**ffective, **R**ugged and **S**afe) was first described by Anastassiades *et al.,* (2003). This relatively new technique aimed to solve the problem of long and labour-intensive analysis with high solvent wastage (Anastassiades *et al.*, 2003). It now appears to have become very popular ever since its introduction in 2003. The QuEChERS technique has demonstrated a good recovery of 150 pesticides and metabolites with good repeatability from within fruit and vegetable matrices. It has also been validated in accordance with the European Union Quality Control Procedures for Pesticide Residue Analysis (Anastassiades *et al.*, 2003). Although originally created for food analysis, many adaptations of QuEChERS have been developed as researchers aim to refine and apply the method to other matrices. Mullin *et al.,* (2010) adapted the technique for the analysis of beebread, comb wax and bees. The technique is able to produce a sample that is compatible to both LC and GC, although the latter requires a more extensive clean-up stage, in the form of SPE, to remove any co-extracted compound which may cause harm to the analytical equipment.

The following description of the QuEChERS technique is taken from Mullin *et al.*, (2010) and is represented as a schematic diagram in Figure 2.2.

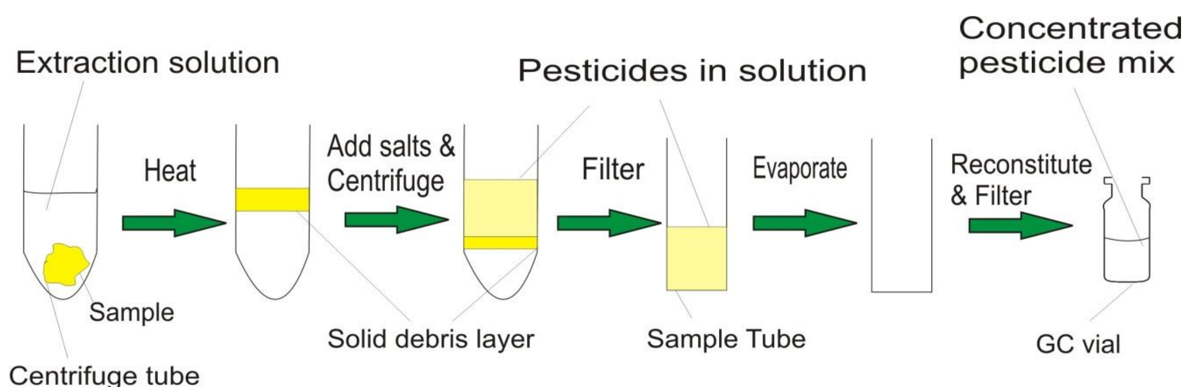


Figure 2.2: A schematic diagram presenting an outline of the QuEChERS procedure as described by Mullin *et al.,* (2010).

A sample is added to a centrifuge tube containing an extraction solution (44 % deionised water, 55 % acetonitrile (ACN) and 1 % glacial acetic acid). Magnesium sulphate and sodium acetate are then added to the solution and shaken vigorously for one minute before being centrifuged. Depending on the nature of the sample it may need to undergo homogenisation (heating or placed in an ultrasonic bath) before centrifuging. Once centrifuged two layers are formed; the organic layer is then removed from the aqueous layer following a clean-up stage using primary-secondary amine (PSA), C18 and $MgSO_4$ within a centrifuge tube, ready for LC analysis. A different clean-up procedure is adopted for GC analysis; here the supernatant is filtered using a dual layer SPE cartridge which contains PSA and CUCARB and eluted using acetone/toluene (7:3 v/v) – this also acts as a solvent exchange step as ACN has a large solvent expansion during vaporisation. The filtered solution is then dried down and is then ready for GC analysis.

QuEChERS favours the use of acetonitrile (ACN) as an extraction solvent, as it does not extract as much fats, wax or lipophilic pigments following SPE clean-up with PSA (Mullin *et al.*, 2010); these co-extractives would negatively affect GC analysis (Anastassiades *et al.*, 2003). ACN is partially immiscible in water, yet it is more easily separated from water

upon the addition of salts than other solvents i.e. acetone. The use of salts i.e. the addition of $MgSO_4$ prior to centrifuging, also helps to induce liquid-liquid phase separation and through varying the amount of salts used it is possible to alter phase polarity. This is important because normally non-polar pesticides would congregate in the organic phase and polar in the aqueous phase. The immiscibility of ACN with water means that polar pesticides would be present in the organic layer where water is present. However, by adding $MgSO_4$ the technique reduces the water phase volume and can precipitate polar pesticides into the organic layer. The heat generated by the hydration of $MgSO_4$ is also believed to aid the extraction process.

Broadly speaking, pesticides are more stable at a lower pH and the addition of glacial acetic acid ensures that basic-sensitive pesticides (i.e. captan) do not break down during extraction. Basic pesticides, however, show poor recovery at low pH and remain within the aqueous layer. This is addressed by the partial immiscibility of ACN with water. Regardless of pH, basic pesticides were found to partition into the semi-polar upper phase (ACN-water mix). A downside is that a higher amount of fatty extracts (i.e. fatty acids) are recovered with a decrease in pH (Anastassiades *et al*., 2003).

PSA is included to remove any co-extracted acids and sugars (Anastassiades *et al*., 2003). However, it must be noted that both CUCARB and PSA can retain both planar and acidic group containing pesticides respectively. An internal standard (ISTD) can be added during the extraction process, in order to determine whether the retention of pesticides is unacceptable. Recoveries of a suitable ISTD, as low as 70 % can be deemed sufficient (Kamel, 2010). It must be further noted that PSA is known to over saturate when exposed to high amounts of polar compounds, such as sugars (Anastassiades *et al*., 2003).

Therefore, not all of these may be removed and consequently seen during analytical analysis. To avoid this loss of planar molecules and acidic metabolites Kamel (2010) also developed a similar technique to that of Mullin *et al*., (2010) but included the use of a C18 SPE cartridge instead of a PSA, CUCARB cartridge. However, Kamel (2010) only looked at the pesticide imidacloprid and this was not intended as a multi-residue extraction method, while the procedure used by Mullin *et al.,* (2010) was.

2.4 Analytical methods

2.4.1 Chromatography

Chromatography is the process of physically separating the analytes of a mixture between two immiscible phases – a stationary phase (gas or liquid) and a mobile phase (liquid or gas) (Niessen 2006). There are five fundamental mechanisms of this chromatographic separation:

1) *Adsorption chromatography*
2) *Partition chromatography*
3) *Ion-exchange chromatography*
4) *Molecular exclusion chromatography*
5) *Affinity chromatography*

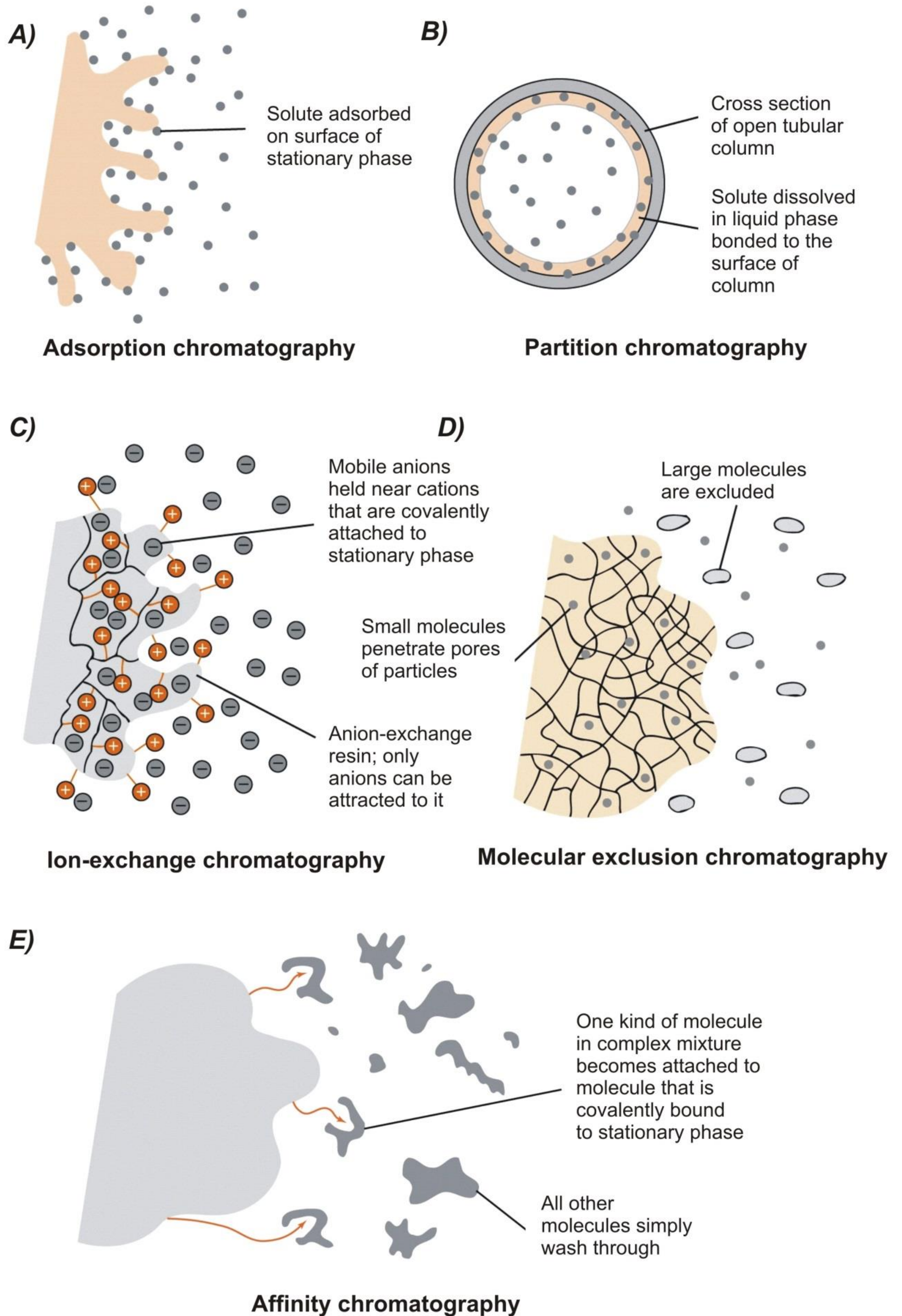


Figure 2.3: A schematic showing the five mechanisms of chromatography: *A)* Adsorption chromatography, *B)* Partition chromatography, *C) I*on-exchange chromatography, *D)* Molecular exclusion chromatography, *E)* Affinity chromatography. Images taken without permission from Harris (2002).

1) Adsorption chromatography

In adsorption chromatography (Figure 2.3 A) analytes are separated according to their polarity, which determines their interaction with the surface of a porous solid stationary phase. A polar stationary phase, such as alumina gel, will have a greater attraction with polar analytes, meaning they are retained for longer on the column; while non-polar analytes will remain in the mobile phase, which can be either a gas or liquid. In this instance non-polar analytes will travel quicker through the column, resulting in the separation of a mixture (McNair & Miller, 2009). The two most commonly used adsorbents are silica gel and alumina. The two most common stationary phases are silica or alumina gel; both of which contain hydroxyl (OH) groups, which can give rise to specific [van der Waals'] interactions (i.e. London forces. hydrogen bonding and/or dipole-dipole interactions) between the stationary phase and analytes (Langford *et al*., 2005). Polar analytes do not always display a good level of separation, meaning that adsorption chromatography is often used to separate relatively non-polar analytes.

2) Partition chromatography

Partition chromatography (Figure 2.3 B) can separate a mixture of analytes according to their respective solubility within both the mobile and stationary phase (Langford *et al*., 2005). A liquid stationary phase, such as silica gel, is chemically bonded to a solid support which has a large surface area. Those analytes which favour the stationary phase will travel at a slower rate than those favouring the mobile phase. By altering the composition/polarity of the mobile phase the retention times of the analytes can also be changed. In liquid chromatography there are two types of partition chromatography, which are based on the relative polarities of the mobile and stationary phases. A non-

polar mobile phase and polar stationary phase is referred to as 'normal-phase' whilst a non-polar stationary phase with a polar solvent system is classed as 'reverse-phase' chromatography (Skoog, West, Holler, & Crouch, 2004).

3) Ion-exchange chromatography

Separation by ion-exchange chromatography (Figure 2.3 C) is achieved according to interaction between the ionic functional groups of the stationary phase and the ionic charge of an analyte. Those analytes with an opposite charge to those of the functional groups will therefore exhibit a greater attraction to the stationary phase, thus travelling slower through the column than those of the same charge (Langford *et al*., 2005).

4) Molecular exclusion chromatography

The basis of molecular exclusion chromatography (Figure 2.3 D) relies on the physical properties of analytes i.e. size. This form of chromatography is useful for separating large molecules from smaller ones, with the latter being retained within the pores of the stationary phase and therefore eluting after the larger molecules. Unlike other chromatographic procedures, there is no physical or chemical interaction between the analyte and stationary phase (Skoog *et al*., 2004).

5) Affinity chromatography

Often used in biological systems, affinity chromatography (Figure 2.3 E) is a liquid purification technique. The technique separates proteins based on a reversible interaction between the protein (or group of proteins) and a specific ligand which is

attached to the stationary phase (e.g. agrose gel). As a specific ligand is used, this technique offers high-selectivity and high resolution (GE Healthcare, 2007).

2.4.2 Instrumental analysis

As noted previously 908 active ingredients exist across the pesticide spectrum, the inclusion of various heteroatoms, i.e. halogens, can help facilitate the analytical detection of various pesticides residues (Mullin *et al*., 2010). Following a successful extraction of pesticide residues, researchers often turn to a chromatographic technique for their analysis. The nature of the mobile phase is often used to describe the chromatographic process in which it is involved; for example, a liquid mobile phase would be liquid chromatography (LC) and a gas - gas chromatography (GC) (Alder *et al*., 2006). Figure 2.4 shows a schematic representation of the chromatographic process; this is also applicable for LC based chromatography.

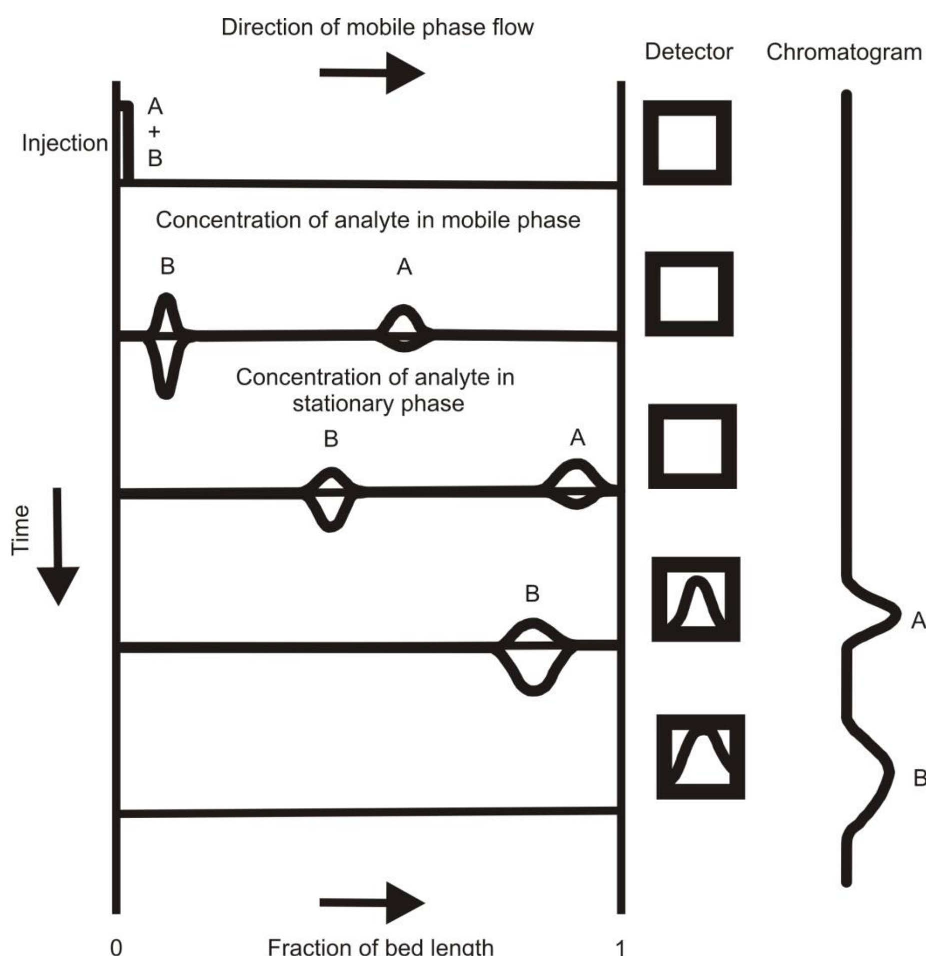


Figure 2.4: Schematic representation of two analytes (A and B) undergoing chromatographic separation. Separation is achieved according to each analytes respective attractions to the mobile phase (above each horizontal line) and stationary phase (below each horizontal line). Redrawn from McNair and Miller (2009).

2.4.2.1 Gas Chromatography

Gas chromatography has the ability to analyse over a hundred compounds within a single run (Rouessac & Rouessac, 2007) which makes it an ideal method for pesticide analysis in which multi-residue samples are now appearing to be common place. A sample is introduced at the injector port of the GC, which is often heated between 250 - 300 °C in order to 'flash vaporise' the liquid sample-solvent mix before it is transferred onto the column using an inert carrier gas (Millar & Haynes, 2000). There are two main methods of

injection onto the column, often referred to as split and splitless modes. In the former a 'split' vent prevents overloading of the column by only permitting a fraction of the originally injected amount onto the column. Splitless mode, however, allows the whole amount of the injected sample to pass onto the column, which is preferred for low concentration amounts of analyte (Millar & Haynes, 2000).

As the sample moves through the column, individual components/analytes interact with the stationary phase and the amount of interaction determines the overall retention time of each analyte (Drijfhout, 2010). The retention time of an analyte refers to how long it takes for the analyte to travel along the length of the column; this is influenced by many factors such as an analyte's volatility and polarity. A highly volatile analyte would elute the soonest since it would have very little interaction with the stationary phase. The polarity of the column will also influence the retention time of the analytes. A general rule of thumb when choosing a column stationary type is that 'like dissolves like'; therefore, polar pesticides are better separated using a polar stationary phase and non-polar pesticides are best separated with a non-polar column (McNair & Miller, 2009).

The column is situated within the oven of the GC, which usually operates between 40 – 325 °C, although temperatures of around 450 °C can be reached (McNair & Miller, 2009); providing the stationary phase of the column is able to withstand higher temperatures (Drijfhout, 2010). Oven temperatures may be left to operate isothermally whilst running a sample. However analysis times are often long and separation of analytes are sometimes incomplete if the temperature fails to reach the boiling point of each component (Baugh, 1993). Alternatively, oven temperatures are programmed to adjust over a set period of time, by a set amount of degrees until a final temperature is met providing an easy and

effective way to induce separation of analytes (McNair & Miller, 2009). Once the vaporised analyte has eluted from the GC column it enters a detector which produces a chromatogram.

Gas chromatography is unfortunately limited by sample volatility and possible thermal degradation of many thermal liable compounds (McNair & Miller, 2009). The issue of volatility, thermal sensitivity or polarity can be addressed via derivatisation, which chemically modifies a compound to produce a compound that has properties suitable for analysis; however, this extra step may introduce handling errors (Matovska, 2004). In this case liquid chromatography can be used as a complimentary technique to GC.

2.4.2.2 Liquid Chromatography

In modern analysis, liquid chromatography refers to the use of a high performance liquid chromatography (HPLC) system; this high performance system uses at least one pump to continuously force the liquid mobile phase through a well packed column (McMaster, 2005). Due to the high pressure needed to propel the mobile phase, the column is much shorter with a wider internal diameter and reinforced outer casing compared to those used in GC (Rouessac & Rouessac, 2007). In order for a sample to be analysed it must be soluble within the mobile phase or similar solvent, the former being pumped from a reservoir into an injector and then onto the column (Rouessac & Rouessac, 2007). Partition chromatography is the most widely used form of chromatography in HPLC systems, which includes both normal-phase chromatography and reversed-phase chromatography. The choice of either normal-phase or reversed phase depends on the nature of the target analytes; however, the latter is believed to be the most popular

(Skoog *et al*., 2004). The polarity of the mobile phase can be altered by changing its composition during analysis. A polar mobile phase (often water) is selected along with a modifying solvent i.e. methanol or acetonitrile – often referred to as the organic modifier. In changing the mixture ratio it is then possible to alter the retention times of analytes (Rouessac & Rouessac, 2007). Increasing improvements in column technologies are allowing for a higher resolution/better separation of analytes through the use of narrow diameter columns and small particle sizes (Skoog *et al*., 2004).

2.4.2.4 Chromatogram

The column of either an LC or GC terminates at a detector. Here eluting analytes are detected, generating an electrical signal which is displayed in the form of a chromatogram (Figure 2.5), which is a plot of the retention time (X-axis) verse the amount of a substance given by its peak area/height (Y-axis). The area under the green peak (32256.53) has been given in Figure 2.5, along with the RT of the peak (3.182 mins).

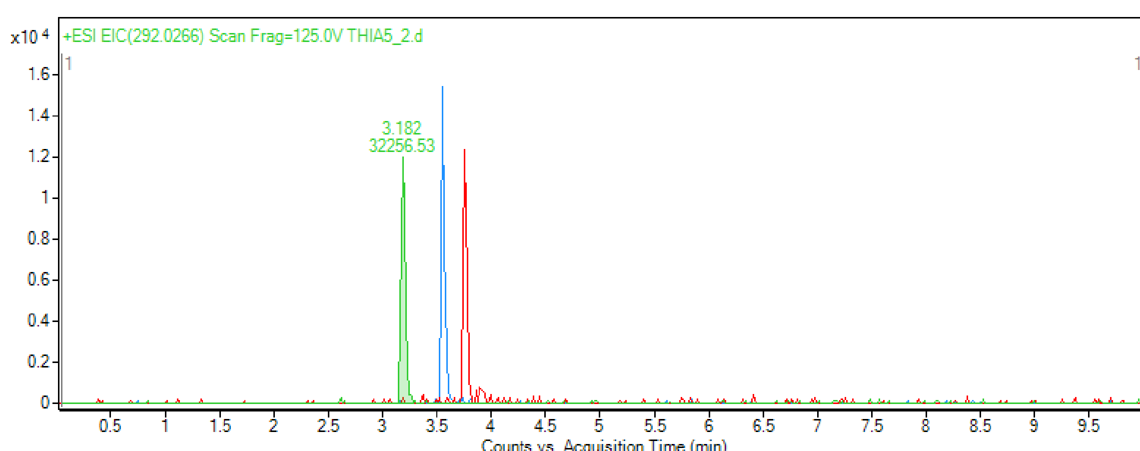


Figure 2.5: An example LC (EIC) chromatogram of three neonicotinoid pesticides: (left to right) thiamethoxam (green) RT = 3.182 mins; clothianidin (blue) RT = 3.554 mins; and imidacloprid (red) RT = 3.759 mins. Thiamethoxam has also been integrated to show the area under the peak.

As already mentioned, the time which it takes for an analyte to elute from the column is known as the retention time, which produces qualitative information. Assuming the same sample parameters are used when comparing runs it is possible to determine the peak's identity by its respective retention time. The area under the peak equates to the total amount of analyte present in a sample (quantitative information). This information can be plotted on to a calibration curve (Section 2.5.5, p114) to determine the concentration of an analyte.

2.5 Detection Methods

A limitation of using any instrumental chromatographic technique is the need for a detector to be coupled in sequence, in order to detect anything from the separated sample. Various detector types exist, for example: nitrogen-phosphorous, flame photometry, mass spectrometry, electron capture detector or flame ionisation (McNair & Miller, 2009).

2.5.1 Mass Spectrometry

Mass spectrometry (MS) can be used as a stand alone analytical technique but it may also be employed as a detector for either GC or LC apparatus. When coupled to a chromatography-based instrument the MS uses the chromatographic effluent as an analyte source (Rouessac & Rouessac, 2007). When analysing natural products, MS has two applications: *(1)* When the identity of studied samples are known, it is possible to confirm their identity using spectral data, *(2)* When the sample's structure is unknown, it is possible to reveal important structural information from either MS alone or in

conjunction with Nuclear Magnetic Resonance (NMR) (Millar & Haynes, 2000). The schematic in Figure 2.6 shows the key components of a mass spectrometer.

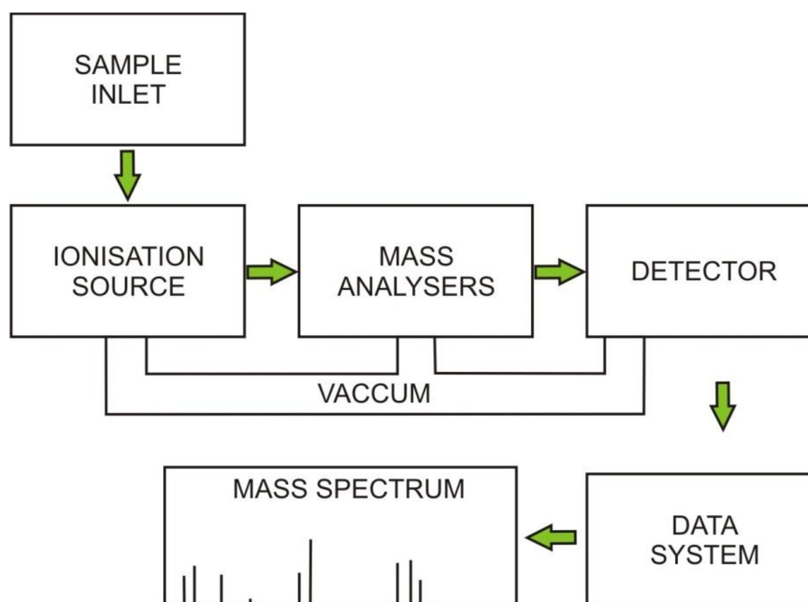


Figure 2.6 Schematic of a mass spectrometer in sequence with a GC (sample inlet). Redrawn and adapted from McNair & Miller (2009).

MS relies on the production and subsequent separation of ions based on the respective mass-to-charge ratio (*m/z*). The relative abundance of detected ions are plotted as a function of *m/z* to produce a mass spectrum (Millar & Haynes, 2000). Ionisation of a molecule is achieved through the use of an ion source. There are many ion sources available such as Electrospray Ionisation (ESI), Matrix Assisted Laser Desorption Ionisation (MALDI), Atmospheric Pressure Chemical Ionisation (APCI), Chemical Ionisation (CI) and Electron Ionisation (EI). Both EI and CI are the two most widely used ionisation techniques in GC-MS (Millar & Haynes, 2000; Niessen, 2006); however, these ionisation techniques are not available for LC systems. Since MS requires samples to be in the gas phase, direct injection of the eluent solvent from the LC column will cause the MS to foul and cause a loss of signal (Baugh, 1993). There are many LC-MS interfaces available to address this

issue; the most common interface is electrospray ionisation (ESI) (de Hoffmann & Stroobant, 2007).

2.5.2 Ionisation

Electron ionisation - *(gas chromatography- mass spectrometry)*

Electron ionisation (EI) is often referred to as a 'hard' ionisation technique which consists of a heated filament which emits electrons that are then accelerated towards an anode; these electrons collide with the gaseous effluent from the GC. A consequence of this is the loss of an electron (ionisation) from the introduced molecule (McMaster, 2005). If this positively charged species is stable then it will appear on the mass spectrum as the highest mass ion, known as the parent molecular ion $[M]^+$ (de Hoffmann & Stroobant, 2007). A beneficial feature of EI mass spectra is its extensive fragmentation pattern, witnessed because the electron beam of 70 eV has enough excess energy to dissipate into the molecule and induce characteristic fragmentation patterns (Baugh, 1993). However, the downside is that in most cases $[M]^+$ is often absent or detected in a low abundance due to an excessive amount of fragmentation (de Hoffmann & Stroobant, 1994).

Chemical ionisation - *(gas chromatography- mass spectrometry)*

Chemical ionisation (CI) is a 'soft' ionisation technique and unlike EI there is very little fragmentation since CI is a lower energy process; as a result $[M+H]^+$ is observed (de Hoffmann & Stroobant, 2007) and this makes CI complementary to EI (Baugh, 1993). CI is not a universal ionisation technique and so is rarely employed for multi-residue pesticide analysis (de Hoffmann & Stroobant, 2007). CI-MS can often be used during analysis of

specific pesticide classes i.e. organo-halogens as CI-MS can give better selectivity over EI-MS; however, the signal intensity of pesticides can vary much more than when analysed using EI-MS (Alder *et al*., 2006).

Electrospray ionisation – (liquid chromatography- mass spectrometry)

Electrospray ionisation (ESI) provides an interface to allow LC systems to be directly coupled to an MS, resulting in an alytical tool with a greater sensitivity than older LC based dection methods i.e. LC-UV (Alder *et al*., 2006). Figure 2.7 shows an ESI setup, whereby the elution product from the LC column undergoes transformation to a charged aerosol (Alder *et al*., 2006). This aerosol subsequently goes through a 'heated curtain' of inert gas which reduces the solvent volume and increases the charge per unit volume of the droplet (Watson, 2005). This results in the eventual formation of single ions with a variable number of charges, before being transferred to the MS (de Hoffmann & Stroobant, 2007).

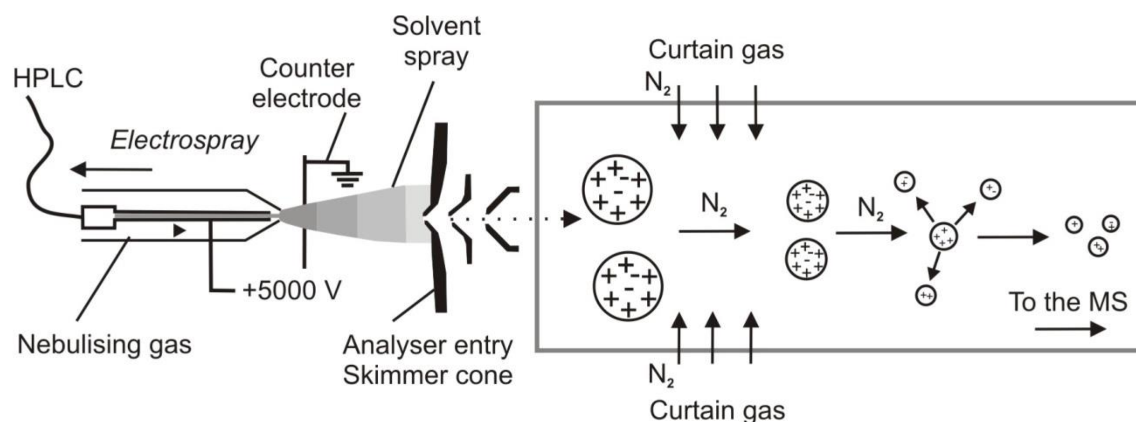


Figure 2.7: Electrospray ionisation – an analyte solution passes through a highly charged tip, producing a charged aerosol which later results in the formation of single ions with variable charges. Redrawn and adapted from Rouessac & Rouessac (2007).

Soft ionisation techniques, such as ESI, primarily generate: molecular ions $[M]^+$, protonated molecules $[M+H]^+$, simple adduct ions $[M+Na]^+$, $[M+K]^+$, or ions with simple mass losses i.e. the loss of water $[M+H - H_2O]^+$. Although the information produced is valuable, it is unable to offer structural information (Agilent Technologies, 2001); which is gained through the fragmentation patterns of an analyte. Having undergone ESI the fragmentation of an analyte can be obtained through the use of collision-induced dissociation (CID).

2.5.3 Mass analysers

Mass analysers are responsible for the separation of ion fragments (formed in the ion source) into individual *m/z* in order to produce a mass spectrum. Several analysers exist to date i.e. time-of-flight (TOF), Ion-tap and quadrupole analyser; each analyser has its own characteristics with the main three being *(1)* the upper mass limit which the analyser is able to measure, *(2)* transmission - ratio between the number of ions reaching the detector compared to the number produced at the source, and *(3)* resolution – ability to distinguish between two masses (Rouessac & Rouessac, 2007).

Quadrupole mass analyser

A quadrupole is made of four perfectly parallel rods through which fixed DC (direct current) and alternating radio frequencies (RF) are applied to each pair of rods (de Hoffmann & Stroobant, 1994); with the paired rods set directly opposite to each other (Figure 2.8). The oscillating electric fields are used to separate ions based on their *m/z* ratio; according to the stability of an ion's trajectory when passing between the rods. As

an ion travels between the rods it is attracted and repelled from each rod according to their respective charges (de Hoffmann & Stroobant, 2007).

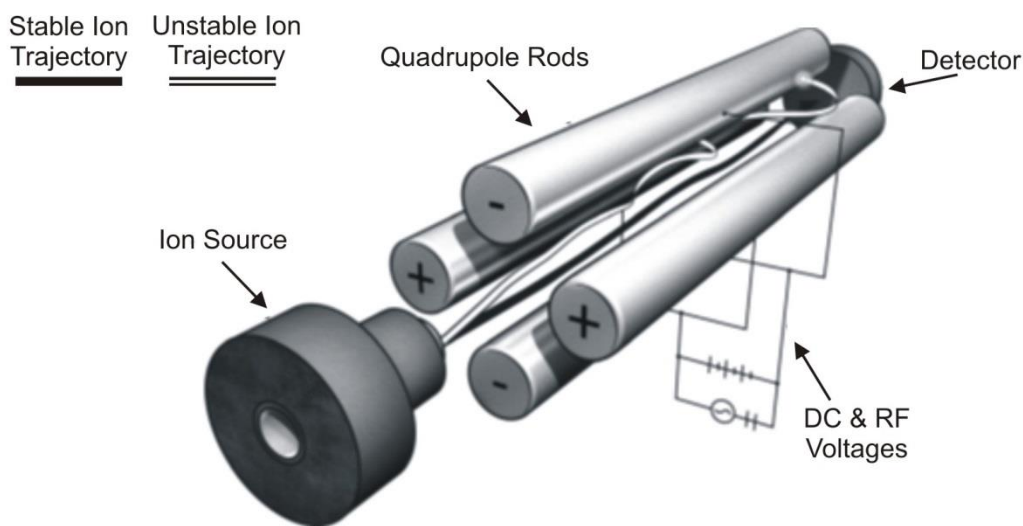


Figure 2.8: A schematic diagram of a quadrupole mass filter system. The rod pairings are denoted by +/- symbols. Ions with a stable trajectory (dark line) traveling between the rods reach the detector, while unstable trajectories (white line) are removed. Adapted from Hart-smith and Blanksby (2012).

Depending on the combination of DC and RF only one *m/z* is able to travel through to the detector (de Hoffmann & Stroobant, 1994); unstable trajectories will cause the ions to collide with the poles and therefore leave the device undetected (McNair & Miller, 2009). Some instruments use six rods (hexapole) for improved mass separation; however, the theory remains similar.

Multiple quadrupoles can be joined in sequence and Figure 2.9 shows a schematic diagram of a triple quadrupole (MS/MS) instrument. Quadrupoles Q1 and Q3 are mass spectrometer quadrupoles, whilst the central quadrupole q2 is an RF-only quadrupole - often referred to as a collision cell (de Hoffmann & Stroobant, 2007).

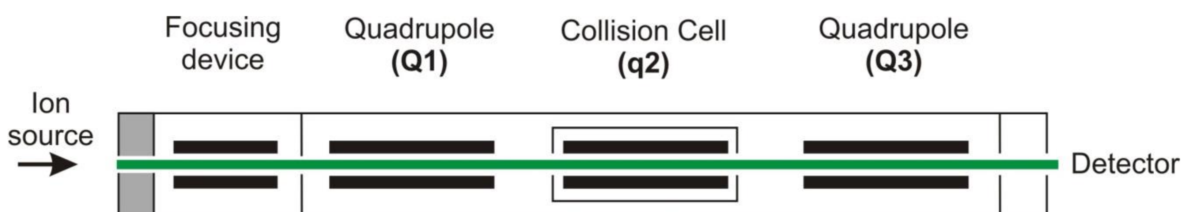


Figure 2.9: A schematic diagram of a triple quadrupole instrument. Quradrupole mass spectrometers are marked by Q (Q1 and Q3) whist an RF-only quadrupole is marked q (q2). Redrawn and adapted from de Hoffmann & Stroobant (2007).

Q1 operates as a mass filtering quadrupole, which separates the selected parent ions before entering q2; where these parent ions are fragmented via collision-induced dissociation (CID), before being further filtered in Q3. This configuration allows for multiple reaction monitoring (MRM) (de Hoffmann & Stroobant, 2007). MS/MS can carry out a number of different scan modes according to what information is required; these are shown in Figure 2.10.

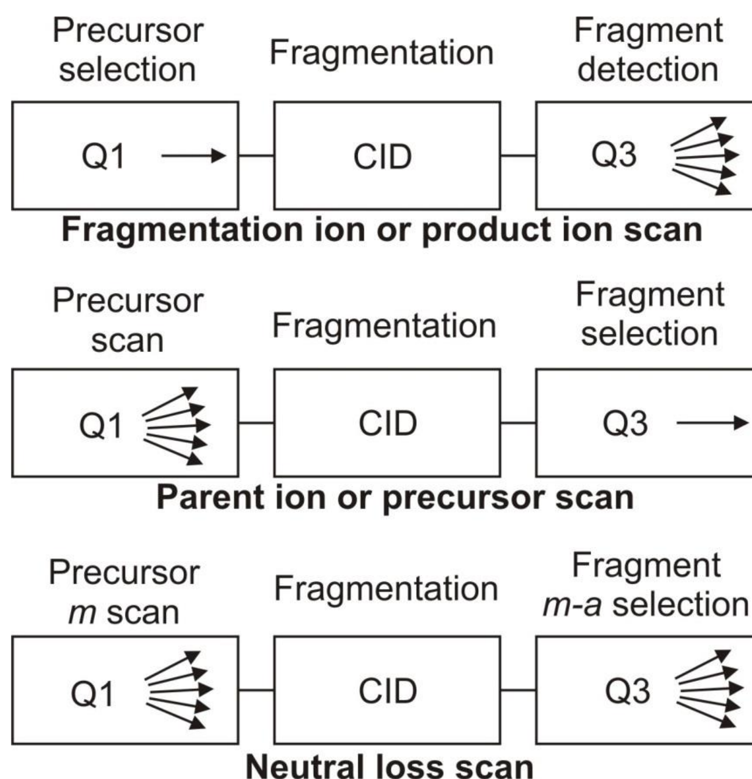


Figure 2.10: A schematic diagram showing the different scan modes of a tandem mass spectrometer. Redrawn and adapted from de Hoffmann and Stroobant (2007).

Fragment ion (product ion) scan mode consists of selecting a single ion (Q1), of a specified *m/z*, which is then fragmented in the collision cell (q2) and fragments detected in Q3. Parent ion scan mode focuses on a particular ion in Q3 whilst scanning a range of masses in Q1. All of those ions which fragment in q2 and produce the target ion (selected in Q3) are detected. During a neutral loss scan, both Q1 and Q3 scan the same mass range, but with a constant mass offset. Therefore, when singly charged ion of mass m travels through Q1 detection only occurs if the fragment ion is of a mass equalling to $m - \Delta m$; where Δm corresponds to the specified mass offset (de Hoffmann & Stroobant, 2007).

CID induces fragmentation of the gaseous ions that originate during ESI. The resulting fragmentation patterns are able to provide structural information of an analyte. CID involves the collision of an ion beam, entering the collision cell (q2), with a neutral collision gas (He, N_2, Ar) which is kept at a higher pressure than the surrounding high-vacuum (Gross, 2004). The kinetic energy of an ion is converted to internal energy, during a collision between analyte and a gas molecule, which results in bond breakage and therefore fragmentation of the ion. CID can be conducted on both MS/MS and quadrupole-time-of-flight instruments (Gross, 2004).

Time-of-flight mass analyser

Time-of-flight (TOF) mass analysis achieves the separation of ions according to an individual ion's velocity during their acceleration through a field-free flight-tube. Smaller/ lighter ions will travel faster down the flight tube than the larger ions and so reaching the detector first. The velocity at which the ions travel can be correlated to their mass and thus making it possible to determine the *m/z* ratio for each ion. Early TOF instruments

often had linear flight-tubes with poor mass resolution; however by lengthening the length of the flight-tube a greater resolution can be achieved (de Hoffmann & Stroobant, 2007). Reflection-TOF uses an electrostatic reflector at one end of the flight-tube to deflect on-coming ions back down the flight-tube to the detector (de Hoffmann & Stroobant, 2007).

A characteristic of a TOF is its easy mass calibration (de Hoffmann & Stroobant, 2007), which can be conducted externally or internally. External calibration uses calibration masses to separate unknown molecules (de Hoffmann & Stroobant, 2007). Internal calibration or internal reference mass (IRM) correction involves the constant infusion of known reference ions during the acquisition of spectral data. The *m/z* of at least two ions, either side of the target *m/z* of interest, is used. By having masses either side of the target mass the instrument software is able to make adjustments to correct the measured masses of unknown samples, should there be any deviation the reference masses. IRM achieves the best degree of accuracy (de Hoffmann & Stroobant, 2007).

Quadrupole – time-of-flight mass analyser

The development of quadrupole – time-of-flight (Q-TOF) mass spectrometer has helped to improve the detection of analytes, as it benefits from the sensitively and selectively capabilities of the TOF MS and quadrupole MS, respectively. Figure 2.11 shows that the third quadrupole has instead been replaced with a TOF mass analyser. Such a combination allows for numerous possible modes of data acquisition: quadrupole, TOF and Q-TOF depending upon the user's preference. By using the quadrupoles to select a

parent ion (Q1) before further fragmenting in q2, the ions are then analysed by the (reflection) TOF; thus providing a high resolution and selectivity of ions.

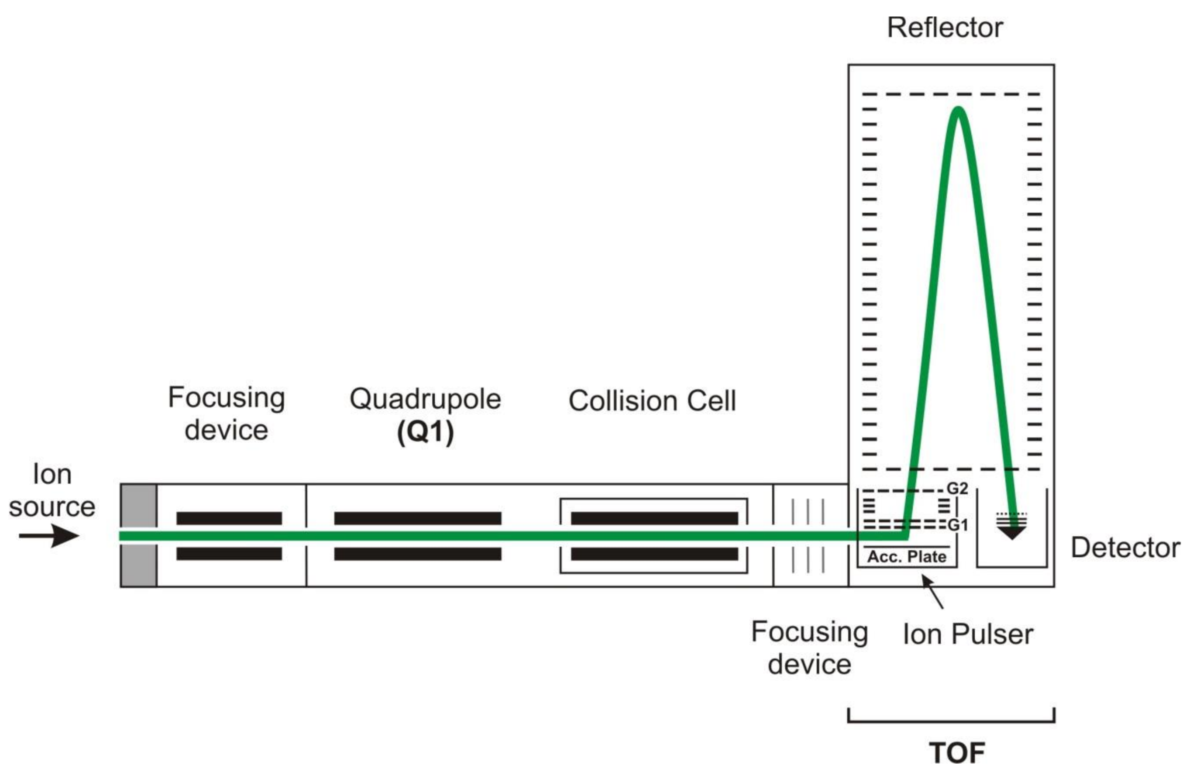


Figure 2.11: Schematic of Q-TOF mass analyser. Redrawn and adapted from (Agilent Technologies, 2011).

The coupling of TOF to a continuous ion source, such as ESI, has been difficult since ESI offers a constant ion beam compared to the pulsed process used by TOF. This coupling has been made possible thanks to the development of orthogonal-acceleration. The first stage of the process involves the continuous ion beam filling the space between the accelerator plate (shown in Figure 2.11) and grid 1 (G1) of the ion pulser. Both the plate and G1 are at a ground state of 0 V. A pulse voltage is then applied to the plate, accelerating the ions up towards grid 2 (G2). The acceleration plate and G1 return to 0 V and the ions begin to collect again. At G2 the ions are further accelerated before entering the flight tube. The flight cycle ends once the highest mass reaches the detector; at this

point the pulse voltage is reapplied to the accelerator plate and the cycle is then repeated (de Hoffmann & Stroobant, 2007).

As already seen in Figure 2.10, an MS/MS requires a precursor ion(s) to be specified prior to analysis. Subsequently ions that are not specified will not be detected. In the case of unknown samples this can be considered a possible disadvantage, unless the aim of analysis was target selected compounds. A Q-TOF detects and records all the ions in a sample as a function of time, producing a total ion chromatogram (TIC) - also known as a total ion current. A TIC allows for the analysts to revisit data and identify other analytes of interest which may not have been necessarily considered prior to analysis. In order to identify target analytes (of a known *m/z*) from the TIC this data must be extracted using the appropriate software. The resulting extracted-ion chromatogram (EIC) is a chromatogram which displays a selected m/z vs retention time. This is useful when retrieving retention time information of a target analyte from a complex data set. Multiple EICs can be generated from one TIC.

Full scan MS Vs SIM Vs MRM

In order to detect rapidly eluting peaks from a GC or LC instrument the rate at which the DC/RF frequencies of the quadrupole(s) are ramped is rapid. During 'full scan mode' a full range of masses (say *m/z* 40-400) is scanned multiple times a second and with only ions of a stable trajectory being detected (Figure 2.12) (McNair & Miller, 2009). Full scan is able to detect all analytes over a large mass range; this can include a number of co-eluted and perhaps unwanted matrix components.

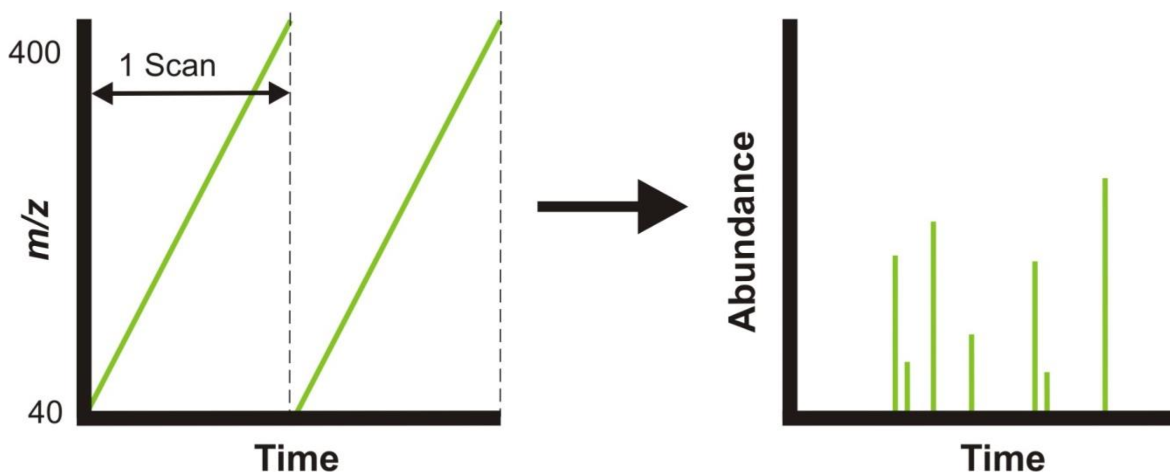


Figure 2.12: During full scan mode the quadrupole mass analyser can be set to scan over a range of *m/z* ratios, resulting in a number of detected ions.

As already discussed, a Q-TOF instrument produces a TIC, which represents the sum of ionic abundances vs retention time. Individual ions of interest as well as other ions can be extracted from a TIC. To produce an EIC, this displays a selected *m/z* vs retention time.

However it is possible to scan specific 'fixed' masses of interest, either for the whole duration of a run or at specified time intervals during analysis. For single quadrupole GC-MS instruments this is called 'selective ion monitoring' (SIM). SIM uses a much slower DC/RF ramp, allowing the dwell time for each mass to be kept for longer and thus collecting more ions of a particular *m/z* (McNair & Miller, 2009). Only selected masses that are detected are then plotted (Figure 2.13).

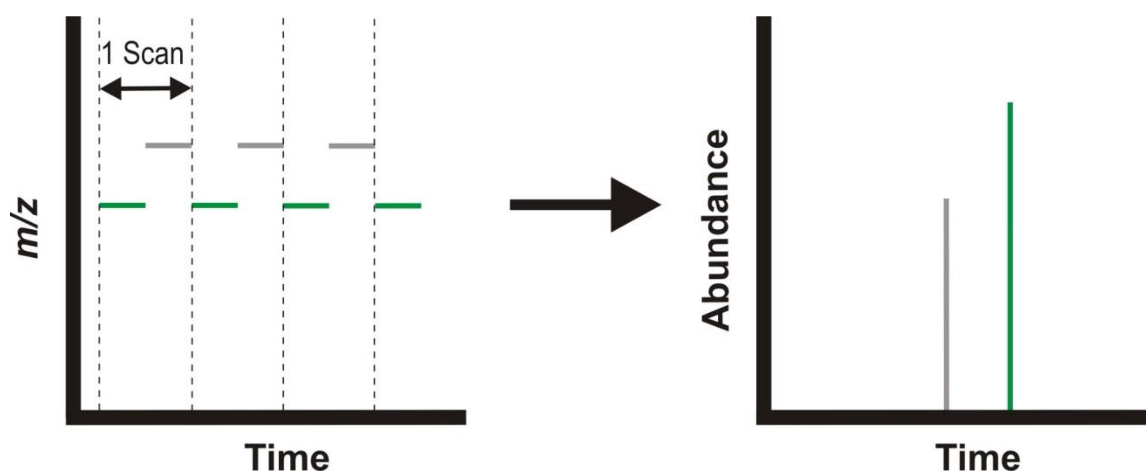


Figure 2.13: During SIM mode the quadrupole mass analyser can be set to alternate between a few *m/z* ratios, resulting in the detection of targeted ions (if present).

Triple quadrupole LC-MS instruments are able to perform a 'multi reaction monitoring' mode (MRM), also referred to as 'selected reaction monitoring' (SRM). Here the mass of a parent compound is specified for MS/MS fragmentation and then defined specifically for a specific fragmentation ion. In the case of the neonicotinoid pesticide imidacloprid, this 'transition' reaction would be represented as (parent mass → transition mass) *m/z* 256.1 → 175.1 (see Figure 2.10: parent ion scan mode). MRM can reduce the chemical background noise of a sample matrix, meaning that even co-extracted compounds can be separated according to their fragmentation ions (Alder *et al.*, 2006). MRM is a very useful technique when specific quantification of samples is needed. The focus on a particular precursor and fragmentation ions over longer time periods allows for an increased in detection rate of these ions. However, a disadvantage of this greater selectivity (de Hoffmann & Stroobant, 2007) is that there is the potential for overlooking unknown compounds, which may be of interest. In this instance, a sample would have to be reanalysed and new target ions selected within the MRM method.

High-resolution mass-spectrometry vs low-resolution mass spectrometry

There are a number of ways in which the mass of an atom, molecule or ion can be derived. For example, the mass of carbon atom can be expressed as the average atomic mass (molecular mass), which is an average of all the isotopes of each element; therefore, the average atomic mass is 12.011 Da. In mass spectrometry, both the nominal mass and, monoisotopic mass are used. The former is equal to the mass of the most abundant isotope rounded to the nearest whole number (C = 12 Da). The monoisotopic mass is derived from the 'exact mass' of the most abundant isotope of each element (C = 12.0000 Da). If the instrument is of a low mass resolution then it may be unable to discriminate between two masses within the mass spectrum; resulting in a single peak with a corresponding 'average mass'. If the resolution of a machine is high enough to resolve between two masses, then the resulting mass is equal to the calculated monoisotopic mass. A greater mass resolution (**R**) also helps to ensure a greater mass accuracy; which refers to the accuracy of the *m/z* as determined by the mass analyser. Mass resolution is defined in Equation 2.1. A narrower peak will result in a large **R** value and therefore a higher resolution. A single quadrupole instrument, for example, has a resolution of around 2000 (de Hoffmann & Stroobant, 2007); whilst an accurate mass Q-TOF can have a resolution >20,000, although this is by no means the highest possible resolution available.

$$R = \frac{(m/z)}{(W_{1/2})}$$

Equation 2.1: Mass resolution (R) is determined by *m/z* divided by the peak width at half height $W_{1/2}$.

Mass measurement error (accuracy) is usually expressed in parts per million (ppm) and if defined in Equation 2.2

$$\text{Mass accuracy (ppm)} = \frac{(m_i - m_a)}{m_a} x10^6$$

Equation 2.2: Mass measurement error (accuracy) in ppm, where m_i is the measured mass and m_a is the calculated mass.

As the molecular mass of compound can be achieved using various elemental compositions and an accuracy of within 5 ppm is deemed sufficient enough to support a proposed formula. High background-noises, which can occur with samples in a complex matrix, can alter the accuracy of the peak centroid ('centre of mass' of the peak) as well as limiting the level of detection thus making the determination of isotopic peaks almost impossible. This can be compensated for by increasing the injection volume of the sample and therefore increasing the number of ions available for detection (de Hoffmann & Stroobant, 2007). The theoretical isotopic pattern, which is calculating according to the natural abundances of each elemental isotope, can be overlaid with the detected mass spectrum to confirm the identity of a compound. This will be conducted later in this thesis.

2.5.4 Electron Capture Detector

An electron capture detector (ECD) is a selective detector and is very sensitive to highly electronegative molecules or those compounds that 'capture electrons'; this includes halogenated compounds, nitro moieties, anhydrides and to a lesser extent ketones (de Hoffmann & Stroobant, 2007). As such, it has become a popular detector for pesticide trace analysis (Baugh, 1993). ECD is classed as an ionisation-type detector and is often coupled with a GC instrument. It differs from other detectors within its class as samples are detected by causing a *decrease* in the level of ionisation as oppose to an *increase* in

ionisation (Baugh, 1993; McNair & Miller, 2009). A schematic of an ECD is shown in Figure 2.14.

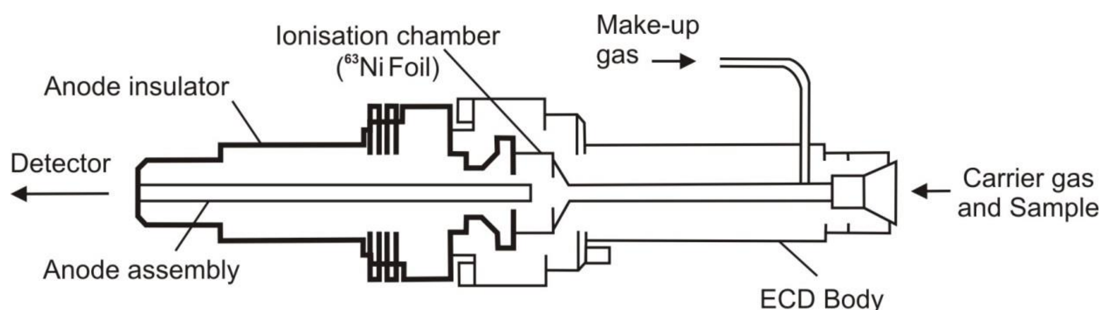
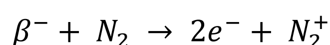


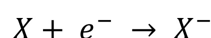
Figure 2.14: Schematic diagram of ECD. Analytes are ionised as they pass through an ionisation chamber containing a radioactive nickel source, before going on to the detector. Redrawn and adapted from Baugh (1993).

A radioactive nickel source (^{63}Ni) is contained within an ionisation chamber with a constant stream of carrier gas i.e. nitrogen (N_2) flowing through the chamber, as shown in Figure 2.14. The carrier gas is ionised by negatively charged beta particles (β^-) released by the ^{63}N source (Equation 2.3). This reaction causes the release of thermal electrons (e^-) which are used for the eventual ionisation of the eluted compounds from the GC column. A positive electrode collects the electrons and this gives a standing electrical current (McNair & Miller, 2009). Any alteration to the standing current is amplified and inverted to give an output signal (Baugh, 1993; McNair & Miller, 2009). Such alterations occur when an electrophilic analyte (X) elutes from the GC column and enters the chamber, it reacts and captures those free electrons used in the standing current (Equation 2.4).



$$\beta^- + N_2 \rightarrow 2e^- + N_2^+$$

Equation 2.3: Beta particle ionisation of nitrogen carrier gas creating free electrons used in ECD.



$$X + e^- \rightarrow X^-$$

Equation 2.4: Electrons produced (above) are then used in the ionisation of eluted electrophilic compound (X) from the GC column.

In order to increase sensitivity, most modern detectors now use a pulsed voltage system which delivers a -50 V pulse at a specific frequency; this helps to preserve the standing current if an analyte is not present. When an analyte is present the frequency increases and this increase is measured as an output signal (Baugh, 1993). ECD is a concentration sensitive detector and therefore a proportional relationship between the amount of electrons captured and the concentration of analyte/peak area is observed (McNair & Miller, 2009).

Other β^- particle sources are available, such as tritium (^{3}H or T), but ^{63}Ni is a safer source that is able to operate at temperatures up to 400 °C with a lower activity level (McNair & Miller, 2009). The carrier gas must be dry and ultrapure without any leaks within the system; as water and oxygen can harm the ECD system. A downside to ECD is that it is often prone to problems and is the most easily contaminated detector available. Therefore samples may be subjected to vigorous clean-up procedures before analysis (McNair & Miller, 2009). The popularity of ECD has declined in recent times due to the ever increasing sensitivity of GC-MS and LC-MS instrumentation (McNair & Miller, 2009).

2.5.5 Limits of detection & quantification and calibrations

An instrument's limit of detection (LOD) is defined as the concentration at which an analyte gives a signal that is *significantly different* from the 'blank' or 'background/ baseline' signal. As represented in Equation 2.5, the current trend is to define the LOD as "an analyte concentration giving a signal equal to the blank (y_B) plus three standard deviations of the blank (s_B)" (Alder *et al.*, 2006).

$$\text{LOD} = y_B + 3s_B$$

Equation 2.5: A popular definition of defining the limit of detection (LOD). Where the LOD of an analyte is equal to the signal of the blank (yB) and three standard deviations (sB) of the blank.

Through use of this definition, analysts can distinguish a genuine signal from the baseline, thus avoiding the misidentification of an analyte when it maybe in fact absent and vice versa (Miller & Miller, 2005). The LOD is used to distinguish a signal from the baseline/background. A less qualitative limit is the limit of quantification (LOQ), which is typically defined as ten standard deviations of a signal significantly different from the baseline (Equation 2.6) and represents the lowest amount of analyte that can be quantifiably determined with suitable precision and accuracy (Miller & Miller, 2005).

$$\text{LOQ} = y_B + 10s_B$$

Equation 2.6: A popular definition of defining the limit of quantification (LOQ). Where the LOQ of an analyte is equal to the signal of the blank (yB) and ten standard deviations (sB) of the blank.

As concentration is directly related to the peak area of an analyte, a calibration graph can be constructed using the peak areas of an analyte over a *known* concentration range. Known as external standard analysis (ESTD), both the calibration standard and unknown sample are analysed separately under the same conditions (Kupiec, 2004). These values are then plotted and a line of best fit is produced. This produces a 'regression coefficient' value (r^2) which provides a statistical measurement of how well the calibration points fit to a straight line (r^2 = 1). The line of best fit can also be described by Equation 2.7 and when satisfied for x can be used to determine concentration of an analyte.

$$y = mx + c$$

Equation 2.7: An equation which describes the line of best fit (straight line); where: *y* = peak area, *x* = concentration, *m* = gradient, and *c* = the intercept of the line at the Y-axis.

Figure 2.15 shows a schematic calibration graph constructed over seven different concentration points. The dashed line displays the relationship between signal response and peak area of a sample with an unknown concentration. Once a calibration plot has been produced, most analytical software automatically will calculate the concentration of each target analyte.

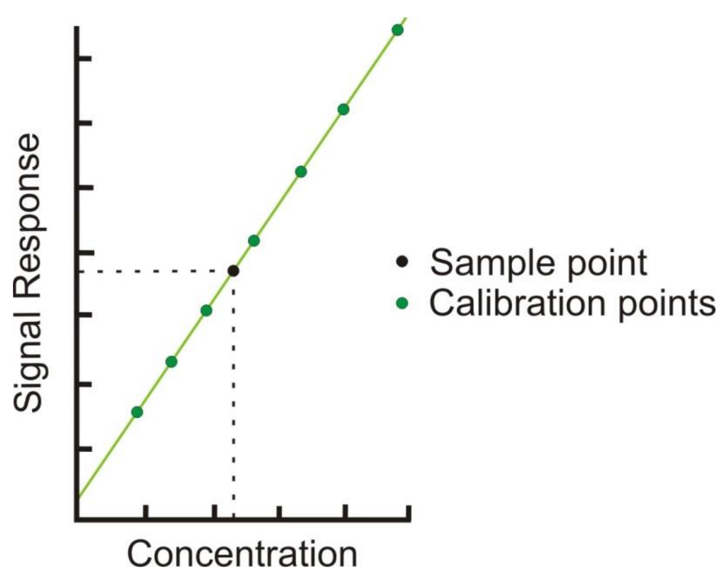


Figure 2.15: A schematic calibration graph being used to determine the concentration of a test sample.

Internal standard analysis (ISTD)

The accuracy of ESTD is influenced by the reproducibility of the injection volume; ISTD eliminates this as it involves the addition of a known amount of a standard to both the calibration and unknown samples. Acting as a standardising factor, this is able to compensate for any losses experienced during sample preparation or instrumental variability. However, for this to work the concentration of the ISTD compound must be

kept the same across the calibration concentration range and within the unknown sample. The compound used must also be chromatographically distinguishable from the calibrated analyte, but similar in its retention time and chemical composition. Should the ISTD compound be isotopically labelled it can then distinguished spectrally, rather than chromatographically (Kupiec, 2004). Isotopically labelled compounds possess the same chemical structure and elemental composition as a target compound; however, one or more atoms have been substituted with an heavy isotope (SANCO/12571/2013). The most commonly substituted isotopes used for radiolabelling are: hydrogen (^{2}H or D), carbon (^{13}C), Nitrogen (^{15}N) and Oxygen (^{18}O) (Gevaert, Impens, Damme, & Lambrechts, 2008). A deuterated standard used within this thesis is imidacloprid-d_4, as shown in Figure 2.16.

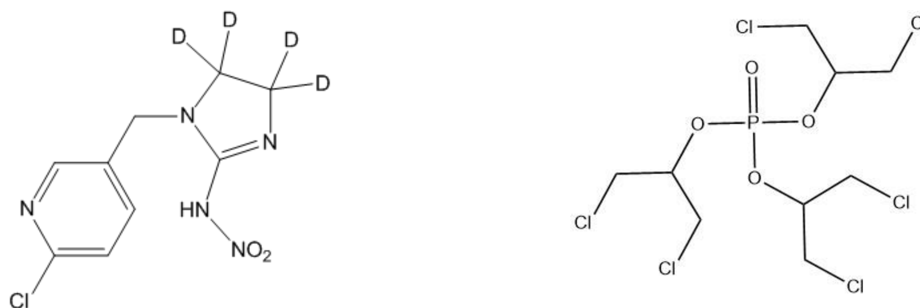


Figure 2.16: the structures or two compounds used as internal standards in this thesis *(left)* imidacloprid-d_4 and *(right)* tris(1,3-dichloroisopropyl)phosphate (TDCPP).

The resulting calibration curve, when employing an ISTD, will still appear the same as the one given in Figure 2.15; however, the values on the Y-axis represent a ratio between the responses of the target analyte over the response of the ISTD.

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CHAPTER 3

Determining pesticide content within honeybee wax samples from across the United Kingdom

3.1 Overview

As introduced in Chapter 1, pesticides have been suggested to play a role in the decline of many bee species and other important pollinators. There are multiple routes to which these beneficial insects can be exposed to such chemicals, they include: apicultural and/or agricultural practices; the latter is also linked to the contamination of pollen and nectar (see Chapter 5). Beeswax consists mostly of hydrocarbons, free acids and various esters (Tulloch, 1980), which are capable of absorbing various materials; as a result it can play host to a number of toxins or pathogens (Berry & Delaplane, 2001). For this reason beeswax has been described as a biological (Berry & Delaplane, 2001) and chemical (Mullin *et al*., 2010) sink. Consequently, beeswax, honeybees and other associated

matrices, for example pollen and nectar, can be considered as good bio-indicators for monitoring environmental contamination (Celli & Maccagnani, 2003; Porrini *et al*., 2003). To date no data has been published on the levels of pesticides contained within the comb wax of the UK honeybee, although data does exist across the USA (Mullin *et al*., 2010; Wu *et al*., 2011) and Europe (Bonmatin, Giorio, Girolami, Goulson, & Kreutzweiser, 2015; Ravoet, Reybroeck, & de Graaf, 2015; Serra-Bonvehí & Orantes-Bermejo, 2010; Wallner, 1999).

3.2 Introduction

3.2.1 The adulteration of beeswax

Beeswax is often recycled and reprocessed for the beekeeping market. In order to make the amount of beeswax go further, it has been known for the wax to be adulterated with cheaper waxes (Reynolds 1998; Tulloch 1980). Traditionally, the waxes used appear to be sourced from plant or vegetables; but as the price of these waxes increased there has been a greater shift towards microcrystalline and petroleum-based waxes (Reynolds 1998). The quality control of wax is set out within European and American Pharmacopeia; these can be used as a guide to determine if beeswax has been adulterated (Bogdanov, 2009). Two European Pharmacopeia definitions exist for white wax and yellow wax; the former is a bleached wax, whilst the latter is considered a natural product without additives (Council of Europe.; European Pharmacopoeia Commission, 2004). These pharmacopeia define the physico-chemical criteria for each type of beeswax, which include: maximum permitted level (%) of hydrocarbons, melting point and acidic number. Without the aid of chromatographic analysis, the latter two properties are considered to

be two of the best parameters to determine adulteration, (Bernal, Jimenez, Toribio, & Martin, 2005). The acidic number of beeswax should be between 17 - 24, while paraffin has no acidic number (Council of Europe.; European Pharmacopoeia Commission, 2004). Therefore, in instances where paraffin has been added to beeswax, there is risk of diluting the amount of naturally occurring compounds (Jimenez, Bernal, Aumente, Toribio, & Bernal Jr., 2003) and so stearic acid is often added in order to keep acidic values within the suggested range (Reynolds 1998). Beeswax used within commercial operations, across the USA and Europe, would typically specify that the wax be sourced from *A. mellifera* (Crane, 1990). From a beekeeping perspective, the higher quality beeswaxes are also said to come from Australia, New Zealand, Europe and the USA, while poor grade waxes are of Chinese or African origin (Reynolds 1998).

A study by Bernal *et al.,* (2005) into the quality of Spanish beeswax revealed that of the 52 different sheets of foundation wax analysed, 27 were rejected by bees. Of these 25 sheets 93 % were found to be adulterated. Adulterated beeswax is said to have negative effects on the structural properties of comb (Bradbear, 2009), as well as possible negative impacts on brood development and increased mortalities (Wallner 2005, as cited in Semkiw & Skubida 2013). However, Semkiw & Skubida (2013) report that wax adulterated with paraffin, by as much as 50 %, had no effect on the construction of comb nor did it have any negative influences on brood mortality. These results are also supported by Medici *et al.,* (2012) who suggest that the inclusion of paraffin may actually dilute the amount of harmful chemicals (i.e. pesticides) found within the wax.

3.3 Routes of pesticide exposure

There are two main ways in which beeswax can become contaminated with pesticides:

1) *Apicultural contamination*
2) *Agricultural contamination*

As considered in Chapter 1, the incorporation of halogens within a pesticide can determine various pharmokinetic parameters, including enhanced lipophilicity. Chlorine (Cl) and fluorine (F), in particular, are important for the delivery of substances between aqueous-based and lipid-based environments (Jeschke, 2010). The lipophilicity of a compound is determined its octanol-water partition coefficient (K_{ow}). This describes that compounds with a higher log K_{ow} will be more soluble within a lipid-based matrix.

1) Apicultural contamination

Apicultural contamination can occur from the direct application of pesticides to a colony by a beekeeper. Table 3.1 shows a list of apicultural hive treatments used within the UK, of which most tend to revolve around the treatment of *Varroa*.

Table 3.1: Hive treatments which were previously and are currently used within the UK.

Product	Active Ingredient	Target Pest	Method of Spread	Application Method	Current UK Status
-	*p*-Dichlorobenzene	Wax moth	E	Crystals	✕
-	Naphthalene	Wax moth	E	Moth ball	✕
Apiguard®	Thymol	*Varroa*	E/C/I	Gel	✔
Apilife-VAR®	Thymol (+ *ess oils*)	*Varroa*	E	Carrier matrix	✔
Apistan®	tau-fluvalinate	*Varroa*	C	Impregnated strips	✔
Apivar®	Amitraz	*Varroa*	C/S	Impregnated strips	Cascade SIC
Bayverol®	Flumethrin	*Varroa*	C	Impregnated strips	✔
Fumidil B®	(Antibiotic)	*Nosema spp*	-	Soluble powder	Expired
(Generic)	Formic acid	*Varroa*	E	Solution	Note 1
(Generic)	Lactic acid	*Varroa*	E	Solution	Note 2
(Generic)	Oxalic acid	*Varroa*	C	Solution	Note 2
MAQS	Formic acid	*Varroa*	E	Impregnated bags	✔
Perizin®	Coumaphos	*Varroa*	C/S	Solution	Cascade SIC
Thymovar	Thymol	*Varroa*	E	Impregnated strip	✔

✕-Not UK authorised; ✔- UK authorised; Cascade SIC – Not UK authorised, but is registered in another EU member state, meaning it can be prescribed by a veterinarian (beekeepers must apply for a Special Import Certificate); Note 1 – Not authorised in EU, except for Germany; Note 2 – Not authorised in EU, but tolerated in many countries. Method of spread: C – contact; E – Evaporate; I –Ingestion.
MAQS – Mite Away Quick Strips. *ess oils* – essential oils. Information adapted from The Food and Environment Research Agency (2013).

Acaricides are often applied directly to the hive in the form of pesticide impregnated strips or through drip applications. The amount of active ingredient present within each treatment will determine the length of time that the application strip, for example, will remain within the hive (Wallner, 1999). Most apicultural treatments, such as coumaphos and tau-fluvalinate, are found to be stable and with a half-life of five years they remain present in the wax long after treatment (Bogdanov, 2006) with the exception of amitraz, which is unstable in beeswax and rapidly degrades (Korta *et al.*, 2001). The active ingredient of the hive treatment Apistan is tau-fluvalinate, which is a half-resolved mixture of four original stereoisomers found in fluvalinate. Figure 3.1 shows the predominant structure of tau-fluvalinate, where (R) and (S) denote the configuration of

each chiral centre; the other stereoisomer is the (R, R)-configuration. The two other stereoisomers of fluvalinate are in the (S, R) and (S, S)-configurations; however, these demonstrate less insecticidal activity than the (R) forms (Jia *et al.*, 2015). Given its use within the beekeeping industry, only tau-fluvainate shall be considered throughout the rest of this thesis.

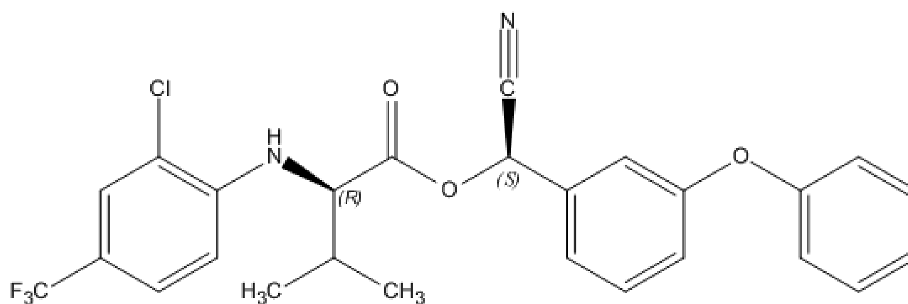

Figure 3.1: The predominant (R, S) stereoisomer of tau-fluvalinate. The other stereoisomer present in tau-fluvalinate is found in the (R, R)-configuration.

It was found that treatments applied at higher concentrations will have a greater distribution throughout the hive than those applied at a lower concentration. Volatile treatments, such as thymol and PDCB, will evaporate from the hive and so their persistence within the comb is limited (Bogdanov *et al.*, 2004; Wallner, 1999), although their residues may still be detected (Bogdanov, 2006). It was reported that foundation sheets spiked with thymol and stored closely packed in a sealed cardboard box retained up to 70 % of the initial concentration after one year, whilst those foundation sheets exposed to a good airflow reduced to almost zero (Bogdanov, Imdorf, & Kilchenmann, 1998). The volatility of PDCB is similar to that of thymol and so the reduction of this chemical from the wax is anticipated to be the same (Bogdanov *et al.*, 2004).

All inner surfaces of a hive are coated in a fine layer of wax, which facilitates the migration of pesticides throughout the hive (Wallner, 1999). The thickness of the comb is

also thought to aid in the rapid diffusion of pesticides throughout the comb (Tremolada, Bernardinelli, Rossaro, Colombo, & Vighi, 2011). Honeybees are capable of distributing fat-soluble pesticides with their legs and bodies whilst walking around the hive (Wallner, 1999); for example nurse bees are considered primarily responsible for transferring pesticides to queen cups and developing queens (Haarmann, Spivak, Weaver, Weaver, & Glenn, 2002).

Newly produced comb has been shown to be quickly contaminated through the migration of pesticides from old existing comb (Wu *et al*., 2011). Continuous pesticide migration has also been confirmed from contaminated comb wax into honey stores via a partition process (Kochansky, Wilzer, & Feldlaufer, 2001; Tremolada, Bernardinelli, Colombo, Spreafico, & Vighi, 2004; Wallner, 1999). Honey is described as a hydrophilic matrix, however, it is the presence of minor lipophilic components such as pollen and wax that are responsible for honey's increased affinity to fat-soluble residues (Tremolada *et al*., 2004).

2) Agricultural contamination

There are multiple ways in which agrochemicals can enter the hive. Honeybees foraging directly on, or near to, agricultural fields are at risk of exposure to agrochemicals. Multiple mechanisms were identified, including contaminated pollen and nectar from both treated crops and from the flowers surrounding the crop, such as dandelions. During sowing talc is often applied to seeds in order to stop them from adhering to each other, thus allowing for a more equal spacing in the ground; this talc is often exhausted from the planter, creating a dust which can drift outside the field boundary. Contaminated soil also

posed a threat (Bonmatin *et al*., 2015; Krupke, Hunt, Eitzer, Andino, & Given, 2012), as surface water can transport chemicals to the roots of non-target plants (Bonmatin *et al*., 2015). The number of agricultural pesticides that can be returned to the hive can be considered alarming (Mullin *et al*., 2010), making beeswax and other matrices bioindicators to environmental pollution (Celli & Maccagnani, 2003; Niell, Hepperle, Doerk, Kirsch, & Kolberg, 2014). Using a national statistics survey on pesticide applications made to arable crops in Great Britain, a number of key agricultural pesticides were identified; based on their frequency of use on honeybee-pollinated crops (Garthwaite, Thomas, Parrish, Smith, & Barker, 2008). These, in addition to a few other pesticides, are listed in Table 3.2.

Table 3.2: A list of some of the key pesticides focused on within this chapter. These were selected according to their frequency of use on honeybee pollinated crops (Garthwaite *et al.*, 2008). Log K_{ow} and LD_{50} values taken from Tomlin (2009).

Compound	Class	Log K_{ow}	LD_{50} (ug/bee) (contact)	LD_{50} (ug/bee) (oral)
Aldicarb	Ins/Acar/Nem	1.15	0.285	-
Amitraz	Ins/Acar	5.5	50	-
Azoxystrobin	Fung	2.5	> 200	> 25
Boscalid	Fung	2.96	200	166
Captan	Fung	2.8	788	91
Carbaryl	Ins/PGR	1.85	1	0.18
Carbendazim	Fung	1.38	>50	-
Chlormequat-chloride	PGR	-1.59	Non-toxic	Non-toxic
Chlorothalonil	Fung	2.92	> 101	> 63
Clothianidin	Ins	0.7	0.0439	0.00379
Coumaphos	Ins	4.13	-	-
lambda-Cyhalothrin	Ins	7[a]	38 ng/bee	909 ng/bee
Cypermethrin	Ins	6.6	0.02	0.035
DDT	Ins	-	-	5
Deltamethrin	Ins	4.6	12 ng/bee	23 ng/bee
Fipronil	Ins	4	-	-
Flusilazole	Fung	3.74	-	-
tau-Fluvalinate	Acar/Ins	4.26	6.7	163
Glyphosate	Herb	< -3.2[b]	>100	100
Imazalil	Fung	3.82	-	40
Imidacloprid	Ins	0.57	-	3.7 ng/bee*
Paraquat-dichloride	Herb	-4.5[a]	70	15 (120 h)
Pendimethalin	Herb	5.2	101.2	-
Permethrin	Ins	6.1[a]	0.029	0.098
Pirimicarb	Ins	1.7	53	4 (24 h)
Thiamethoxam	Ins	-0.13	0.024	0.005
Triadiamenol	Fung	3.08	Non-toxic	Non-toxic

[a] 20 °C; [b] 20 °C, pH 2-5.

Acar – acaricide; Fung – fungicide; Herb – herbicide; Ins – insecticide; Nem – nemocide; PGR – plant growth regulator. * Data taken from Schmuck *et al.*, (2001).

As seen with apicultural applications, agricultural pesticides can be distributed throughout the hive by bees walking around combs (Pettis, Collins, Wilbanks, & Feldlaufer, 2004; Wallner, 1999). Likewise, this can be achieved by placing contaminated pollen and nectar within a cell, thus exposing the immediate area to a particular pesticide

(Wallner, 1999). Pollen and nectar are known to be deposited randomly within a comb before being relocated outside of the brood nest (Johnson, 2009), thus giving the typical brood pattern seen in Figure 1.7. This relocation of food stores could therefore offer multiple sites of contamination.

3.4 Health effects of pesticide-contaminated comb wax

Exposure to contaminated comb wax during the early stages of honeybee development can be detrimental, as the developing brood may demonstrate a greater sensitivity to certain contaminants than during adulthood (Zhu, Schmehl, Mullin, & Frazier, 2014). Honeybees exposed to highly contaminated combs have been shown to have a reduced level of survival, compared to those exposed to relatively uncontaminated combs. Workers raised in lower contaminated combs were found to live four days longer than those reared in a comb of high contamination (Wu *et al*., 2011). A large reduction in the number of foragers can have major repercussions on the size and sustainability of a colony. 'Precocious foraging' is a response to replace the loss of foragers with progressively younger and ineffective hive bees (Perry, Søvik, Myerscough, & Barron, 2015). This will eventually reduce the number of nurse bees to brood ratio and thus the amount of replacement adults over time (Desneux, Decourtye, & Delpuech, 2007). The inability to sustain food levels within the colony can result in colony failure (Perry *et al*., 2015). Contaminants within the comb may also conceal nestmate recognition cues found within the comb; this can be problematic for returning foragers as they may fail to recognise their own colony (Berry & Delaplane, 2001).

During the study by Wu *et al*., (2011) there was found to be delays in adult emergence from highly contaminated brood comb. The delay in adult emergence is thought to be advantageous in the reproductive cycle of the *Varroa* mite (Fraizer 2007, as cited in Wu *et al.,* 2011).

Wu *et al.,* (2011) also found that 23 % of the honeybee eggs laid in contaminated combs failed to hatch into larvae (day 4 of development cycle). At day 8 the failure rate of larvae increased to 46 %. This can have a number of negative effects on the colony, including energetic stress on nurse bees, who must eject failed eggs and larvae from the hive (Wu *et al*., 2011). Those reared in contaminated combs were found to have a greater susceptibility to *Nosema ceranae* (Wu, Smart, Anelli, & Sheppard, 2012). High levels of acaricides within comb wax were also found to negatively affect developing queens. This included reduced body weight as well as an increased rejection by nurse bees during their development (Pettis *et al*., 2004). In addition to the topical exposure from contaminated comb wax, there is a possibility of pesticide migration into other matrices, such as honey stores (Kochansky *et al*., 2001; Tremolada *et al*., 2004). Contaminated food stuffs also pose a risk of oral exposure to pesticides (Rortais, Arnold, Halm, & Touffet-Briens, 2005). Synergistic interactions between multiple pesticides within the comb can also have a greater overall toxic effect on developing larvae than would be seen for a single pesticide (Johnson, Pollock, & Berenbaum, 2009; Zhu *et al*., 2014).

3.5 Aim of investigation

The aim of this chapter is to describe which pesticides are present in comb wax samples obtained from around the UK.

3.6 Materials and Methods

3.6.1 Reagents and standards

All solvents and chemicals used in the study were of HPLC grade or analytical grade, with the exception of *para*-dichlorobenzene (acquired from J. Routh, FERA). Alidicarb PESTANAL®, Amitraz PESTANAL®, Azoxystrobin PESTANAL ®, Boscalid PESTANAL®, Captan PESTANAL®, Carbaryl PESTANAL®, Carbendazim PESTANAL®, Chlormequat-chloride PESTANAL®, Chlorothalonil PESTANAL®, Clothianidin PESTANAL®, Coumaphos PESTANAL®, λ-cyhalothrin PESTANAL®, Cypermethrin PESTANAL®, Deltamethrin PESTANAL®, tau-Fluvalinate PESTANAL®, Glyphosphate PESTANAL®, Imidacloprid PESTANAL®, Pendamethlin, PESTANAL®, Pirimicarb PESTANAL®, Thiamethoxam PESTANAL®, purchased from Fluka Analytical (Germany); DDT, Thymol (Aldrich, Gillingham, UK). Ammonium formate (NH_4HCO_2) ≥99 %, (Fluka Analytical, Germany). Magnesium sulphate (Sigma-Aldrich, Germany). Acetone, Acetonitrile, Glacial acetic acid (Fisher Scientific, Loughborough, England). Toluene and dichloromethane CHROMASOLV® (Sigma-Aldrich, Germany). The deionised water was purified at 18.2 MΩ with a Purelab Option-Q DV25 purification system. QuEChERS kits 60105-205 and 60105-210 were bought from Thermo Scientific (Hemel Hempstead, England) and the dual layer ENVI™ – Carb II/PSA 300/600 mg solid phase extraction (SPE) cartridges were supplied by Supelco Analytical (USA).

3.6.2 Sample collection

In order to obtain comb wax samples from around the UK, an advert was placed within the British Beekeepers Association (BBKA) monthly newsletter (Appendix A); this informed readers about the aim of the project, general background and the need for

volunteers to donate comb wax. A stand/collection point was also arranged at the 34th Spring Convention (16th April 2011), Stoneleigh (Warwickshire), which acted as a distribution point for the specially created sample collection packs. Each collection pack contained a self-addressed envelope, a cut comb container, disposable gloves, alcohol wipes, blank label, a set of instructions (Appendix B) and a questionnaire (Appendix C). In order to reach out to a greater demographic, contact was made with northern (England) beekeeping associations, in addition to Dr Christopher Connolly (University of Dundee). This allowed for approximately 120 collection packs to be distributed between Northern England and throughout Scotland. In total, 500 sample packs were distributed; of which 152 of these were returned (30.4 %): England n = 98 (including Isle of Man, n = 3), Scotland n = 46 and Wales n = 8. The approximate location of these returned samples is shown in Figure 3.2.

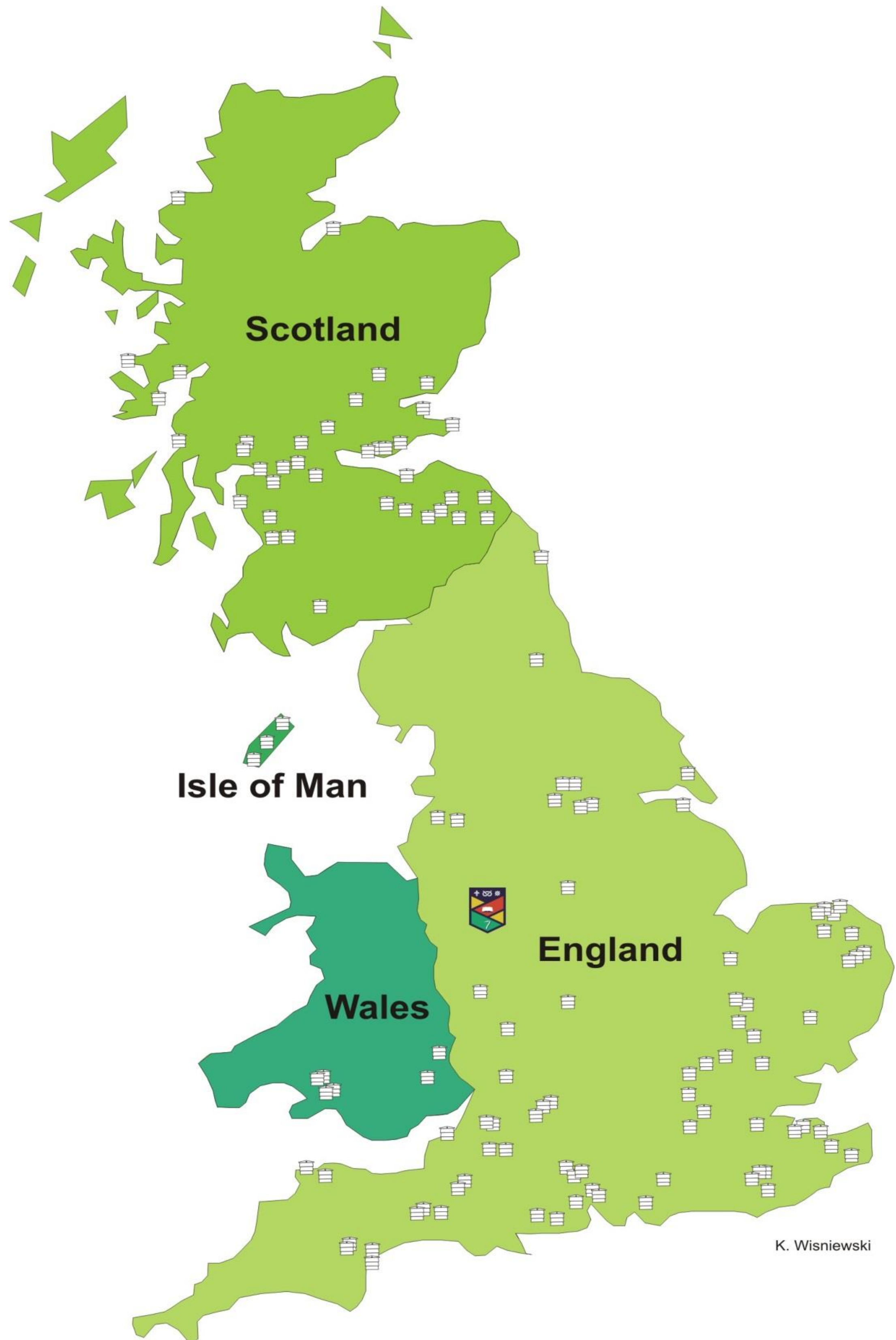

Figure 3.2: A schematic diagram showing the approximate locations of 152 comb wax samples obtain in 2011 from England (n = 98), Scotland (n = 46) and Wales (n = 8) in relation to Keele University (logo).

3.6.3 Sample extraction

A modified QuEChERS was used (Mullin *et al*., 2010). Here, 3 g of comb wax was added to a 50 ml centrifuge tube containing 27 ml of extraction solution (55 % acetonitrile, 44 % deionised water, 1 % glacial acetic acid) and heated at 80 °C for 20 min. Once at room temperature, 6 g anhydrous magnesium sulphate ($MgSO_4$) and 1.5 g anhydrous sodium acetate (QuEChERS kit 60105-210, Thermo Scientific, Hemel Hempstead, England) were then added to the centrifuge tube and shaken vigorously for 2 min. Finally, the mixture was centrifuged (5 min @ 4000 rpm) and the organic layer (**A**) removed and placed in a 15 ml centrifuge tube.

Clean-up for GC-MS analysis. A dual layered SPE cartridge containing 350 mg graphitized carbon black (CUCARB) and 600 mg primary secondary amine (PSA) was prepared by adding 160 mg $MgSO_4$ to the cartridge and conditioning with 4 mL acetone/toluene (7:3 v/v) under positive pressure and eluted to waste. 2 mL of **A** was then added to the cartridge and eluted with 4 mL acetone/toluene (7:3 v/v) into a glass sample tube. The elutant was evaporated to dryness under a stream of nitrogen gas, before being dissolved in 350 µl dichloromethane and transferred into a 2 ml autosampler vial ready for analysis.

Clean-up for Q-TOF LC/MS analysis. 10 ml **A** was transferred to a centrifuge tube containing 900 mg $MgSO_4$, 300 mg PSA and 150 mg graphitised carbon black (CUCARB) (QuEChERS kit 60105-205, Thermo Scientific, Hemel Hempstead, England). After vortexing (1 min) and centrifuging (4 min @ 4000 rpm), 10 ml supernatant was removed and dried down under a stream of nitrogen gas, then dissolved in 500 µl water/acetonitrile (95:5 v/v) and added to a 2 ml autosampler vial ready for analysis.

3.6.4 Instrumentation

GC-MS analysis: samples were analysed on an Agilent 7890A GC equipped with a Zebron Inferno ZB-5HT column (30 m x 0.32 mm, 0.25 µm) connected to an Agilent 5975C MSD (quadrupole) mass spectrometer (70 eV electron impact ionisation); mass range 40 - 800 *m/z*. Samples were injected in splitless mode (injection volume: 2 µl) and the oven was programmed from 25 °C to 110 °C at 40 °C/min and then 110 °C to 320 °C at 20 °C/min. The injection port was held at 250 °C. Helium was used as a carrier gas at a constant flow of 1 ml/min.

Q-TOF LC/MS analysis: samples were injected into an Agilent 1260 Infinity LC system (injection volume: 15 µl) equipped with autosampler, thermostatted column compartment (set to 35 °C) and 1290 Infinity in-line filter (0.3 µm) Agilent ZORBAX Extended-C18 Rapid Resolution HD (2.1 x 50 mm, 1.8 µm) column was used with a ZORBAX Eclipse Plus C18 (2.1 x 5 mm, 1.8 µm) guard column connected to an Agilent 6530 Accurate-Mass-Q-TOF LC/MS. The LC mobile phases were (A) water with 5 mmol ammonium formate and (B) acetonitrile. The elution gradient, at a flow rate of 0.6 ml/min, was as follows: 0 – 0.5 min (95 % A/ 5 % B), 0.5 – 9 min (0 % A/ 100 % B), 9 – 9.5 min (0 % A/ 100 % B), 9.5 – 10 min (95 % A/ 5 % B). The Q-TOF settings were as follows: acquisition mode MS with MS range 100 – 1000 *m/z*; MS scan rate 1 spectrum/s; electrospray ionization (ESI) source – gas temperature: 300 °C; gas flow: 11 L/min; nebulizer: 50 psig, positive ion polarity; scan source parameters: Vcap 4000 V; fragmentor, 125 V; skimmer, 65 V; octapole RF (OCT RF Vpp), 750 V.

Compound identification

Q-TOF LC/MS: Using the Agilent MassHunter Qualitative Analysis Workstation Software (version B.06.00), compounds were identified using the 'find by molecular feature', locating any distinguishable peaks which correspond to a distinct $[M+H]^+$ ion. The obtained accurate mass of the $[M+H]^+$ was then used to identify possible molecular formulae for these peaks. The 'mass filter' tab was selected to use these proposed molecular formulae to search a 'pesticide database' which was created in-house. In short, this feature scans each selected TIC in order to extract and identify any $[M+H]^+$ and related isotopic information and compare the findings to the database. Mass deviations (mass accuracy) of less than 5 ppm were investigated further. Based on the natural isotope abundance of each element, a theoretical isotopic pattern was overlaid with each match and the differences compared. The RT of each peak was also compared to previously analysed standards.

GC-MS: Compounds were detected by extracting the parent molecular ion, where available, or the base peak of the compounds of interest. The resulting mass spectrum was then compared to the NIST08 mass spectral library.

3.7 Results

3.7.1 Questionnaire results

A summary of the questions answered is given in Appendix D; however, some of the questions have not been tabulated due to their qualitative nature.

Of the 152 samples received, 53.29 % were from a rural environment, 29.61 % semi-rural and 15.13 % from an urban environment (1.97 % not specified). The top three apicultural treatments used within England (according to those surveyed) were thymol (37.11 %), oxalic acid (20.13 %) and Fumidil B (10.06 %). The most popular treatments in Scotland (n = 46) were oxalic (26.19 %), Apistan/tau-fluvalinate (22.62 %) and thymol (17.86 %). The number of samples received from Wales was small (n = 8), with no reports of synthetic chemicals used. Thymol and oxalic acid (both 46.15 %) were the only treatments used. 22.62 % of Scottish beekeepers were found to use Apistan/tau-fluvalinate, compared to only 7.55 % of English beekeepers. The samples received from England were of varied ages, whilst the samples received from Scotland tended to be older in comparison (Figure 3.3).

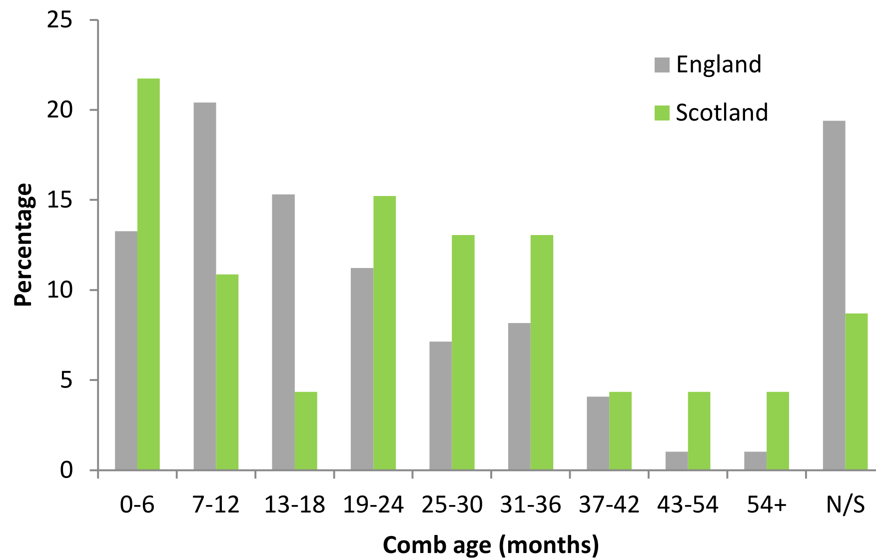

Figure 3.3: The percentage distribution of comb age from across England (n= 98) and Scotland (n = 46).

3.7.2 GC-MS analysis

In total 152 comb wax samples were analysed using GC-MS. Thymol was detected in 50% of English, 62.5 % Welsh and 36.96 % of Scottish samples analysed, respectively. No other pesticides were detected. Butylated hydroxytoluene (BHT), a synthetic stabiliser, was also identified within 32.65 % of English, 50% Welsh and 78.26 % of Scottish samples analysed, respectively. See Appendix E for raw data.

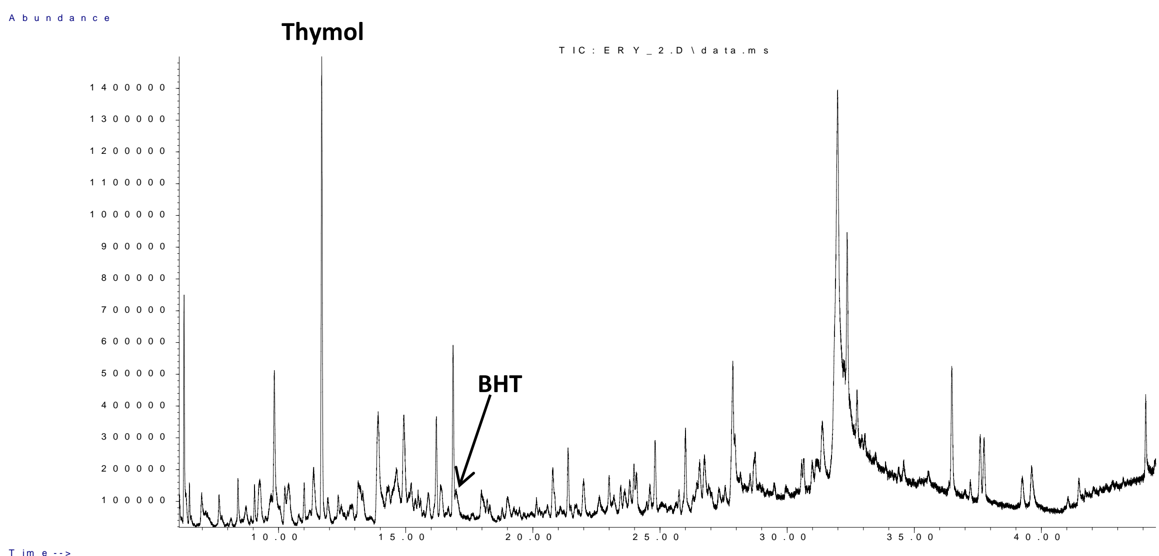

Figure 3.4: An example of a GC-MS chromatogram of an analysed wax sample (ERY_2_15/07/11). Both thymol and butylated hydroxytoluene (BHT) have been annotated. All other detected compounds are not the focus of this thesis.

Figure 3.4 shows the detection of a (undetermined) number of compounds found within a single wax sample, analysed by GC-MS. Only thymol and BHT have been annotated, as they are considered to be of interest. This particular comb wax sample was reported not to have been treated with thymol, although clearly detected and supported by the mass spectrum presented in Figure 3.5. Other compound classes detected include hydrocarbons, fatty acids and alcohols.

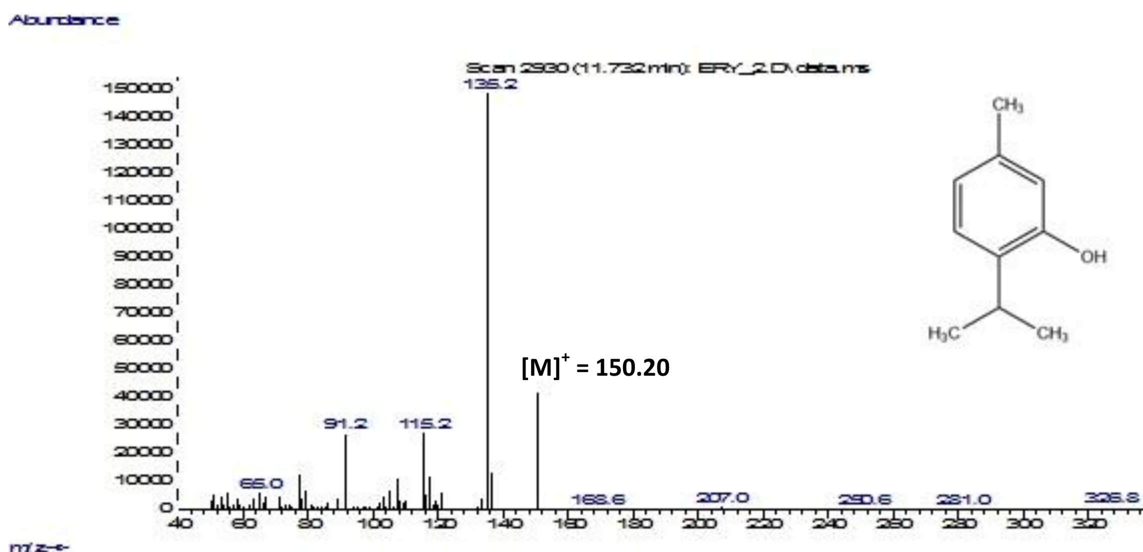

Figure 3.5: An example of a mass spectrum for thymol detected in an analysed wax sample (ERY_2_15/07/11). The structure of thymol is also provided.

3.7.3 Q-TOF LC/MS analysis

Q-TOF LC/MS analysis of 15 comb wax samples revealed that 11 of the 15 samples were found to be contaminated with tau-fluvalinate; regardless of comb age. As tau-fluvalinate exists as a mixture of two stereoisomers, two peaks can often be detected at 8.4 minutes and 8.7 minutes; the latter is the more predominant and therefore is the only stereoisomer quoted in Table 3.3. Only one of the three samples from the Isle of Man contained tau-fluvalinate. No other chemicals were detected across all samples.

Table 3.3: The results of Q-TOF LC/MS analysis of comb wax samples (n = 15).

Sample I.D.	Location	Compound	RT	Score	Difference (ppm)	Age (months)
CAM_1_06/07/11	Cambridge	tau-Fluvalinate	8.769	98.02	-0.79	12
DEV_6_06/07/11	Devon	tau-Fluvalinate	8.784	99.19	-1.17	24
DOR_3_12/07/11	Dorset	-	-	-	-	12
HAM_4_07/07/11	Hampshire	tau-Fluvalinate	8.777	94.63	0.06	14
IOM_1_05/07/11	Isle of Man	-	-	-	-	42
IOM_2_05/07/11	Isle of Man	-	-	-	-	30
IOM_3_05/07/11	Isle of Man	tau-Fluvalinate	8.738	98.82	0.29	12
KEN_2_05/07/11	Kent	tau-Fluvalinate	8.760	95.24	0.65	-
KEN_5_08/07/11	Kent	-	-	-	-	36
KEN_6_08/07/11	Kent	tau-Fluvalinate	8.754	85.96	0.50	2
LON_1_06/07/11	London	tau-Fluvalinate	8.795	95.29	0.04	12
NFK_3_06/07/11	Norfolk	tau-Fluvalinate	8.760	98.17	0.31	14
SOM_4_06/07/11	Somerset	tau-Fluvalinate	8.726	89.81	0.63	3
SRY_1_08/07/11	Surry	tau-Fluvalinate	8.776	96.04	0.05	5
WYK_4_06/07/11	W. Yorkshire	tau-Fluvalinate	8.782	81.76	0.52	15

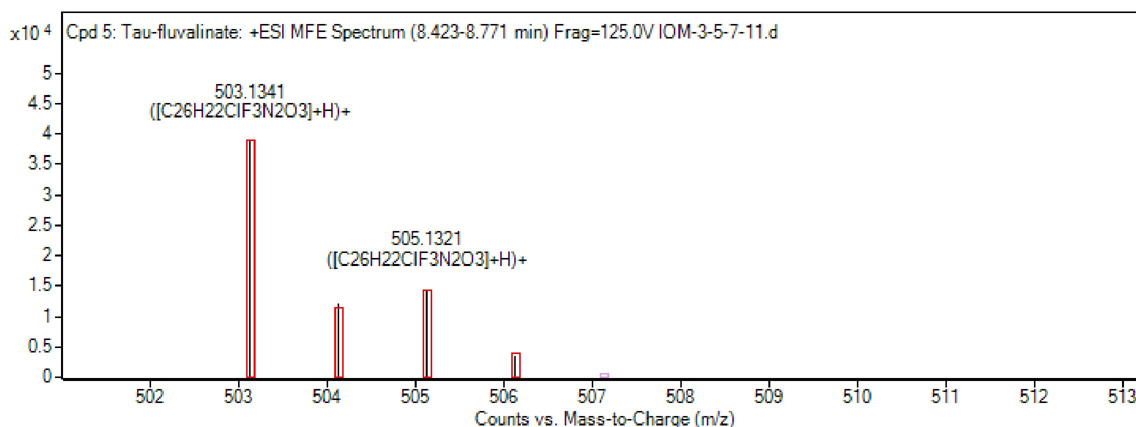

Figure 3.6: An overlay comparison between tau-fluvalinate, detected in a comb wax sample, to its theoretical isotopic pattern (red boxes).

Using the theoretical isotopic pattern of tau-fluvalinate (Figure 3.6), its presence was confirmed in addition to comparing RT values with a previously analysed tau-fluvalinate standard.

3.8 Discussion

3.8.1 Sample collection

A great amount of effort was put into finding beekeepers to participate and although a 100 % response was not expected the return of 152 samples (30.4 %) was very satisfactory. The majority of English samples came from the South West, South East and Eastern regions of the country; which correlates to the majority of registered BBKA members being within these areas (David Aston, personal communication, 2011). Returns from Northern beekeepers were more limited.

3.8.2 Comb wax analysis

The aim of this study was to analyse samples with a 'broad-brush' approach and to qualitatively identify any pesticides contained within the comb wax, as the age and histories of the wax samples could not be accurately determined - although beekeepers were asked to provide an approximate age of returned samples. This chapter therefore asks the question 'to what pesticides are UK honeybees potentially exposed?'. The answer to this question would then guide future investigations, conducted in Chapter 4.

The analysis of comb wax, as conducted by Mullin *et al.,* (2010), was quoted to cost in the region of $175,000; however, the work presented here was limited to a much smaller and modest budget. Consequently, it was not possible to obtain a standard for all pesticides used within the UK. Therefore, in order to increase the likelihood of detecting agricultural pesticides within each sample, it was decided that only the top 20 agricultural pesticides would be focused on, taking into account their use on insect pollinated crops i.e. oilseed rape, as well as the total weight of active ingredient applied in addition to their use on

multiple crop types. This information was obtained using a pesticide usage survey (Garthwaite *et al*., 2008). Those pesticides listed in Table 3.2 are the result of this search. This table does include some relatively hydrophilic compounds; however, chemicals of this nature have been previously detected in comb wax (Mullin *et al*., 2010). Despite their low log K_{ow} values, there is still expected to be a relative migration of these chemicals between comb wax and honey plus any other matrices contained within wax cells. Table 3.1 lists a number of in-hive treatments which were also of interest during analysis. The use of some of these compounds, within a particular hive (prior to sample collection) can be traced through the use of the returned questionnaires.

The questionnaire results (Appendix D) found that thymol was used by 37.11 % of English beekeepers (n = 98), Welsh beekeepers (n = 8) 46.15 % and Scottish (n = 46) only 17.86 %. Analysis of the comb wax (Appendix E) revealed a positive match of 63.33 % (English), 66.67 % (Welsh) and 80 % (Scottish) with those beekeepers who reported using thymol. However, thymol was also detected in a number of instances whereby thymol usage was not declared (England: 11 detections; Wales: 0; Scotland: 2 detections). Figure 3.4 shows an example of where thymol was stated not to be used on a particular hive, yet its presence can clearly be seen and confirmed with the corresponding mass spectra (Figure 3.5) ($[M]^+$ 150.20 *m/z*) and comparison to the NIST08 mass spectral database. The detection of thymol may come from natural sources, as it is a naturally occurring aromatic compound that is present in a number of plant and tree species, including: thyme *(Thymus vulgaris)* (Kosalec, Mastelic, Pieckova, & Pepeljnak, 2007) lime (*Tilia* spp.) and chestnut (*Castanea sativa*) (Guyot, Bouseta, Scheirman, & Collin, 1998). Consequently, thymol has been detected in lime tree honeys at concentrations between 18 – 161 ppbv

(Guyot *et al*., 1998). Given the lipophilic nature of thymol there may be a possibility that its inclusion in wax is the result of a preferable migration from honey, although this cannot be confirmed by this study. Interestingly, thymol was found not to be removed during thermal treatment and so it may already be present in foundation wax (Bogdanov *et al*., 1998).

In those instances whereby thymol was not detected, despite being used, it may mean that the levels within the wax may be below the LOD (~ 1 ppmv) of GC-MS, as a result of natural evaporation. However, its popularity as a *Varroa* treatment within the UK means that depending on the application method, the exposure of this compound to honeybee colonies can be continuous (Wallner & Fries, 2003). Thymol levels within honey comb, typically located within the supers, was found to be lower (average 21.6 ppmv) than brood comb (average 516.8 ppmv) (Bogdanov *et al*., 1998). Therefore, depending on the location of the submitted sample, the chances of detecting this compound will be more favourable in brood comb. This information was not considered in the questionnaire.

It is possible that beeswax can be adulterated during the recycling process (Semkiw & Skubida, 2013) and although not the focus of this chapter, the compound butylated hydroxytoluene (BHT) was detected in 46 % of the samples analysed. Scotland was found to have the highest incidence of BHT as it was found in 76 % of the 46 samples; Wales 50 % and England 33 %. BHT is a synthetic antioxidant, often added to food as a preservative (E 321) (Race, 2009). Originally developed for use in petroleum and rubber based products (Race, 2009), BHT can be found in food grade paraffin wax (CDS Analytical Inc, n.d), which is known to be used in the adulteration of beeswax (Semkiw & Skubida, 2013). BHT is also used as a stabiliser within various plastics (Race, 2009) and in order to

eliminate the possibility of cross-contamination from plastic equipment used during the extraction process, a small investigation was conducted. Here five centrifuge tubes were exposed to the QuEChERS extraction procedure and the solvent then analysed. No BHT was detected following this experiment; thus it is suspected that some of the foundation wax used by beekeepers may have been adulterated - this claim, however, cannot be proven. It is more plausible that its detection may result from its addition to pesticide formulations as a chemical stabiliser.

Samples KEN_6_08/07/11, SOM_4_06/07/11 and SRY_1_07/07/11 are considered newly drawn comb at around 2, 3 and 5 months, respectively; however, the detection of tau-fluvalinate within these samples, using Q-TOF LC/MS, suggests that the foundation used already contained this chemical prior to being used within the hive. Alternatively, the drawn-out comb may have been quickly contaminated. Apicultural treatments were found to be the most persistent compounds found within comb wax, unlike agrochemicals (Bogdanov *et al*., 2003; Bogdanov 2006; Serra-Bonvehí & Orantes-Bermejo 2010; Ravoet et al. 2015), which echoes the findings seen in Table 3.3. Interestingly, IOM_3_05/07/11 also contained tau-fluvalinate, which is significant as the Isle of Man is not affected by *Varroa* infestations; therefore, there is no reason why the chemical should be detected other than from its use as an agricultural treatment. However, several studies suggest that the occurrence of tau-fluvalinate is most likely due to apicultural treatments (Bogdanov *et al*., 2003; Bogdanov *et al*., 1999; Wallner 1999; Wu *et al*., 2011). Determining the actual source, however, remains difficult (Serra-Bonvehí & Orantes-Bermejo, 2010).

IOM_1_05/07/11, KEN_5_08/07/11 and IOM_2_05/07/11 are the three oldest samples analysed by Q-TOF LC/MS at 42, 36 and 30 months, respectively; despite this no chemicals were detected. This suggests that older combs do not necessarily demonstrate greater levels of contamination, although it is known that residues will gradually fall over time (Bogdanov, 2004). In Switzerland, residues of tau-fluvalinate found in comb wax dropped following a decline its use due to *Varroa* resistance in the 1990s. It is thought that another 20 years are needed before the levels finally vanish from beeswax (Bogdanov 2006). A longer study would need to be conducted in order to make a definite conclusion.

The results presented in this chapter offer an insight into the contamination of comb wax from the UK. The wax recycling processes often adopted by beekeepers only physically removes debris, meaning certain chemicals will remain persistent in wax (Bogdanov, 2006). However, success in attempting to remove compounds via chemical means has been limited (James, Ellis, & Duehl, 2013). Of those surveyed, 33.55 % (21.05 % not specified) stated that they trade in old comb wax for recycled foundation sheets, which could have implications on the health of future colony generations, if found to be contaminated (Wu *et al*., 2012; Wu *et al*., 2011). The levels of tau-fluvalinate found within comb wax were determined to have a greater correlation to the levels of this chemical detected in bee bodies, as oppose to its content in pollen. This was also true for coumaphos; however, amitraz showed no significance between the levels in bee bodies to either comb wax or pollen samples (Mullin *et al*., 2010). These three chemicals were found to account for the majority of detected residues in comb wax (Mullin *et al*., 2010;

Wu *et al*., 2011; Simon-Delso *et al*., 2014), thus leading researchers to regard comb wax as the primary source for bee contamination (Mullin *et al*., 2010).

A significant finding is the lack of detected agrochemicals, which distances the findings of this study away from the severely contaminated combs of North America (Mullin *et al*., 2010). A study from France, investigating the long-term effects of colony exposure to thiamethoxam treated crops, reports that in tested wax samples both thiamethoxam and clothianidin fell below their LOQ (1 ppbv). It was concluded that these chemicals were not persistent in comb wax (Pilling, Campbell, Coulson, Ruddle, & Tornier, 2013). These findings agree with the results of this study, as neither thiamethoxam nor clothianidin were found in UK comb wax. The small sample size use within this chapter is not very representative of the whole UK population. Rural locations increase the likelihood of exposure to agrochemicals and thus the detection of these compounds (Simon-Delso *et al*., 2014); however, to focus solely on a cropped area is again not truly representative of a whole country.

It is perhaps no surprise that the application of a chemical treatment, directly to a hive, should result in the contamination of the immediate surroundings (comb wax). However, the method in which agrochemicals reach the hive is a little more complex as it relies on multiple factors. For example: the successful return of a forager to the hive; whether the chemical may have undergone some sort of metabolism during transit or storage in the honey stomach; the lipophilicity of the compound and its initial concentration within pollen or nectar. The application of agrochemicals to a crop is conducted in a way to minimise the exposure of honeybees and other beneficial pollinators to harmful chemicals. Such measures can include evening applications, as well as prior warnings to

beekeepers and thus giving them plenty of time to relocate their colonies, if necessary; this is also confirmed through questionnaire responses.

3.9 Conclusion

The aim of this chapter was to determine which pesticides are present in comb wax samples obtained from around the UK, of which no data currently exists. Using GC-MS analysis, only thymol was detected. Thymol was detected in samples which were reported not to be treated with this compound, suggesting persistence in comb wax over an undetermined amount of time. Given the detection limit of the GC-MS instrumentation (LOD ≥ ppmv levels), it can be assumed that if any pesticides are present within comb wax then their levels will be in sub-ppmv range. Later analysis using Q-TOF LC/MS (LOD ≤ ppbv levels) revealed the presence of tau-fluvalinate in 12 out of the 15 samples analysed; which is one of the most commonly detected *Varroa* treatments. The possibility of other chemicals being present within comb wax cannot be excluded. GC-MS analysis also revealed the presence of butylated hydroxytoluene (BHT), a synthetic anti-oxidant which may originate from pesticide formulations or it may suggest the adulteration of comb foundation wax during the recycling process, although this cannot be confirmed.

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CHAPTER 4

The accumulation of pesticides within comb wax over a two year time period

4.1 Overview

The lipophilic nature of some agrochemicals and apicultural treatments, along with their respective stabilities in comb wax, means that these pesticides can potentially accumulate over time. This chapter looks to investigate what pesticides accumulate in comb wax over a two year period.

4.2 Introduction

As indicated in the literature mentioned in Chapter 3, it is already known that the detection of lipophilic pesticides in beeswax is possible through the use of sensitive analytical equipment (Lambert *et al*., 2013; Mullin *et al*., 2010; Ravoet, Reybroeck, & de Graaf, 2015), thus revealing the alarming amounts of pesticides that honeybees are

chronically exposed to (Mullin *et al.*, 2010). The levels and chemical nature of the pesticides found in beeswax appear to vary, according to both apicultural and [local] agricultural practices (Mullin *et al.*, 2010; Wu, Anelli, & Sheppard, 2011) associated with a particular hive. A good example which includes both of these practices is migratory beekeeping. America is renowned for its migratory beekeeping due to its large industrial scale. As already mentioned in Chapter 1, the US almond industry alone sees as many as a million colonies being relocated to aid in pollination. Throughout the course of a growing season, colonies may be relocated between two and five times a year (Johnson, 2010) or more (Bond, Plattner, & Hunt, 2014). Each move increases the likelihood of exposure to a number of biological and environmental stressors, including pesticides (Cox-Foster 2007 as cited in Johnson 2010).

A survey into the levels of pesticides within American apiaries revealed high levels of agrochemical and acaricides contained within comb wax. The survey analysed both migratory and non-migratory comb wax; however, it did not make a distinction between either of these wax types within the results. Of the 259 wax samples analysed, 60 % contained at least one systemic pesticide. Amazingly, 98 % of the foundation and comb wax analysed contained beekeeper applied tau-fluvalinate and coumaphos with a concentration range of 2 - 204 ppmv and 1 - 94 ppmv, respectively. Over 47 % of these samples also contained chlorothalonil (fungicide) (Mullin *et al.*, 2010); this combination was linked to a phenomena described as ‘entombed pollen’ (vanEngelsdorp *et al.*, 2009). Amitraz is known to degrade rapidly within wax (Korta *et al.*, 2001); though, its major metabolite DMPF (2,4-dimethylphenylformamide) was detected within 60 % of the samples at a range of 9.2 - 43 ppmv. In total 87 different pesticides were detected overall

with an average of 6 pesticides per comb and a high of 39 within a single comb (Mullin *et al*., 2010). Similarly, Wu *et al.,* (2011) also studied comb wax used with migratory beekeeping practices. The findings of this study revealed that tau-fluvalinate and coumaphos were again the most detected pesticides residues, ranging from 0.164 – 24.34 ppmv and 0.281 – 22.1 ppmv, respectively. A total of 39 different pesticides were detected; averaging 10 pesticides per comb, with a high of 17 pesticides contained in a single comb.

As considered in Chapter 3, there are a number of ways in which agrochemicals can enter the hive (Krupke, Hunt, Eitzer, Andino, & Given, 2012). Due to their (stored) abundance within the comb, the biggest contributors are contaminated pollen and nectar (Chauzat *et al*., 2006); consequently, different chemicals can be brought back to the hive, depending on the source of forage. A single crop has been shown to be treated with a number of agrochemicals (see Chapter 5, p195); this is reflected in the findings of both Mullin *et al.,* (2010) and Wu *et al.,* (2011). The scale of American migratory beekeeping is perhaps an extreme exaggeration of those practices seen across the UK and indeed Europe, where beekeeping is mainly reserved for the hobbyist, who does not normally transport their hives (Grunewald, 2010). Despite this, the methods of exposure and principles of accumulation still remain the same.

A French study, which included samples from commercial beekeepers (> 150 colonies) also revealed that both coumaphos and tau-fluvalinate to be the most detected pesticides found in French comb wax; with mean detections of 647.5 ppbv and 220 ppbv, respectively (Chauzat *et al*., 2009). Although looking at a narrower range of pesticides, this agrees with the findings those studies previously considered (Mullin *et al*., 2010; Wu

et al., 2011). Tau-fluavlinate was also found at a range of 0.27 – 88.66 ppmv in 93.6 % of wax samples, taken from commercial Spanish colonies (n = 147). Interestingly, coumaphos was only detected within 3.7 % of the wax samples analysed (n = 134) at a range of 13.6 – 22.7 ppbv. The most detected acaricide was chlorfenvinphos (95.9 %, n = 197) at a range of 0.196 – 10.64 ppmv (Serra-Bonvehí & Orantes-Bermejo, 2010). In Belgium, tau-fluvalinate was detected within all wax samples analysed (n = 10), with a range of 11 - 83 ppbv, whilst coumaphos was detected (90 % of samples, n = 10) with a range of 6 – 66 ppbv (Ravoet *et al*., 2015). Within these European studies, the rate of detection and the levels of agrochemicals were found to be relatively low, compared to the discussed apicultural treatments.

Agricultural pesticides tend to be unstable and degrade quickly after application (Bogdanov, Imdorf, Charrière, Fluri, & Kilchenmann, 2003), although their persistence can be prolonged; for example, UV-sensitive neonicotinoids can have half-lives of over 1000 days in certain soil conditions and their levels can accumulate through repeated applications (Bonmatin, Giorio, Girolami, Goulson, & Kreutzweiser, 2015). Despite this, apicultural applications are still considered to pose a greater threat to the colony (Bogdanov, Imdorf, *et al*., 2003).

4.3 Accumulation of pesticides in wax

In this thesis, accumulation refers to the progressive increase in the amount of an active ingredient in a material i.e. comb wax. The composition of comb wax has been covered in Chapter 1, Section 1.3.2.5; its lipid nature means that it is capable of housing many lipophilic substances, particularly acaricides - these tend to be the most prevalent

chemical in comb wax (Wallner, 1999; Wu *et al*., 2011). The distribution and accumulation of an acaricide throughout a hive depends on its lipophilicity (log K_{ow}), frequency of use and the amount of applied active ingredient (Bogdanov, Imdorf, & Kilchenmann, 1998b; Wallner, 1999). The activity of the bees during applications should also be considered, as their movement can distribute pesticides throughout the hive (Wallner, 1999).

The accumulation of agrochemicals in beeswax receives less attention than those used in apiculture (Chauzat & Faucon, 2007). Interestingly, in-hive applications were found to result in a higher level of these pesticides in comb wax than found in pollen, while pollen was found to contain a greater or equal amount or agrochemicals compared to comb wax (Johnson, Ellis, Mullin, & Frazier, 2010).

The application of acaricides can vary (Table 3.1); nevertheless, a long contact exposure and a high initial concentration of an active ingredient will result in a greater uptake and distribution of the compound. However, low levels of an active ingredient, even with a long exposure, can fail to produce unmeasurable levels (Wallner, 1999). Once applications stop the levels of acaricide within comb wax will begin to fall (Bogdanov, 2004a). The high levels tau-fluvalinate and coumaphos and the persistence of these chemicals in comb wax, as reported by multiple studies (Bonmatin *et al*., 2015; Mullin *et al*., 2010; Serra-Bonvehí & Orantes-Bermejo, 2010; Wu *et al*., 2011) is perhaps no surprise; as it is already known that these chemicals are particularly stable within comb wax (Bogdanov, 2006), with an approximate half-life of five years (Bogdanov, 2004a). This can be as a result of the migration of pesticides from one comb to another, as it was found that the active ingredient from the initial comb fell and built up within newly produced comb (Wu *et al*., 2011); consequently, comb wax is considered “uniformly contaminated” (Mullin *et al*.,

2010). The migration of pesticides can also occur between comb wax and the contents within each cell (Stuart *et al*., 2008; Tremolada, Bernardinelli, Colombo, Spreafico, & Vighi, 2004); this can result in similar concentrations of pesticides in both comb wax and honey stores (Stuart *et al*., 2008). The partition between honey and comb wax will decrease during the honey maturation process, as the water content begins to drop (Chauzat & Faucon, 2007). Not all fat-soluble pesticides are stable as wax was found to accelerate the degradation of amitraz; which is also known to have poor stability in honey. Volatile and semi-volatile fat-soluble compounds, such as essential oils, are also known to decrease in concentration through evaporation, which occurs as a result of the temperature within the hive. Hydrophilic chemicals, such as oxalic acid and formic acid, do no accumulate in wax, although they can be detected in honey giving rise to an unpleasant taste (Wallner, 1999).

The removal of acaricides can be difficult, as there are limited options available to achieve this (Chauzat & Faucon, 2007), although an attempt to reduce pesticide residues using Ozone was found to be partially successful (James, Ellis, & Duehl, 2013). Acaricides were found to be persistent in wax even after heating at 140 °C for 2 hours (Bogdanov 1998) resulting in the contaminated foundation wax. Exposing wax to such high temperatures can have a negative effect on its condition (Bogdanov, 2009). Interestingly, thymol, although volatile, was found not to evaporate during comb wax recycling (Bogdanov, Imdorf, & Kilchenmann, 1998a). Contaminated foundation wax, therefore, presents another mechanism by which pesticides are introduced in to the hive (Adamczyk, Lázaro, Pérez-Arquillué, Bayarri, & Herrera, 2010; Bogdanov, 2004a; Wallner, 1999).

4.4 Contaminants of foundation wax

Wallner (1999) considers comb wax, in countries where (stable-synthetic) substances are used, to be “permanently damaged”. Only the complete destruction (for example, burning) of the comb wax can destroy the active ingredient (Wallner, 1999). This is based on the fact that certain chemicals, for example, tau-fluvalinate, coumaphos and DMPF, are not removed after thermal treatment (Bogdanov, 2004a; Korta *et al*., 2001; Tremolada *et al*., 2004). As a result, internationally traded wax can contain substances not used or approved in the country to which it was imported (Wallner, 1999). 62.5 % of the foundation sheets analysed in Germany and 20 % of imported foundation were found to contain coumaphos (Wallner, 1999). Interestingly, although high levels of coumaphos was detected in Italian foundation wax, it was found to show a decreasing trend in colonies which were kept organically (no synthetic chemical used) (Lodesani, Bigliardi, & Colombo, 2003), supporting the notion that concentrations will fall once applications have ceased (Bogdanov, 2004a).

4.5 Aim of investigation

The aim of this chapter is to analyse distributed sheets of organic foundation wax to determine which pesticides, if any, accumulate in the resulting comb over a two year time frame in five selected regions of the UK.

4.6 Experimental design (accumulation over time)

In order to monitor an accumulation of pesticides over a two year period, a frame of (certified) organic foundation wax (EH Thorne (Beehives) Ltd) was distributed to participating beekeepers, along with a set of instructions which set out the requirements of this study (Appendix F). The instructions stated that the foundation wax was to be left in the hive (if possible) for two complete seasons (2012 – 2014). After one year, a section of this comb is then to be returned to Keele University, whilst the remainder of the frame is to be left within the hive. A final sample will then be collected again in 2014 (July-August) and returned along with a second questionnaire (Appendix G). During instances whereby beekeepers may chose not to use the supplied foundation wax, or where the foundation was inadequate for their hive (due to incorrect sizing), a sample of their own wax was requested prior to installation. This was to provide a baseline on which the accumulation of residues can be monitored, as in some cases their wax had been recycled continuously for decades, so any initial contaminants were accounted for. Sheets of blank organic foundation were also analysed.

4.7 Materials and Methods

4.7.1 Sample collection

Beekeepers from Chapter 3 who originally registered interest in continuing with further studies, were contacted along with beekeeping associations in areas of interest. Sheets of blank organic foundation were distributed to those beekeepers who responded. The foundation used was deemed to be "beeswax obtained from hives which were operated organically" in accordance with Article 29(1) of Regulation (EC) No 834/2007 (EH Thorne

(Beehives) Ltd, Personal communication), although no paperwork was ever provided on request.

Sample locations were initially targeted based on the results of Chapter 3, whereby the regions of the UK with highest levels of comb wax contamination would be the main focus of the study. However, as the GC-MS analysis failed to reveal pesticides at levels greater than 1 ppmv, the approach to determining these locations was therefore altered. Figure 4.1 shows a comparison of the percentage distribution of pesticides (Garthwaite, Barker, Smith, Chippindale, & Pietravalle, 2010) and the locations of beekeepers (from Chapter 3) who agreed to participate in future studies.

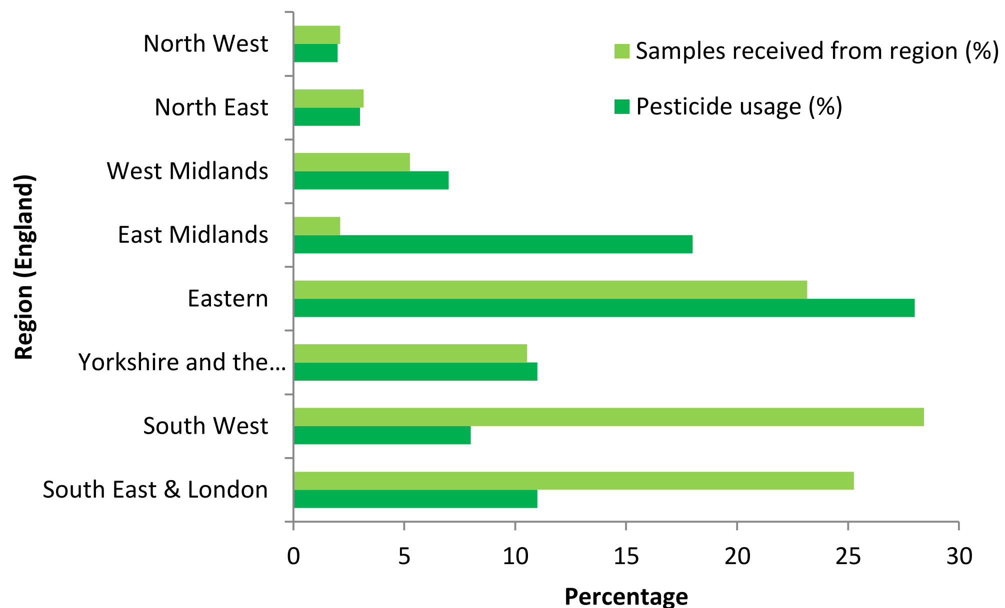

Figure 4.1: A comparison of the pesticide usage (Garthwaite *et al*., 2010) and percentage location of participating beekeepers (from Chapter 3) based on each region across England.

Using the information from Figure 4.1, five key regions were selected:

1) Eastern
2) South West (SW)
3) South East (SE) & London
4) Yorkshire and the Humber
5) North Staffordshire/ South Cheshire

In total 43 sheets of foundation were distributed (Figure 4.2). Most foundation sheets were placed within the hives between July/August 2012. First season samples were returned between July and August 2013, whilst second seasons samples were received August/September 2014. All returned samples were stored at -40 °C.

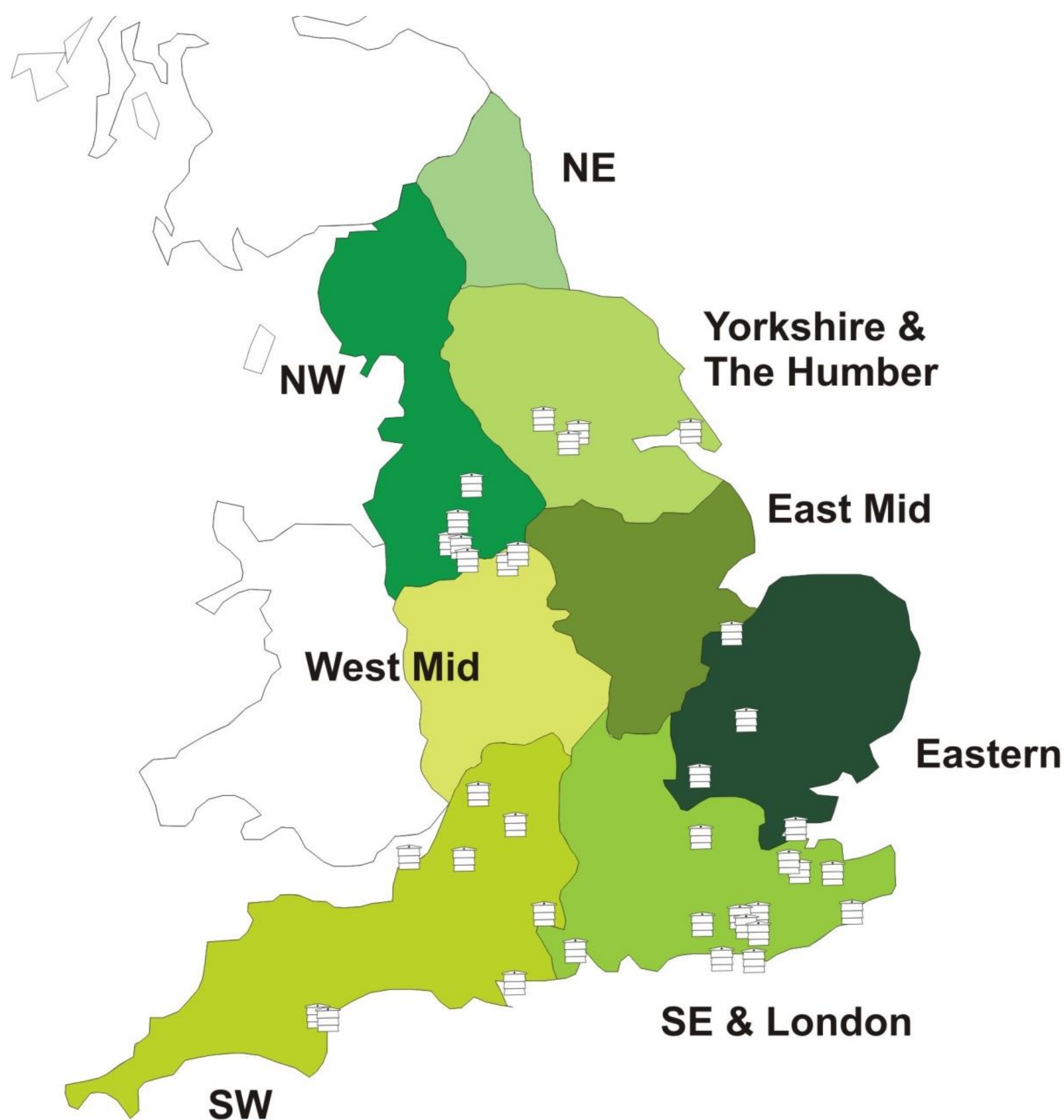

Figure 4.2: The (approximate) locations of distributed sheets of organic foundation. No distinction is shown between the locations where multiple sheets have been sent to the same beekeeper. SW – South West; SE – South East; NW – North West.

4.7.2 Sample extraction

The QuEChERS extraction and Q-TOF LC/MS clean-up methodology were conducted in the same manner as described in Chapter 3.6.3, p135.

4.7.3 Instrumental analysis

Q-TOF LC/MS analysis was conducted in the same manner as described in Chapter 3.6.4, p136.

4.8 Results

4.8.1 Questionnaire results

A summary of the questionnaire results given by beekeepers during each season of collection is given in Appendix H; however, some of the questions have not been tabulated due to their qualitative nature. Only one questionnaire was completed for the second season and has therefore not been summarised below.

First season questionnaires results

Of the 16 samples received, 56.25 % were from a rural environment and 43.75 % were from a semi-rural environment. No samples were received from areas considered to be urban. The top three (specified) apicultural treatments used were thymol (75 %), oxalic acid (50 %) and Apistan (tau-fluvalinate) (12.50 %). 18.75 % of the samples received were specified as not being treated. Of the samples received, 81.25 % used the organic foundation wax provided by Keele University; the remaining 12.5 % used foundation obtained from other distributors.

4.8.2 Analysis of foundation wax

As shown in Table 4.1, none of the four organic foundation sheets analysed were found to contain any hive treatments or agrochemicals; thus establishing a blank starting point,

within the limits of detection. The three foundation sheets provided by beekeepers for analysis, two were found to contain tau-fluvalinate.

Table 4.1: Results of organic foundation wax analysis (n = 4), plus beekeeper supplied foundation sheets (n = 3).

Sample I.D.	Compound	RT (min)	Score	Difference (ppm)
Organic foundation (1)	-	-	-	-
Organic foundation (2)	-	-	-	-
Organic foundation (3)	-	-	-	-
Organic foundation (4)	-	-	-	-
CAM_12/07/13 (F)	tau-fluvalinate	8.807	99.07	0.43
KEN_22/07/13 (F)	-	-	-	-
KEN_02/09/13 (F)	tau-fluvalinate	8.825	98.93	0.86

(F) = foundation

4.8.3 Analysis of first season comb wax samples

As seen in Table 4.2, tau-fluvalinate was detected in 8 out of the initial 16 comb wax samples analysed (50 %). Only a single sample (KEN_17/07/13) was identified to contain more than one detectable pesticide; both of the compounds detected in this sample (boscalid and pyraclostrobin) were also the only chemicals which were believed to have originated from outside the hive. Seven of the samples analysed were found to contain no detectable pesticides.

Table 4.2: First season Q-TOF LC/MS results for analysed comb wax samples (n = 16).

Sample I.D.	Compound	RT (min)	Score	Difference (ppm)	Acc. age (months)
CAM_12/07/13	tau-fluvalinate	8.809	94.57	0.001	14
CHS_29/07/13	tau-fluvalinate	8.813	91.39	1.400	12
DEV_02/07/13[a]	tau-fluvalinate	8.875	99.51	0.570	11.5
DOR_12/07/13[c]	tau-fluvalinate	8.760	88.47	3.110	13
DOR_30/07/13	tau-fluvalinate	8.823	76.70	1.970	10
HRT_09/07/13[a]	-	-	-	-	13
KEN_17/07/13	Boscalid	5.773	96.50	1.870	12
	Pryaclostrobin	6.625	99.56	0.050	
KEN_22/07/13	tau-fluvalinate	8.785	98.24	0.640	15
KEN_02/08/13	-	-	-	-	12
KEN_02/09/13[b]	tau-fluvalinate	8.821	79.17	2.690	12
SOM_09/07/13[a]	-	-	-	-	11
STS_02/09/13	-	-	-	-	10
STS_11/09/13[b,c]	-	-	-	-	10
SXE_02/07/13	-	-	-	-	11
SXW_12/07/13	-	-	-	-	11.5
WYK_10/7/13	tau-fluvalinate	8.818	81.71	0.610	11.5

[a] tau-fluvalinate used; [b] no hive treatments used; [c] colony reported to have failed to over winter.

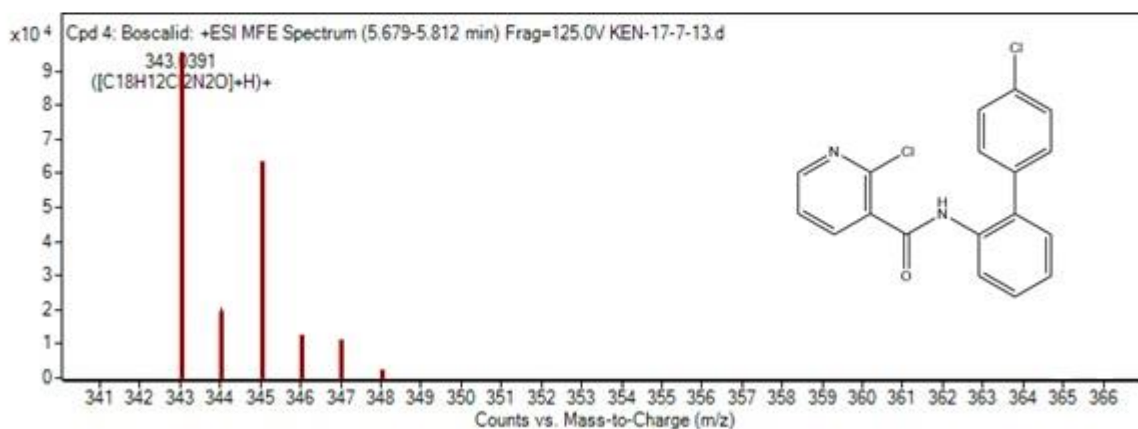

Figure 4.3: An overlay comparison between boscalid, detected in KEN_17/07/13, to its theoretical isotopic pattern (red boxes).The structure of boscalid has also been provided.

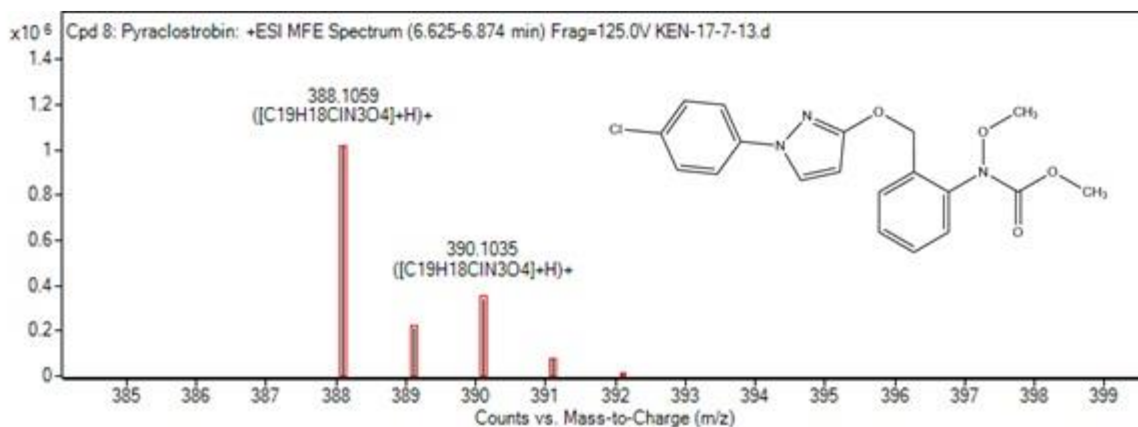

Figure 4.4: An overlay comparison between pryaclostrobin, detected in KEN_17/07/13, to its theoretical isotopic pattern (red boxes). The structure of pryaclostrobin has also been provided.

Using the theoretical isotopic pattern both boscalid (Figure 4.3) and pryaclostrobin (Figure 4.4) were identified in KEN_17/07/13 with 96.50 % and 99.56 % matches, respectively.

4.8.4 Analysis of second season comb wax samples

No pesticides were detected in either of the two samples received during the second season of this study (Table 4.3), despite previously detecting tau-fluvalinate in CAM_12/07/13 and both boscalid and pryaclostrobin in KEN_17/07/13.

Table 4.3: Second season Q-TOF LC/MS results for analysed comb wax samples (n = 2).

Sample I.D.	Previous sample	Compound	RT	Score	Difference (ppm)	Acc. age (months)
CAM_05/09/14	CAM_12/07/13	-	-	-	-	26
KEN_22/08/14	KEN_17/07/13	-	-	-	-	24

4.9 Discussion

4.9.1 Sample collection

The significant drop in beekeeper participation is partially due to limiting the sampled areas to five regions of England as opposed to monitoring potential accumulations from across the country and indeed the rest of the UK. However, this does not explain the return of only 16 samples from the originally distributed 43 sheets of foundation wax. Some beekeepers had been in touch to explain that their colony had been destroyed in order to control the spread of American foulbrood. Comb wax was also reported to have been discarded in instances where colonies had failed to survive over the winter period. The GC-MS results of Chapter 3 may have also dissuaded further interest in the study, as a failure to detect any 'harmful' chemicals may have left beekeepers feeling that their colony(s) were not at risk of any detrimental chemical exposure. Another explanation is perhaps the slow return of results from Chapter 3 back to the beekeepers, due to unforeseen complications.

4.9.2 Pesticide residues within foundation wax

No pesticides, including those applied by beekeepers, were found following the analysis of four blank organic foundation sheets. Sampled foundation sheets were taken from the middle of every newly opened packet (10 pieces); this was conducted in order to maximise the chance of detecting any pesticides should there be any migration between the sheets. The initial negative results were satisfactory enough to assume that all foundation sheets were of the same purity and that any pesticides detected within the returned combs can be assumed to be a result of external contamination.

4.9.3 Season one: returned comb wax samples

GC-MS analysis was not conducted within this chapter, due to its limited success in the detection of pesticides in Chapter 3. However, it is expected that thymol would again be detected in the samples of this chapter as this is a popular *Varroa* treatment used within the UK and depending on the application method, its exposure to honeybee colonies can be continuous (Wallner & Fries, 2003). However, HRT_09/07/13, KEN_02/09/13 and STS_11/09/13 all reported that no treatments were used on their hives. As these samples were constructed on the initially supplied organic foundation, it therefore limits the introduction of chemicals into the comb by external sources, or from pre-contaminated foundation wax which may also be contained within the hive. Of these samples, only KEN_02/09/13 was found to contain tau-fluvalinate.

According to the questionnaire results, two participating beekeepers in this chapter used tau-fluvalinate as a hive treatment: DEV_02/02/13 and SOM_09/07/13; this chemical was positively identified in the former whilst it was not detected in the latter. It is known that the accumulation of chemicals in the hive can result from their frequency of use and the amount of active ingredient applied (Wallner, 1999); unfortunately neither of this information was requested from or supplied by beekeepers. Interestingly, SOM_09/07/13 (11 months old on organic frame) arrived as a dark comb, suggestive of multiple brood generations. The same beekeeper supplied a comb in Chapter 3 (SOM_4_06/07/11), which was only three months old and recorded as pale in colour; this was later found to contain tau-fluvalinate. It was regularly observed that pale wax (including foundation) completely melted during QuEChERS, whereas the darker combs only partially melted, thus leaving behind exuviae or 'cell ghosts' (Hepburn, 1986). It is known that the

percentage ratio of wax within the comb will decrease with age (Hepburn & Kurstjens, 1988), which raises the question how much wax is there in a 3 g sample of old comb?

Interestingly, although the foundation wax from KEN_02/09/13 (F) was found to contain tau-fluvalinate, the detection of the compound in drawn-out comb (KEN_02/09/13) was of a poorer percentage match (79.71 %) than compared to the initially detected chemical in the foundation (99.07 %). A possibility for this reduction is as the amount of wax increases through comb-building, or the migration of the chemical into other combs/matrices. It will then have a lower concentration per unit area, meaning it becomes closer to the LOD, resulting in low-quality mass spectral data.

Although a foundation sheet was distributed to CAM_12/07/13, it appears that an alternative foundation sheet was preferred and this was returned for analysis. It was stated that tau-fluvalinate was not used and relating back to the sample questionnaire provided by the same beekeeper in Chapter 3 (CAM_3_06/07/11), only thymol was reported to have been used. Therefore, it is likely that the source of tau-fluvalinate is from the foundation wax used by the beekeeper, as two of the three foundation sheets analysed were found to contain this chemical. Unless approached quantitatively, it is difficult to monitor the accumulation, or indeed the reduction of a pesticide within a comb wax sample, if the foundation used already contains a compound of interest. Although valuable information can still be derived from qualitative observations, this highlights a weakness of this study.

STS_11/09/13 was described as a very small and weak colony, which had unfortunately died very early on in the season and although the comb was partially drawn, no pesticides were found to have accumulated. As pesticides are said to distribute about the hive

during honeybee movement around the colony (Wallner, 1999), perhaps the small amount of hive activity was not substantial enough to result in a significant transfer between combs. This, of course, is based on the assumption that other combs in the hive contained pesticides. However, DOR_12/07/13 also failed to over winter, yet tau-fluvalinate was detected in the sampled comb. The comparative strength of both colonies is not known, so it is not possible to draw conclusions of the role of hive activity, although it is suggested to be a major contributor to the spread of pesticides around the hive (Wallner, 1999).

Even though originally not targeted as a pesticide of interest, the fungicide pryaclostrobin (log K_{ow} 3.99 (Tomlin, 2009)) was detected in KEN_17/07/13; boscalid was also identified as being present in the same sample. The questionnaire relating to KEN_17/07/13 acknowledged that the local flora included fruit trees and field beans, amongst others. Pyraclostrobin and boscalid are found together in two UK approved pesticide applications: *1)* Bellis® (BASF) – for use on fruit trees; and *2)* Signum® (BASF) which is used on many crops including field beans (BCPC, 2014). Consequently, it is not unreasonable to assume that these sources of forage could be the origin of the agrochemicals detected. The low levels of agricultural pesticides detected in the hive may be attributed to the 'filtering' effect of bees (Bogdanov, 2006), as well as the relatively short period of time for these chemicals to sufficiently accumulate. Thiamethoxam treatments were not found to be persistent in wax (Pilling, Campbell, Coulson, Ruddle, & Tornier, 2013).

4.9.4 Season two: returned comb wax samples

In season one, CAM_12/07/13 was found to contain tau-fluvalinate, which was considered to be a consequence of an already contaminated foundation wax; however, the second collected sample (CAM_05/09/14) contained no detected pesticides. With such a limited sample size (n = 2) it is not possible to determine any trends or to make any definite conclusions regarding accumulation. What is seen here is a potential reduction in a pesticide, within the space of around 12 months; which contradicts suggestions of long term persistence (Bogdanov, 2006). Yet, given that this comb wax was not actively treated with the previously detected substance, this does support the gradual reduction of a compound over time (Bogdanov, 2004b). The fact that tau-fluvalinate is not detected after a relatively short amount of time, compared to its five year half-life (Bogdanov, 2004a), may suggest that it has been 'diluted' as more wax is added or lost to other matrices and may have, as a result, fallen below the limit of instrumental detection.

It is expected that, for a comb demonstrating a typical brood pattern, the majority of hive activity would be concentrated at the centre of the comb with the peripheral areas being reserved for honey stores. This 'hot-spot' of hive activity would surely mean there is a potential for a greater concentration of chemicals in this area. Indeed, this was found true for tau-fluvalinate, as residual levels were higher in brood combs than honey combs (Tsigouri, Menkissoglu-Spiroudi, Thrasyvoulou, & Diamantidis, 2003).

There are also a number of other possibilities for not detecting a previously found chemical, which lies with the instructions provided to beekeepers. The instructions (Appendix F) state that the supplied comb should be used to fit in line with their existing apicultural practices. The initial idea of this was to not to inconvenience beekeepers,

whilst also ensuring that the comb was not paid any special attention, which may result in biased results; for example increasing its likelihood of coming into contact with any chemical treatment. As a result, it is not known how the comb was used within the hive, including its location.

The failure to detect boscalid and pryaclostrobin within KEN_22/08/14 suggests that the previously analysed sample may have contained pollen which was contaminated with these chemicals. It is not known whether this hive was relocated during each season nor if the crops which are the source of contaminated pollen were on a rotation system, meaning that there would be no need for this chemical to have been used.

Although very speculative, it is a possibility that the sampled comb may have been taken from the same position in the frame as the previous year, as this section of wax would not contain any reinforcing wire from the initial foundation sheet. Consequently, the sampled comb would only be 12 months old. Unfortunately the inclusion of wire within comb wax samples was not recorded.

4.10 Conclusion

This chapter presents the first known attempt to monitor the accumulation of pesticides in UK comb wax samples. Unfortunately, the aims of this study have not been met; as with such a limited response in the second season of this study (n = 2) it was not possible to fully appreciate the accumulation of pesticides over a two year period. Although the results presented here were not quantified, the detection of tau-fluvalinate in beekeeper supplied foundation wax and in combs drawn-out from blank organic foundation wax was confirmed. This suggests that contaminated foundation wax may be the probable source

of this chemical. As only two agricultural pesticides were detected, albeit in a very small sample set, it is perhaps possible to distance the UK from the severe levels of agrochemical contamination found in American migratory honeybee hives (Mullin *et al*., 2010). The findings of this study are therefore more inline with European findings (Bogdanov *et al*., 2003). In order to improve it would be best to monitor accumulation over a greater number of years than used here, whilst also securing a greater number of samples for analysis.

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CHAPTER 5

Determining the levels of three seed-applied neonicotinoids in oilseed rape nectar and pollen

5.1 Overview

The demand for oilseed rape oil for use in biodiesels has seen a rise in the popularity of this crop grown in the UK. As such, the UK was considered to be a leading figure in the export of this product (Berry, Cook, Ellis, Gladders, & Roques, 2014). Being described as having a medium honey flow, oilseed rape offers a valuable source of forage for many pollinators (Farkas & Zajácz, 2007). However, scientific research published in the spring of 2012 (Henry *et al*., 2012; Whitehorn *et al*., 2012), in addition to a report from the European Food Safety Authority (EFSA, 2013), saw an imposed two year restriction on the use of neonicotinoid seed-treatments typically applied to oilseed rape. This was due to concerns of these chemicals having negative impact on pollinator health. This restriction is up for review before December 2015, with calls for more information regarding the

impact of neonicotinoids on pollinators. This study presents the amount of these neonicotinoids found within the nectar and pollen of oilseed rape.

5.2 Introduction to oilseed rape

The *Brassica* genus (*Brassicas*) includes many crop species such as: cabbage, sprout, kale, cauliflower, turnip, mustard and oilseed rape. This assortment of crops derives from much interbreeding, in order to amplify and exaggerate the size of roots, buds and seed pods; these are then exploited, often for human consumption (Kirk, 1992). Oilseed rape is a general term used for several species of oil-seed crops within the *Brassica* genus, most commonly *Brassica napus* L. (swede rape), *Brassica rapa* L. (turnip rape) and *Brassica juncea* (mustard). Within the United Kingdom, *B. napus* is the predominantly grown oilseed crop and therefore most commonly referred to as oilseed rape (Bunting, 1986; Kirk, 1992). It is in this sense in which the name will be referred to throughout this chapter – unless otherwise stated. There are spring-sown and autumn-sown varieties of oilseed rape, often referred to as spring and winter rape, respectively. With a typical yield of 20 % more than the spring-sown variety, winter rape (autumn-sown) is the predominate variety within the UK (Bunting, 1986) and will also be the focus of this chapter.

5.2.1 A brief history of oilseed rape

A parliamentary Bill introduced in 1572 entitled 'making oil out of seeds grown in England, equal to Spanish or foreign oils' was the first documented reference of oilseed being used in England. Oilseed had important economic value and production expanded

rapidly until the end of the seventeenth century; so much so that oil production became protected by tariff barriers. Around this time the residual oil meal, a by-product of oil production, had started to be used for cattle feed. By the mid-eighteenth century mineral oils had started to replace rapeseed oil and through free trade policies oilseed had all but disappeared from British farming and similar trends were observed across Europe (Bunting, 1986). Rapeseed oil has been used as an edible oil in Eastern cultures for thousands of years (Thompson & Hughes, 1986). With the exception of 1940s wartime Germany, western countries considered rapeseed oil to be unsuitable for human consumption (Thompson & Hughes, 1986). This is perhaps due to its unpleasant bitter taste and odour, brought about by the natural occurrence of glucosinolates and acidic compounds found within the seed (Kirk, 1992; Thompson & Hughes, 1986).

The commercial reintroduction of oilseed into Britain began in 1950, on a moderate scale. It was not until the 1970s that the amount of oilseed grown in the UK increased dramatically, owing to the use of oilseed as a break crop for cereals in addition to the new demand for poly-unsaturated vegetable fats. Consequently, this saw a rise in value of oilseed on the world market (Kirk, 1992).

However, the natural occurrence of some chemicals in rapeseed oil and meal have been found to have adverse effects on the health of humans and livestock (Kirk, 1992). For example, erucic acid has been found to cause cardiac abnormalities in laboratory studies (Beare-Rogers & Nera, 1972; de Wildt & Speijerst, 1984; Renner, Innis, & Clandinin, 1979). A high content of glucosinolates in the meal used for livestock can also produce undesirable effects (Kirk, 1992; Mithen, 1992), although, glucosinolates also have been associated with anti-carcinogenic properties (Hillman, Ratcliffe, Lynn, & Collins, 2006).

During the 1970s intensive breeding programs led to the production of various varieties of oilseed rape, which were considerably lower in glucosinolates and erucic acid. Low–erucic varieties of spring-sown rape were first registered in Canada in the 1974. It was not until 1977 that winter varieties were later registered (Bunting, 1986). Oilseed containing low levels of erucic acid and high levels of glucosinolates are referred to as "single-low" varieties (Kirk, 1992); nevertheless, high erucic acid varieties are used within industrial processes, such as inks and lubrication (Berry *et al*., 2014; Bhardwaj & Hamama, 2000; Bunting, 1986). Oilseed with low levels of both erucic acid and glucosinolate are known as "double-low" varieties; these became extensively grown towards the end of the 1980s (Kirk, 1992). Those varieties of *B. napus,* with an acidic content lower than 5 % (Thompson & Hughes, 1986), came to be known as 'Canola' – '**C**anadian **o**il, **l**ow **a**cid' (Office of the Gene Technology Regulator, 2008). Both of the terms oilseed rape and canola appear to be used interchangeably in the literature. The former is predominately used within the UK, whilst the latter tends be more commonly adopted across North America and Australia.

5.2.2 The role of oilseed rape in modern agriculture

During fallow periods, which occur between cropping systems, the land is often left bare and exposed. Consequently, the soil is at risk of erosion as well as losing various nutrients and organic matter, which may not be replaced; this can ultimately reduce the productivity of the soil (Kaspar & Singer, 2011). Cover crops, as the name suggests, are crops which cover the soil, aiding in the reduction of soil erosion. There are various types of cover crop available and their use depends on the duty they are required to perform. For example, 'green manures' are nitrogen (N) fixing crops (such as legumes), which

ensure that nitrogen is available for the succeeding cash crop. ‘Catch crops’, however, take up the nutrients which would otherwise be lost if the land was left fallow. ‘Living mulch’ cover crops tend to occupy the spaces between cash crops and this helps to provide weed suppression as well as regulating soil temperatures. However, the growth of living mulches is controlled so not to compete with the more valuable crop (Kaspar & Singer, 2011). Cover crops are also important in the control of pests and disease (Baldwin, 2006; Kirk, 1992). Oilseed rape is often used as a cover crop and is frequently included in a rotation cycle with wheat, potatoes and sugar beet, for example (Berry *et al*., 2014). Crops of the same family should not immediately follow each other on the same land during rotation cycles, as this can result in a build-up of soil-borne pathogens (Baldwin, 2006). Oilseed was originally grown in one in every five rotations, although this has since reduced to one in every two or one in every three; this is a result of economic pressures, as the demand for biodiesels remains strong. A reduction in the time between rotations has seen a smaller yield for each subsequent oilseed crop, as a result of accumulating diseases or through soil compaction – which can affect the growth of the plant. The chemical treatments of oilseed also provide protection for future crop generations (Berry *et al*., 2014), as they will remain persistent within the soil (Goulson, 2013).

5.2.3 Plant structure and physiology

The flower of the oilseed has six stamen and four nectaries in total. The stamen can be separated into two distinct types; a group of four inner stamen and two outer stamen. The inner stamen extend beyond the petals and release their pollen outwards; whilst the outer stamen, which are shorter, are found below the level of the petals and release their pollen inwards (Kirk, 1992). This morphological arrangement would suggest that the

shorter, inner stamen are not involved in self-pollination, as pollen grains are released below the level of the stigma throughout the whole anthesis process (Rosa, Blotchtein, Ferreira, & Witter, 2010).

There are a total of four different nectary arrangements within the Brassicaceae family (Bender *et al*., 2012). *Brassica napus* has a 'four-nectary' type morphology consisting of two pairs of nectaries, which are commonly bilobed in shape and are located at the base of the flower behind the sepals (Bender *et al*., 2012; Kirk, 1992). The nectary arrangement is shown in Figure 5.1.

The nectaries, like the stamen, can also be separated into two groups: the inner and outer nectaries. The outer nectaries are exposed, whilst the inner nectaries are situated between the stigma and short stamen. Nectaries appear as small green 'bumps' which can be found between the gaps of the inner stamen and are often found surrounded by clear drops of freshly secreted nectar (Kirk, 1992), a complex mixture of compounds which includes sugars and amino acids dissolved in water (Calder, 1986).

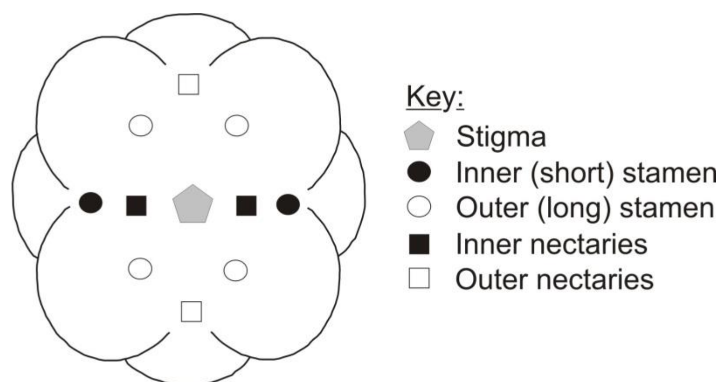

Figure 5.1: Schematic drawing (view from above) showing the locations of the reproductive features of the B. *napus* flower. Adapted from Rosa *et al*., (2010).

In regards to sugar content, the inner nectaries secrete less concentrated nectar but in greater volumes than that of the outer nectaries (Eisikowitch, 1981; Kirk, 1992). The inner nectaries receive a much greater vascular supply of phloem compared to the outer nectaries. This results in an unbalanced secretion of nectar, with 96 - 100 % of nectar being secreted at the inner nectaries (Davis, Pylatuik, Paradis, & Low, 1998).

Examples of the simplest sugars found in nectar are the monosaccharides: glucose and fructose; these and their combination product, sucrose, are the principal components of nectar (Calder, 1986). The ratio of these three sugars varies between species. Glucose and fructose are predominately found within oilseed rape nectar (Calder, 1986; Davis *et al*., 1994; Kołtowski, 2007), although small quantities of sucrose can be found in some varieties (Pierre, Mesquida, Marilleau, Pham-Delègue, & Renard, 1999). A characteristic of honey made from oilseed rape nectar is its rapid setting (crystallisation) within the hive, owed to its high glucose content (Calder, 1986) and it must therefore be extracted from the hive within weeks of it being collected (Kirk, 1992).

Oilseed rape is sown as a large monoculture, which provides a medium honey flow during a valuable developmental stage of the honeybee (Farkas & Zajácz, 2007). Beekeepers value the honey yields produced from oilseed nectar, a single hive may collect up to 27 kg of honey whilst foraging on oilseed rape (Kołtowski, 2007). Therefore, honeybees are encouraged to forage on the crop, which is achieved by relocating hives to as close to the crop as possible (Kirk, 1992).

5.2.4 Growth and development

The reproductive process of oilseed rape is out of the scope of this thesis and shall not be described; however it is useful to be aware of the various growth stages of a developing oilseed crop. That been said, there have been difficulties producing a growth stage key that is universally accepted (Almond, Dawkins, & Askew, 1986). For the purpose of this thesis, seven growth stages of the oilseed rape plant are considered (Almond *et al*., 1986; Eisikowitch, 1981); these can be separated under two headings: 1) vegetative and 2) reproductive stages (Figure 5.2).

Figure 5.2: Seven stages of oilseed rape growth and development. Information taken from Sylvester-Bradley and Makepeace, (1984) as cited in Almond *et al*., (1986).

The vegetative stages concern the growth and development of an oilseed plant from seed to the point prior to flowering; this is of no interest to pollinators as there is no floral rewards available i.e. nectar, and as such these stages will not be covered further.

The reproductive stages of the oilseed plant start at the point of flowering (anthesis) until the development of seeds. Anthesis consists of six stages (Table 5.1): from the time the bud opens (stage A) through to a continued flowering period (Stage F) (Eisikowitch, 1981). The bloom time of an individual flower is said to last for approximately five days, or

around 50 day-light hours (Cresswell, 1999), while the crop can remain in bloom for over a number of weeks (Cresswell, 2011).

Table 5.1: The relationship between the features of an oilseed flower during the various stages of anthesis. Data taken and adapted from Eisikowitch (1981).

Stage	Flower stage	Stigma reception	Pollen shed	Chance of spontaneous self-pollination	Inner nectaries	Outer nectaries
A	Bud opening	-				-
B	All petals (corolla) and anthers continue to open	- +	+		+	-
C	Anthers continue to open until the end of flower opening	+	++	-	+	-
D	Anthers directed towards stigma	+	+++	+	++	++
E	Anthers almost touching stigma	+	+++	++	+++	+++
F	Continued flowering until pod formation	+	+++	+++	++	+++

Oilseed rape is described as partially (70 %) self-fertile (autogamous) (Kirk, 1992; Mesquida, Marilleau, Pham-Delegue, & Renard, 1988). However, the abundant production of nectar and pollen, in addition to bright yellow petals, suggests that oilseed is also developed for insect pollination, as these features encourage insects to visit the flowers (Kirk, 1992; Mesquida *et al.*, 1988).

Nectar secretion is first observed at the inner nectaries (stage B) before the outer nectaries begin to secrete at stage D. The amount of nectar secreted by the oilseed flower varies according to a number of variables which are considered later on. Pollen is available for forage at stage B, until the end of stage F. Observation show that pollen is often collected by a greater number of the bees in the morning, possibly due to its

greater abundance at the beginning of the day (Free & Nuttall, 1968). The stigma is partially-receptive to pollen almost as soon as the flower opens, before becoming fully receptive at stage C (Eisikowitch, 1981). Self-pollination occurs when pollen from the outer stamen falls on to the stigma of the oilseed flower (Kirk, 1992) or direct contact is made between the anther and stigma (Eisikowitch, 1981). It is not likely that the flower will be self-pollinated until the anthers begin to direct themselves towards the fully-receptive stigma (Stages D – F). Self-pollination is unpredictable and only likely to happen during blustery conditions (Eisikowitch, 1981). A high amount of pollen is also carried within the air by the wind and can result in (wind) pollination (Williams, 1984). However, during greenhouse trials, wind alone was not able to dislodge pollen grains from anthers of any flower and it was only until the anther was brushed with a needle that clouds of pollen were released. A similar pollen-cloud phenomenon was observed to be created by foraging bumblebees, suggesting that pollen dislodged by insects is only truly able to be dispersed by wind (Eisikowitch, 1981).

At temperatures above 15 °C (Eisikowitch, 1981), honeybees are commonly found foraging on oilseed rape (Kirk, 1992) and are considered to be the main insect pollinator of the crop (Mesquida *et al*., 1988). Bumblebees are also key pollinators as they are more tolerant of poorer weather conditions than honeybees. Both honeybees and bumblebees will visit flowers throughout anthesis (stages B – F), where each visit leads to pollination (Eisikowitch, 1981). This can increase a farmer's profits through accelerated pollination, resulting in a uniform pod-ripening and thus a more efficient harvest (Kirk, 1992; Williams, Martin, & White, 1986). A shortened flowering period can also allow for the post-flowering chemical treatments to be conducted sooner (Williams *et al*., 1986). The

seed yields obtained through self-pollination are equal to and as high as wind-pollinated yields (Williams *et al*., 1986), although it is thought that insect-pollination is unlikely to increase these yields significantly (Free & Nuttall, 1968; Pierre *et al*., 1999).

5.2.4.1 External influences on nectar secretion

It has been discussed that the amount of nectar produced is influenced by the age of the flower (Table 5.1); however, the quality and quantity of nectar can be influenced by a number of factors (Alekseyeva & Bureyko, 2000; Kenoyer, 1916; Shuel, 1952). Those factors that are most applicable to this chapter are:

1) *Time of day*
2) *Weather conditions*
3) *Soil type*

1) Time of day

Time of day is an important factor in nectar secretion that closely relates to temperature. There is an accumulation of sugar in a plants tissues during cool periods (e.g. night time), followed by the secretion of nectar during warmer spells (e.g. day time). There is, however, a minimum temperature at which individual species of plant excrete nectar and this varies according to the individual species. It is thought the optimum temperature for most UK species is around 26.6 – 29.4 °C, although this value is lower for spring-flowering species (Butler, 1954). It was found that nectar secretion varied on a day to day, or even hour to hour basis (Butler, 1954; Shuel, 1952). Nectar production was at its highest during the morning, lowest at midday, before returning to a high level around mid-afternoon

(Meyerhoff, 1958). Generally, nectar production at the inner nectaries begins to decline with flower age (Eisikowitch, 1981).

2) Weather conditions

Nectar is described as being hygroscopic meaning that, depending on the atmospheric humidity, it can absorb moisture from the air and thus dilute the nectar (Butler, 1954; Edge *et al*., 2011; Farkas, Molnar, Morschhauser, & Hahn, 2012). Rainfall can also dilute the nectar of unprotected nectaries in an open flower. In both instances the attractiveness of nectar is reduced (Butler, 1954). Equally, rainfall can wash away nectar from the flower, reducing the amount of nectar available (Kenoyer, 1916). In drier and warmer conditions nectar can lose moisture to the atmosphere and become more concentrated (Butler, 1954; Jaric, Durdevic, Macukanovic-Jocic, & Gajic, 2010), particularly at the outer nectaries (Eisikowitch, 1981), even to the point of forming sugar crystals (Butler, 1954). Extended periods without water were shown to have a negative (decreasing) effect on nectar volume and concentration over time (Edge *et al*., 2012). Despite this, some plant species will continue to excrete nectar even when wilting (Butler, 1954). Importantly, the water content of nectar determines its overall viscosity and hence presents an upper limit at which bees can efficiently uptake the nectar (Nicolson 2011); the maximum limit was found to be around 60 % sugar (Roubik & Buchmann, 1984). Should there be a suitable level of rainfall before the flowing period, carbohydrate levels can often be exhausted during instances of vigorous plant growth; this can actually reduce the amount of sugars available for nectar secretion (McLachlan as cited in Butler (1954). However, during observations made over a 30 year period, it was concluded that the best seasons for honey production were wetter than average, proceeded by higher

than average levels of precipitation in the following months. These conditions were thought to stimulate plant growth, as well as providing enough surplus water needed for good nectar production (Kenoyer, 1917). The concentration of sugar secreted in nectar was found to be related to the quality illumination (i.e. direct sunlight), which influences plant metabolism (photosynthesis). Thus the amount of sugar available for secretion and the volume of nectar secreted influence the final concentration of nectar (Shuel, 1952).

3) Soil type

Nutrient-rich soil can result in greater plant growth, which correlates to an increase in secreted nectar volumes (Kenoyer, 1916). High levels of phosphorus were responsible for good flower production, whilst high potassium levels benefited the vegetative stages of plant development (Shuel, 1952). For very dry and water logged soils the sugar concentration of nectar in snapdragons (*Antirrhinum majus* L.) were high, although the volume of nectar reduced (Shuel & Shivas, 1953). Similarly, oilseed rape growth is known to be restricted by poor drainage (Berry *et al*., 2014). The same is true for compacted soils, as these can limit rooting capabilities of the crop, thus impacting nutrient and water uptake (Berry *et al*., 2014).

5.3 Bee visitations

From the prospective of a honeybee forager, the profitability of a nectar reward is assessed based on a number of factors including the rate of food retrieval, weather conditions, the return distance to and from the hive (Seeley, 1985), as well as sugar concentration and nectar volume (Butler, 1954; Shuel, 1952). A more dilute sample of nectar will require a greater expansion of energy to concentrate it down to produce

honey (Butler, 1954). It is thought that the sugar concentration of nectar needs to be above 20 % for there to be a net gain in energy (Butler, 1954). For this reason, honeybees will rarely collect nectar with a sugar content below 20 %, even if their colony is starving (Butler, 1954). Therefore, higher quality nectar will increase the number of visits by pollinators to a flower, which will in turn increase crop yield (Alekseyeva & Bureyko, 2000). Based on a number of environmental factors, the concentration and volume of nectar produced by a flower can vary; for example, the sugar content of raspberry nectar was found to be between 5.5 – 72 % (Butler, 1954), whilst sugar levels in oilseed rape is commonly between 30 – 40 %, but levels can be found as high as 60 % (Eisikowitch, 1981).

Nectar is considered to be the preferred floral reward collected from oilseed rape (Rosa *et al*., 2010), while only a small minority of foragers exclusively focus on pollen collection (Calder, 1986). Observations have recorded that nectar is preferably taken from the inner nectaries (Free & Nuttall, 1968; Kołtowski, 2007; Rosa *et al*., 2010), whereas the outer nectaries were infrequently visited (Eisikowitch, 1981). Observations made by Free and Nuttall (1968), during what is considered to be stages D to F, revealed that honeybee foragers spent an average of 4.1 seconds per flower visit. Those foragers collecting nectar would inadvertently become dusted with pollen; in most cases this pollen was also collected. Pollen collection was found to be most frequent during the morning and decreased as the day progressed. In contrast, this trend was reversed for nectar foragers, whose frequency increased as the day advanced. Towards the end of the flowering period nectar collection declined as pollen collection increase suggesting a limited availability of nectar as the flowering period draws to an end (Free & Nuttall, 1968). It is worth noting

that unfavourable weather conditions can also deter honeybees from foraging (Winter *et al*., 2006).

5.4 Pest and disease control

The threats to oilseed rape include weeds, various diseases, including: phoma leaf spot (*Leptosphaeria maculans*), clubroot (*Plasmodiophora brassicae*) and Sclerotinia stem rot (*Sclerotinia sclerotiorum*), as well as numerous pests, including: slugs (*Cylindrobulla* spp.), cabbage stem flea beetle (*Psylliodes chrysocephala*), seed weevil (*Ceutorhynchus* spp.), brassica pod midge (*Dasineura brassicae*) and pollen beetle (*Meligethes* spp.) (Berry *et al*., 2014; Kirk, 1992). The details of these are briefly discussed elsewhere (Berry *et al*., 2014; HGCA, 2003; Kirk, 1992), although some the treatments of these pests and diseases will be covered below.

5.4.1 Natural pest control

Glucosinolates provide a natural defence against non-brassica insects (insects that have not become specialised to cope with chemical defences of brassica plants) (Kirk, 1992). Glucosinolates are sulphur-containing compounds that are characteristic of the cruciferae family (Kirk, 1992) and were first observed at the beginning of the seventeenth century, during an effort to understand the origin of the sharp taste associated with mustard seeds (Fahey, Zalcmann, & Talalay, 2001). There are over 120 different glucosinolate (*S*-β-thioglucoside *N*-hydroxysulfate) compounds that have been described; the majority of which have been isolated from cruciferous plants (Dinkova-Kostova & Kostov, 2012). Glucosinolates are stored within tissues of the plant along with enzymes known as

myrosinases (β-thioglucosidase). These enzymes are kept physically separated and only come into contact if the tissue of the plant is damaged typically through chewing or cutting (Dinkova-Kostova & Kostov, 2012; Kirk, 1992). Should the enzyme and glucosinolate substrate come into contact, unstable intermediates (aglucones) and glucose are formed through rapid hydrolysis. The aglucone intermediates, depending on the original glucosinolate structure, rearrange to create a variety of biologically reactive compounds (Figure 5.3) - predominantly isothiocyanates (Dinkova-Kostova & Kostov, 2012). These toxic products are used in the defence against insects and slugs, whilst also preventing the growth of bacterial and fungal diseases (Kirk, 1992).

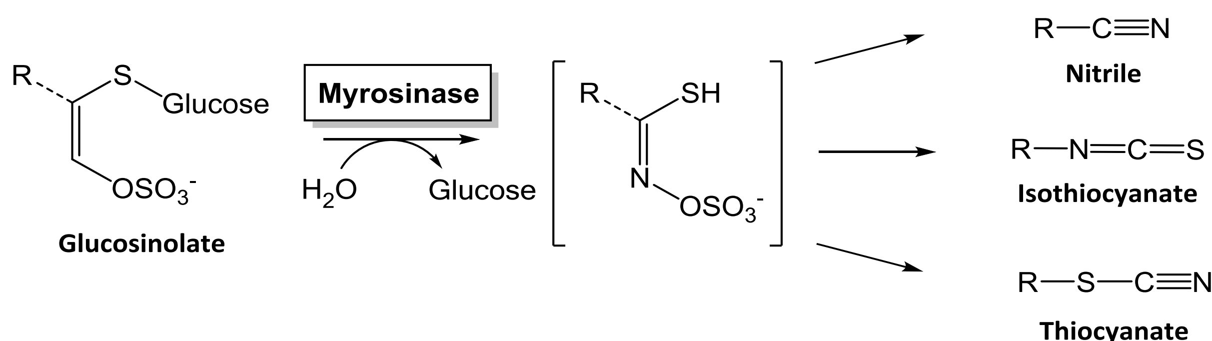

Figure 5.3: The possible reaction products of glucosinolate hydrolysis, via an unstable intermediate (aglucone). Hydrolysis occurs following cellular disruption, whereby glucosinolate is catalysed by the enzyme myrosinase. A number of products can be formed, depending on original glucosinolate side chain (R). Adapted from Dinkova-Kostova & Kostov, (2012).

There was concern that the introduction of single- and double-low varieties would leave them more vulnerable to attack from pests and disease. However, there were no clear differences of pest infestation level on either variety (Mithen, 1992; Williams, Doughty, Bock, & Rawlinson, 1991), although the seedlings of double-low varieties were found to be more susceptible to attack (Glen, Jones, & Fieldsend, 1990).

5.4.2 Synthetic pest control

Glucosinolates alone cannot fully control pests and disease, since resistance to glucosinolates is known (Hopkins, Dam, & Loon, 2009). Oilseed rape has been shown to withstand and compensate for damage (Pinet, Mathieu, & Jullien, 2015). However, the use of synthetic agrochemicals is adopted in order to maximise crop yields. Table 5.2 shows the number of treatments used on an oilseed rape crop in the UK (August 2012 – June 2013). Thirteen separate pesticide applications were made, in addition to five non-pesticide applications.

Table 5.2: An example of the agrochemical treatments used on oilseed rape during a flowering season (2012 - 2013). A total of 13 agrochemical applications were made, these include: 1 molluscicide, 3 herbicide, 4 insecticide and 5 fungicide treatments. The seed treatment, thiamethoxam, is highlighted in bold. Other application made to the crop is done so in order to avoid nutrient deficiencies. Table supplied from a farmer involved with this project.

Date	Treatment	Active Ingredient(s)	Class
Aug-12	Cruiser®	**Thiamethoxam** + Fludioxonil + Metataxyl	Ins
"	Matrix®	Phosphite + Natural bioactive complex	Nut
Sep-12	Novall®	Metazachlor + Quinmerac	Herb
"	Tempt	Metaldehyde	Mollus
Oct-12	Falcon®	Propaquizafop	Herb
"	Mac Cypermethrin	Cypermethrin	Ins
"	Harvesan	Carbendazim + Flusilazole	Fung
"	Boron	Boron	TE
Dec- 12	Pizza 400	Propyzamide	Herb
Apr-13	Harvesan	Carbendazim + Flusilazole	Fung
"	Boron	Boron	TE
Jun-13	Proline®	Prothioconazole	Fung
"	Euro Lambda 100CS®	Lambda-cyhalothrin	Ins
"	Bittersalz	Magnesium	TE
"	Proline®	Prothioconazole	Fung
"	Euro Lambda 100CS®	Lambda-cyhalothrin	Ins
"	Bittersalz	Magnesium	TE
"	Delsene®	Carbendazim	Fung

Ins – insecticide; Herb – herbicide; Fung – fungicide; Mollus – molluscicide; TE – trace element; Nut – nutrient.

5.5 The suggested routes of neonicotinoid exposure

It must not be forgotten that neonicotinoids are, of course, insecticides, which are designed to eradicate unwanted pests from various crop species. The improper use of any agrochemical can result in the death of beneficial pollinators (Everts, 2008). Indeed, pollinator deaths can occur even when farmers carefully time their pesticide applications in order to minimise the chance of pesticide exposure. Seed treatments are said to offer a relatively safe alternative compared to other methods of application (Jeschke & Nauen, 2008; Wollweber & Tieyen, 1999); despite this, bees are still being exposed to neonicotinoids during foraging. The systemic nature of neonicotinoids means that the active ingredient is transported to all parts of the plant, which includes nectar and pollen (Cutler & Scott-Dupree, 2007). As previously shown in Figure 1.24, p63), seed-treatment forms a 'disinfectant halo', as the active ingredient begins to leach into the surrounding soil; this is later absorbed by the roots. Some of the active ingredient can remain behind in the soil (Ainsley, Harrington, Turnbull, & Jones, 2014), for in excess of 1,000 days (Goulson, 2013); this can provide protection to future crops (Berry *et al*., 2014), in a practice known as no-till (HGCA, 2012). The progressive accumulation of neonicotinoids in soil can lead to wash towards field edges, contaminating various plant species, thus providing an addition route of exposure, all year round (Goulson, 2013).

Oilseed has a good flow of nectar and it is calculated to produce around 100 kg of sugar per hectare (Kołtowski, 2007). Consequently, beekeepers often 'take bees to the rape' in order to boost honey supplies (personal communication with beekeepers). It has also been explained that honeybees can travel, on average, up to 4.5 km during foraging (Seeley, 1985), which may also include oilseed rape should it be within this radius. The

return of contaminated pollen and nectar to the hive means that not only are foraging bees directly exposed during collection, but so are the rest of the colony via the migration of pesticides to various hive products, resulting in the increased mortality of honeybee brood (Wu, Anelli, & Sheppard, 2011).

5.6 Aim of investigation

The aim of this chapter is to determine the level of neonicotinoid pesticides in the nectar and pollen of oilseed rape flowers.

5.7 Materials and Methods

5.7.1 Sample collection

Between May and July 2013 and April and May 2014, nectar and pollen samples were collected from oilseed rape fields across various locations in England (Lincolnshire, Oxfordshire, Staffordshire and Worcestershire (2013 only) and sites in Denbighshire (North Wales). The location of these counties are presented in Figure 5.4.

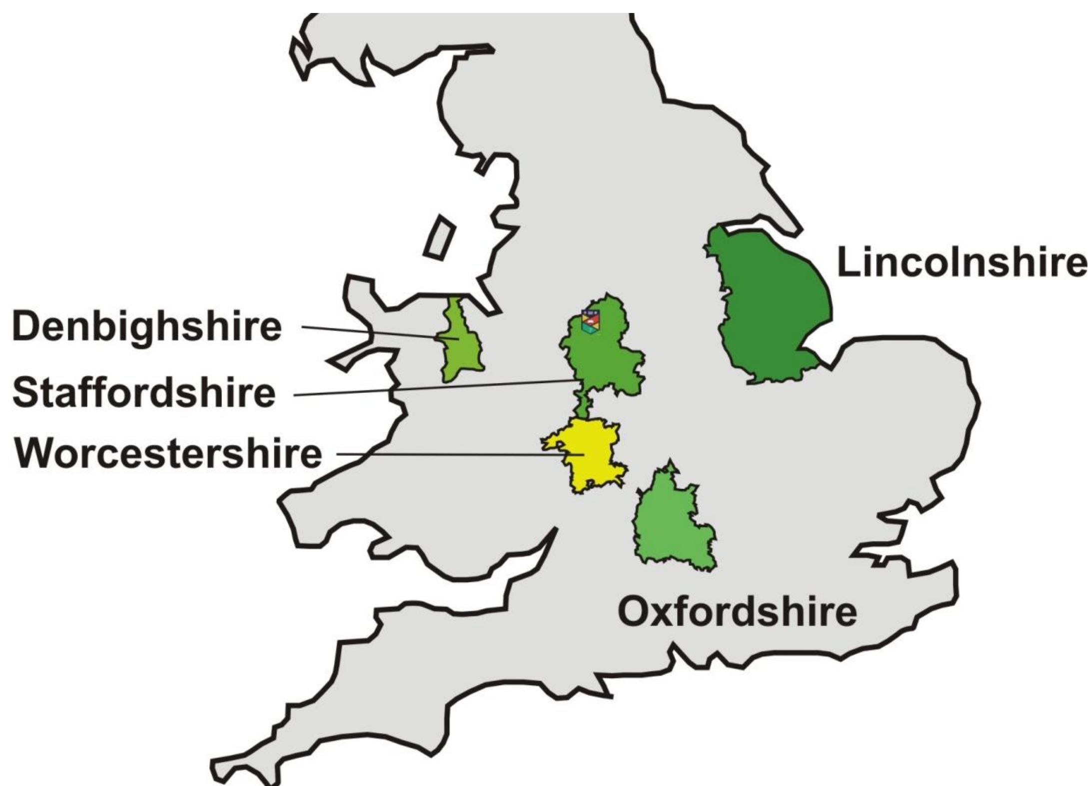

Figure 5.4: The counties from which oilseed nectar and pollen were sampled during 2013 and 2014. Worcester was only sampled during 2013. Field locations have been omitted in order to maintain farmer anonymity.

Nectar was extracted, by hand, using 10, 25, 50 or 100 µl glass micro-capillaries (Blaubrand® intraMARK, Germnay) (Figure 5.5) - depending on the viscosity of the nectar. Each nectar sample had a corresponding collection sheet (Appendix I), which included data such as flower height and the percentage level of sugar within nectar; the latter was determined using a sugar refractometer (0 - 50 % & 40 – 85 %) (Bellingham & Stanley Ltd, Tunbridge Wells, England). Nectar was then stored in inserts contained within GC vials at 4 ºC until analysis. Pollen traps (E.H. Thorne (Beehives) Ltd, Market Rasen, England) were also fitted to hives (Figure 5.6) at locations corresponding to where nectar was collected. In most cases, hives were located on the oilseed rape fields being sampled or within very

close proximity. Confirmation of pollen species was confirmed using a pollen load colour chart (Kirk, 2006). Pollen was stored at -40 °C until analysis.

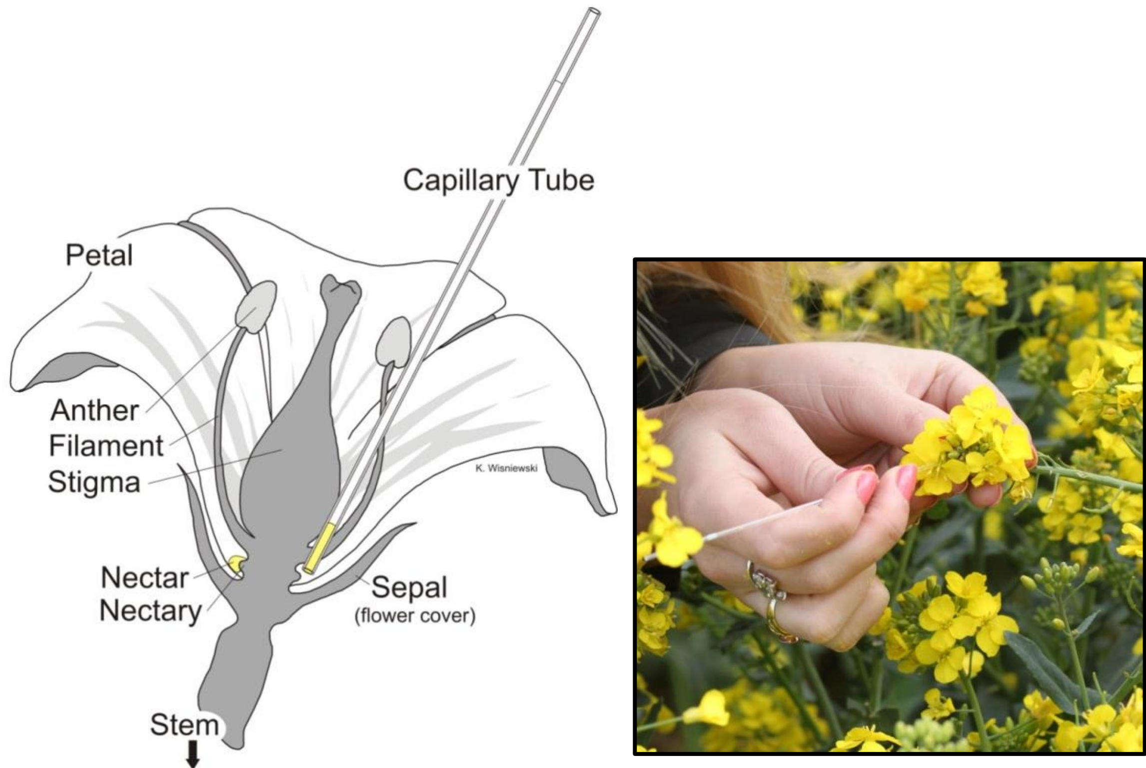

Figure 5.5: ***(left)*** **A schematic diagram of nectar being collected from the nectary of an oilseed rape flower using a micro-capillary tube. Image adapted from (Clipart Pal, 2014).** ***(right)*** **A photograph demonstrating the nectar collecting procedure; the image shows the angle at which the micro capillary was held in order to help the nectar to be drawn up the tube via capillary action. Photograph by K.D. Wisniewski (2014).** (Clipart Pal, 2014)

Figure 5.6: *(left)* A pollen trap fitted to a hive located opposite an oilseed rape field in North Staffordshire. *(right)* A typical example of oilseed rape pollen loads (yellow), along with other (unidentified) pollen loads, collected using a pollen trap. Photographs by K.D. Wisniewski (2013).

In total, 50 nectar and 9 pollen samples (including one mixed pollen sample) were collected in the first season (2013); while 48 nectar and 6 pollen samples were collected during the second season (2014).

Reagents and standards

All solvents and chemicals used in the study were of HPLC and analytical grade. Clothianidin PESTANAL®, Imidacloprid PESTANAL®, Imidacloprid-d_4 PESTANAL®, Thiamethoxam PESTANAL®, Tri(2,3-dichloropropyl)phosphate OEKANAL® (TDCPP) and Ammonium formate (NH4HCO2) ≥99 % were purchased from Fluka Analytical (Germany). Both acetonitrile and glacial acetic acid were from Fisher Scientific (Loughborough, England). Deionised water was purified at 18.2 MΩ with a Purelab Option-Q DV25 purification system. QuEChERS kits 60105-205 and 60105-210 were obtained from Thermo Scientific (Hemel Hempstead, England).

5.7.2 Preparation of standard stock solutions

Standards of thiamethoxam, clothianidin, imidacloprid, imidacloprid-d_4 and TDCPP were prepared at 1000 ppmv in water. Standards were stored in amber volumetric flasks at 4 °C. Working pesticide standard solutions were made at 1000 ppbv in water, with the exception of TDCPP which was at 300 ppbv in water.

5.7.3 Calibration and recoveries of standards

Working solutions of clothianidin, thiamethoxam and imidacloprid (each at 1000 ppbv) were appropriately diluted in water/acetonitrile mix (95:5) to create a series dilution from 40, 20, 10, 5, 2.5, 0.125 and 0.625 ppbv. Each dilution was fortified with TDCPP and imidacloprid-d_4, both to a final concentration of 30 ppbv; this concentration was kept constant throughout the analysis of both pollen and nectar samples. The calibration graphs were found to be linear with correlation coefficients (R^2) greater than 0.99 for each compound (Appendix J). Both the LOD and LOQ values were estimated from calibration levels, corresponding to a signal-to-noise ratio of about 3 and 10, respectively; in addition to the presence of the $[M+H]^+$ ion (within a mass accuracy of 5 ppmv) for each compound. The LOD/LOQ of each neonicotinoid are as follows: clothianidin, 1/3 ppbv; thiamethoxam, 0.625/1.25 ppbv and imidacloprid, 0.625/2.5 ppbv. Honey was diluted to give a sugar content of around 50 %; this was then spiked with a neonicotinoid. Five replicates were then extracted and the percentage recovery determined, along with the percentage relative standard deviation (% RSD). The mean recoveries are as follows: thiamethoxam, 78.02 % ± 14.03 %; clothianidin, 88.07 % ± 2.05 % and imidacloprid, 102.36 % ± 10.70 % (see Appendix K for raw data).

5.7.4 Sample extraction

5.7.4.1 Nectar

A modified QuEChERS method was used (Anastassiades, Lehotay, Stajnbaher, & Schenck, 2003) that was adapted in order to accommodate smaller quantities. 100 µl of nectar was measured into 2 ml Eppendorf Safe-Lock® tube (Eppendorf AG, Hamburg, Germany), after adding 900 µl extraction solution (44 % distilled water, 55 % acetonitrile and 1 % glacial acetic acid) each sample was fortified with 3 µl of the internal standard (imidacloprid-d_4 @ 1000 ppbv). To each sample is then added 150 mg of anhydrous magnesium sulphate ($MgSO_4$) and anhydrous sodium acetate (NaOAc) 4:1 (w/w) which had been weighed from QuEChERS kit 60105-210 (Thermo Scientific, Hemel Hempstead, England). Eppendorf tubes were shaken for 1 minute, centrifuged (2.5 minute @ 4000 rpm) and supernatant **A** transferred to a 2 mL Eppendorf tube containing 25 mg of primary secondary amine (PSA), graphitised carbon black (CUCARB) and $MgSO_4$, 6:2:1 (w/w/w), which had been weighed from QuEChERS kit 60105-205 (Thermo Scientific, Hemel Hempstead, England). After centrifuging (2.5 minute @ 4000 rpm), the resulting supernatant is dried under a stream of nitrogen gas and then reconstituted to a final volume of 100 µl, fortified with TDCPP at a final concentration of 300 ppbv. This is then ready for Q-TOF LC/MS analysis.

5.7.4.2 Pollen

Pollen (3 g) was added to a 50 ml centrifuge tube and homogenised in an ultra-sonic bath for 2 minutes. The same QuEChERS extraction procedure used in Chapters 3 and 4 (Q-TOF LC/MS clean-up procedure) was then applied to the pollen samples.

5.7.5 Instrumentation

Samples were injected into an Agilent 1260 Infinity LC system (injection volume: 15 µl) equipped with an autosampler, thermostatted column compartment (set to 35 °C) and 1290 Infinity in-line filter (0.3 µm); with an Agilent ZORBAX Extended-C18 Rapid Resolution HD (2.1 x 50 mm, 1.8 µm) column, with ZORBAX Eclipse Plus C18 (2.1 x 5 mm, 1.8 µm) guard column connected to an Agilent 6530 Accurate-Mass-Q-TOF LC/MS. The LC mobile phases were (A) water with 5 mmol ammonium formate and (B) acetonitrile. The elution gradient, at a flow rate of 0.6 ml/min[2], was as follows: 0 – 0.5 min (95 % A/ 5 % B), 0.5 – 9 min (0 % A/ 100 % B), 9 – 9.5 min (0 % A/ 100 % B), 9.5 – 10 min (95 % A/ 5 % B). The Q-TOF settings were as follows: acquisition mode MS; with MS range 100 – 1000 *m/z*; MS scan rate 1 spectrum/s; electrospray ionization (ESI) source – gas temperature: 300 °C; gas flow: 11 L/min; nebulizer: 50 psig, positive ion polarity; scan source parameters: Vcap 4000 V; fragmentor, 125 V; skimmer, 65 V; OCT RF Vpp, 750 V.

5.7.6 Meteorological data

Temperature and humidity information was collected using iButton temperature and iButton humidity data loggers (Maxim Integrated, USA). The loggers were placed on each of the sampled oilseed rape fields away from direct sunlight and set to take readings at 30 min intervals. The weather conditions at the time of collection were also noted. Alternative weather data were obtained from the University of Oxford and Keele University.

[2] Nectar samples 78 – 98 were analysed at a column temperature of 60 °C and a flow rate of 0.535 ml/min in order to reduce column back-pressure.

5.8 Results

5.8.1 Season 1

The raw data for collected nectar samples, which shows the breakdown of results for individual locations, are provided in Table 5.3 and are summarised in Table 5.4. Of the 50 samples analysed, thiamethoxam was detected the least, both quantifiably and less than the LOQ, whilst clothianidin was detected the most. The highest concentration detected was for imidacloprid at 474.15 ppbv, whilst the lowest quantifiable was thiamethoxam at 4 ppbv. An attempt to calculate the standard deviation and mean from quantifiable results has been conducted and is shown in the table.

Table 5.3: Raw data for 50 oilseed rape nectar samples obtained during the first season of collection (2013). Samples were extracted using a micro-scale QuEChERS technique and analysed using Q-TOF LC/MS. Concentrations were determined using matrix-matched calibration curves, given in Appendix J, with results adjusted according to each detected compounds respective recoveries (Appendix K) during extraction.

County	Sample date	Time of day	Sample name	Imidacloprid			Clothianidin			Thiamethoxam			% Sugar (mean +/- SD)	Vol (µl)	Mean height (cm)
				RT (min)	Detected conc (ppbv)	Assumed conc (ppbv)	RT (min)	Detected conc (ppbv)	Assumed conc (ppbv)	RT (min)	Detected conc (ppbv)	Assumed conc (ppbv)			
Oxfordshire 1	12/06/2013	am	N33	3.673	<LOQ	-	3.491	11.8952	13.50653	3.076	<LOD	-	56 ±0	0.86 ±0.26	100.4 ±4.90
Oxfordshire 2	12/06/2013	am	N35	ND	ND	-	3.499	<LOQ	-	3.084	<LOQ	-	51.2 ±4.44	1.25 ±0.45	97.6 ±5.19
	12/06/2013	pm	N34	ND	ND	-	3.501	<LOQ	-	3.103	<LOD	-	47.4 ±1.95	N/A	97.6 ±5.19
	26/06/2013	pm	N62	3.683	50.0458	50.0458	3.517	5.4061	6.138413	3.136	3.1417	4.026792	N/A	N/A	118.5 ±3.24
Oxfordshire 3	26/06/2013	pm	N61	ND	ND	-	ND	ND	-	3.100	<LOD	-	N/A	N/A	67.7 ±5.03
Staffordshire 1	22/05/2013	am	N73	ND	ND	-	3.5	<LOD	-	ND	ND	-	23.8 ±5.24	0.75 ±0.26	113 ±3.07
	22/05/2013	pm	N40	ND	ND	-	ND	ND	-	ND	ND	-	N/A	N/A	113 ±3.07
	30/05/2013	am	N51	ND	ND	-	ND	ND	-	ND	ND	-	10.6 ±1.34	0.64 ±0.25	132.4 ±6.38
	30/05/2013	pm	N76	ND	ND	-	ND	ND	-	ND	ND	-	25.4 ±10.69	N/A	132.4 ±6.38
	06/06/2013	am	N39	3.688	<LOQ	-	3.522	<LOQ	-	ND	ND	-	46 ±3.67	N/A	140.3 ±6.04
	06/06/2013	pm	N71	ND	ND	-	3.516	<LOQ	-	3.118	<LOD	-	73 ±1.58	N/A	140.3 ±6.04
	20/06/2013	am	N70	3.672	<LOQ	-	3.489	<LOQ	-	ND	ND	-	6.2 ±2.49	N/A	N/A
	21/06/2013	pm	N72	3.681	<LOQ	-	ND	ND	-	ND	ND	-	N/A	N/A	N/A
Staffordshire 2	22/05/2013	pm	N49	ND	ND	-	3.492	<LOQ	-	ND	ND	-	61.2 ±6.34	0.51 ±0.24	100.7 ±6.82
	30/05/13 (am)	am	N48	ND	ND	-	3.485	<LOQ	-	ND	ND	-	17.4 ±5.55	1.1 ±0.82	129.4 ±11.34
	30/05/2013	pm	N78	ND	ND	-	ND	ND	-	ND	ND	-	27 ±15.37	N/A	129.4 ±11.34
	06/06/2013	am	N47	3.689	<LOQ	-	3.506	<LOQ	-	ND	ND	-	50.2 ±3.19	N/A	140.4 ±9.18
	06/06/2013	pm	N46	3.687	<LOQ	-	3.487	<LOQ	-	ND	ND	-	69 ±1.41	N/A	140.4 ±9.18
	14/06/2013	am	N45	3.688	<LOQ	-	3.506	<LOQ	-	ND	ND	-	26.4±4.16	0.29 ±0.12	159.4 ±8.19
	20/06/2013	am	N77	ND	ND	-	3.551	35.8976	40.7603	ND	ND	-	N/A	N/A	N/A
Staffordshire 3	22/05/2013	am	N44	ND	ND	-	3.501	<LOQ	-	ND	ND	-	N/A	0.69 ±0.22	118.6 ±14.04
	22/05/2013	pm	N79	ND	ND	-	3.496	<LOQ	-	ND	ND	-	33.2 ±6.87	N/A	118.6 ±14.04
	30/05/2013	am	N43	ND	ND	-	ND	ND	-	ND	ND	-	19.8 ±3.49	0.89 ±0.19	141.3 ±10.06
	30/05/2013	pm	N75	3.687	<LOQ	-	ND	ND	-	ND	ND	-	31 ±4.53	N/A	141.3 ±10.06
	06/06/2013	am	N41	3.689	<LOQ	-	ND	ND	-	ND	ND	-	54.4 ±4.56	N/A	147.6 ±8.75
	06/06/2013	pm	N42	3.688	<LOQ	-	3.522	3.4728	3.943227	ND	ND	-	64.6 ±1.67	N/A	147.6 ±8.75
	14/06/2013	am	N74	3.598	<LOQ	-	3.499	<LOD	-	ND	ND	-	38.6 ±2.19	0.24 ±0.09	156.7 ±6.17
	20/06/2013	am	N55	3.676	258.1211	258.1211	3.51	33.1756	37.66958	3.095	13.1183	16.81404	4.2 ±1.48	N/A	N/A

Lincolnshire 1	23/05/2013 (am)	am	N52	3.688	< LOQ	-	3.506	< LOQ	-	ND	ND	-	3.57 ±2.64	1.76 ±1.72	96.1 ±9.86
	23/05/2013 (pm)	pm	N57	3.669	135.3322	135.3322	3.503	28.779	32.67742	3.104	13.5769	17.40184	3.2 ±6.61	N/A	96.1 ±9.86
	29/05/2013 (am)	am	N50	3.651	4.9304	4.9304	3.485	< LOQ	-	ND	ND	-	12.2 ±6.53	0.19 ±0.10	98.3 ±5.20
	13/06/2013 (am)	am	N56	3.684	474.1465	474.1465	3.502	35.2461	40.02055	3.103	95.135	121.9368	37.2 ±11.19	0.4 ±0.15	125.2 ±4.08
	13/06/2013 (pm)	pm	N67	ND	ND	-	3.488	< LOD	-	3.107	< LOQ	-	50 ±0	N/A	125.2 ±4.08
Lincolnshire 2	29/05/2013 (am 1	am	N31	3.667	< LOQ	-	3.501	15.5533	17.66016	ND	ND	-	4.4 ±2.40	0.19 ±0.67	73 ±8.19
	29/05/2013 (am 2	am	N30	3.686	< LOQ	-	ND	ND	-	ND	ND	-	4.4 ±2.40	0.19 ±0.67	73 ±8.19
	29/05/2013 (am 3	am	N60	3.693	39.1999	39.1999	3.511	11.9852	13.60872	3.113	4.3003	5.511797	4.4 ±2.40	0.19 ±0.67	73 ±8.19
	13/06/2013 (pm)	pm	N65	3.664	230.1747	230.1747	3.482	22.9004	26.0025	3.100	67.9203	87.05508	57.6 ±2.30	0.19 ±0.10	95.4 ±4.55
Lincolnshire 3	23/05/2013 (am)	am	N54	ND	ND	-	3.51	< LOQ	-	ND	ND	-	14.2 ±12.89	0.4 ±0.90	102.5 ±7.91
	23/05/2013 (pm)	pm	N66	3.676	289.8764	289.8764	3.51	31.2345	35.46554	3.111	19.7447	25.30725	24.6 ±15.40	N/A	102.5 ±7.91
Worcestershire	11/06/2013 (am)	am	N53	ND	ND	-	ND	ND	-	ND	ND	-	24.8 ±5.02	2.7 ±0.75	100.6 ±5.36
	11/06/2013 (pm 1	pm	N38	ND	ND	-	3.517	< LOQ	-	ND	ND	-	23.8 ±2.17	N/A	100.6 ±5.36
	11/06/2013 (pm 2	pm	N37	ND	ND	-	3.527	< LOQ	-	ND	ND	-	23.8 ±2.37	N/A	100.6 ±5.36
	21/06/2013 (am)	am	N68	3.683	< LOQ	-	3.501	< LOQ	-	ND	ND	-	16.8 ±1.10	N/A	120.7 ±3.33
	21/06/2013 (pm)	pm	N69	ND	ND	-	ND	ND	-	ND	ND	-	32.4 ±11.28	N/A	120.7 ±3.33
Denbinghshire 1	31/05/2013 (am)	am	N36	3.69	< LOQ	-	ND	ND	-	ND	ND	-	N/A	N/A	N/A
	31/05/2013 (pm)	pm	N32	3.664	< LOQ	-	3.498	13.4058	15.22176	ND	ND	-	N/A	N/A	N/A
	07/06/2013 (am)	am	N58	3.672	12.8492	12.8492	3.506	< LOQ	-	ND	ND	-	N/A	N/A	N/A
	07/06/2013 (pm)	pm	N64	3.663	125.6584	125.6584	3.48	13.6815	15.5348	3.098	11.1328	14.26918	N/A	N/A	N/A
Denbinghshire 2	07/06/13 (am)	am	N63	3.675	106.3917	106.3917	3.493	13.8855	15.76644	3.127	12.5739	16.11627	N/A	N/A	N/A
	07/06/13 (pm)	pm	N59	ND	ND	-	3.516	< LOQ	ND	ND	ND	-	N/A	N/A	N/A

Table 5.4: A summary of the neonicotinoid residues detected in nectar samples (n = 50) from various locations around the UK in 2013, as presented in Table 5.3. Mean value calculated assuming LOD values for each respective neonicotinoid where <LOD and 0 ppbv where ND.

		Number of samples (n = 50)			Residue concentration (ppbv)			
Neonicotinoid	**ND**	**< LOD**	**Detections (< LOQ)**	**Quantified**	**Low**	**High**	**Mean**	**S.D**
Imidacloprid	22	-	17	11	4.93	474.15	35.38	92.10
Clothianidin	13	3	20	14	3.095	40.021	7.77	11.68
Thiamethoxam	35	4	2	9	4.03	87.06	6.27	21.30

The EIC of the sample found to contain the highest concentration of imidacloprid (474.15 ppbv) has been provided in Figure 5.7 and is represented as the green trace. This has been overlaid with the EIC of imidacloprid-d_4, shown by the black trace, which was added at 30 ppbv as an internal standard for imidacloprid.

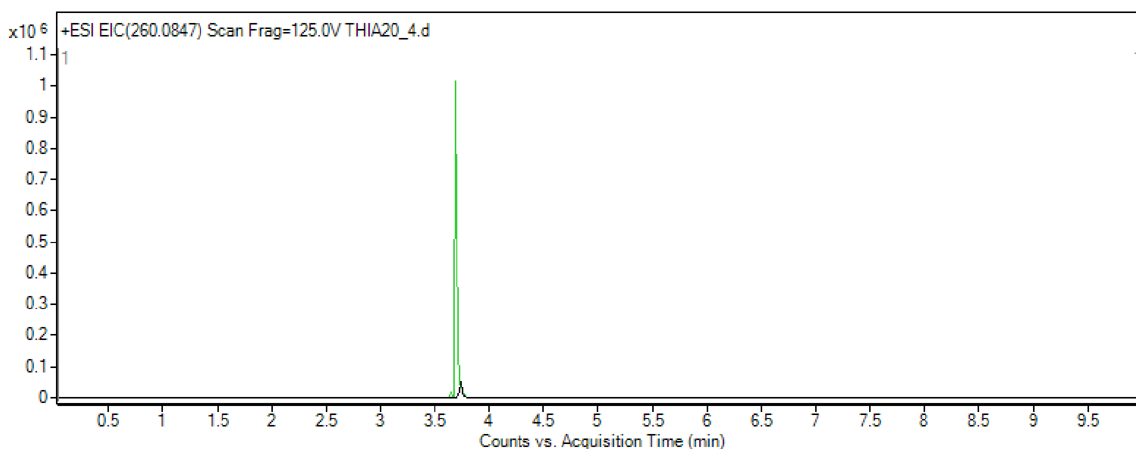

Figure 5.7: Overlaid EICs of imidacloprid detected at 474.15 ppbv in nectar sample N56 (green trace), compared to the 30 ppbv ISTD, imidacloprid-d_4 (black trace).

Table 5.5 shows the raw data for 9 pollen samples collected during the 2013 season. This information is summarised in Table 5.6, where it can be seen that no quantifiable amounts of neonicotinoids were detected in the nine pollen samples analysed. However, thiamethoxam was found to be below the limit of detection on two occasions.

Table 5.5: Raw data for 9 pollen samples obtained during the first season of collection (2013). Samples were extracted using QuEChERS and analysed using Q-TOF LC/MS. Sample P9 was regarded as a mixture of a number of pollens.

			Clothianidin		Thiamethoxam		Imidacloprid	
County	**Collection date**	**Sample I.D.**	**RT**	**Cal conc (ppbv)**	**RT**	**Cal conc (ppbv)**	**RT**	**Cal conc (ppbv)**
Staffordshire 2	03/06/2013	P1	ND	-	ND	-	ND	-
Staffordshire 3	07/06/2013	P2	ND	-	ND	-	ND	-
	17/06/2013	P3	ND	-	ND	-	ND	-
Lincolnshire 2	25/05/2013	P4	ND	-	3.235	< LOD	ND	-
	13/06/2013	P5	ND	-	ND	-	ND	-
Oxfordshire 3	26/06/2013	P6	ND	-	3.26	< LOD	ND	-
Denbinghshire 1	13/06/2013	P7	ND	-	ND	-	ND	-
Denbinghshire 2	10/06/2013	P8	ND	-	ND	-	ND	-
	10/06/2013	P9 (MIX)	ND	-	ND	-	ND	-

Table 5.6: A summary of the neonicotinoid residues detected in pollen samples (n = 9) from various locations around the UK in 2013. Mean value calculated assuming LOD values for each respective neonicotinoid, where <LOD and 0 ppbv where ND.

	Number of samples (n = 9)				Residue concentration (ppbv)			
Neonicotinoid	**ND**	**< LOD**	**Detections (< LOQ)**	**Quantified**	**Low**	**High**	**Mean**	**S.D**
Imidacloprid	9	-	-	-	-	-	-	-
Clothianidin	9	-	-	-	-	-	-	-
Thiamethoxam	9	2	-	-	0.625	0.625	0.139	0.276

5.8.2 Season 2

Table 5.7 shows the raw data for the 48 nectar samples collected during the 2014 season. A total of six quantifiable detections were made for all neonicotinoids, as summarised in Table 5.8.

Table 5.7: Raw data for 48 oilseed rape nectar samples obtained during the second season of collection (2014). Samples were extracted using a micro-scale QuEChERS technique and analysed using Q-TOF LC/MS. Concentrations were determined using matrix-matched calibration curves, given in Appendix J, with results adjusted according to each detected compounds respective recoveries (Appendix K) during extraction.

				Imidacloprid			Clothianidin			Thiamethoxam					
County	Sample date	Time of day	Sample name	RT (min)	Detected conc (ppbv)	Assumed conc (ppbv)	RT (min)	Detected conc (ppbv)	Assumed conc (ppbv)	RT (min)	Detected conc (ppbv)	Assumed conc (ppbv)	% Sugar (mean +/- SD)	Vol (µl)	Mean height (cm)
Oxfords hire 1	02/05/2014 (1)	pm	N5	3.686	7.1385	7.1385	ND	ND	-	3.072	3.2556	4.172780	17.4 ± 1.95	N/A	126 ± 8.39
	02/05/2014 (2)	pm	N21	ND	ND	-	ND	ND	-	ND	ND	-	13.75 ± 4.5	N/A	126 ± 8.39
	03/05/2014	pm	N7	ND	ND	-	ND	ND	-	ND	ND	-	58.6 ± 4.04	N/A	N/A
	16/05/2014 (1)	am	N2	ND	ND	-	ND	ND	-	ND	ND	-	49 ± 2	N/A	N/A
	16/05/2014 (2)	am	N29	ND	ND	-	ND	ND	-	ND	ND	-	49 ± 2	N/A	N/A
	21/05/2014	pm	N4	ND	ND	-	ND	ND	-	ND	ND	-	40.67 ± 3.00	N/A	N/A
Oxfords hire 4	02-03/05/2014	pm	N6	ND	ND	-	ND	ND	-	ND	ND	-	46 ± 3.46	0.2 ± 0.09	157.6 ±
	16/05/14 (1)	pm	N1	ND	ND	-	ND	ND	-	ND	ND	-	63.8 ± 2.28	0.35 ± 0.27	140.7 ± 6.80
	16/05/2014 (2)	pm	N20	ND	ND	-	ND	ND	-	ND	ND	-	63.8 ± 2.28	0.35 ± 0.27	140.7 ± 6.80
	21/05/2014	am	N3	ND	ND	-	ND	ND	-	ND	ND	-	N/A	N/A	N/A
Staffordshire 1	23/04/2014	am	N24	ND	ND	-	ND	ND	-	ND	ND	-	42.6 ± 3.65	0.48 ± 0.19	133.6 ± 5.87
	28/04/2014	am	N8	ND	ND	-	ND	ND	-	ND	ND	-	38.4 ± 10.41	0.68 ± 0.24	113.9 ± 8.28
	30/04/2014	pm	N98	ND	ND	-	ND	ND	-	ND	ND	-	57.8 ± 1.64	N/A	121.5 ± 4.81
	15/05/2014	pm	N11	ND	ND	-	ND	ND	-	ND	ND	-	N/A	1.11 ± 0.24	125.5 ± 2.46
	18/05/2014	am	N9	ND	ND	-	ND	ND	-	ND	ND	-	N/A	N/A	N/A
	19/05/2014	am	N10	ND	ND	-	ND	ND	-	ND	ND	-	N/A	N/A	N/A
	20/05/2014	pm	N96	ND	ND	-	3.193	6.0673	6.8891791	ND	ND	-	N/A	N/A	N/A
Staffordshire 2	24/04/2014	pm	N12	ND	ND	-	ND	ND	-	ND	ND	-	N/A	N/A	N/A
	29/04/2014 (1)	am	N13	ND	ND	-	ND	ND	-	ND	ND	-	N/A	N/A	N/A
	29/04/2014 (2)	pm	N97	ND	ND	-	ND	ND	-	ND	ND	-	N/A	N/A	N/A

Lincolnshire 1	30/04/2014 (1)	am	N26	ND	ND	-	ND	ND	-	ND	ND	-	N/A	N/A	N/A
	30/04/2014 (1)	am	N27	ND	ND	-	ND	ND	-	ND	ND	-	N/A	N/A	N/A
	01/05/2014 (1)	am	N17	ND	ND	-	ND	ND	-	ND	ND	-	N/A	N/A	N/A
	01/05/2014 (2)	am	N28	ND	ND	-	ND	ND	-	ND	ND	-	N/A	N/A	N/A
	01/05/14 (1)	pm	N84	ND	ND	-	ND	ND	-	ND	ND	-	N/A	N/A	N/A
	01/05/14 (2)	pm	N85	3.742	< LOD	-	ND	ND	-	ND	ND	-	N/A	N/A	N/A
	02/05/2014	pm	N86	ND	ND	-	3.49	< LOQ	-	ND	ND	-	N/A	N/A	N/A
	14/05/14 (1)	am	N87	3.726	< LOQ	-	ND	ND	-	ND	ND	-	N/A	N/A	N/A
	14/05/14 (2)	am	N88	ND	ND	-	ND	ND	-	ND	ND	-	N/A	N/A	N/A
	16/05/14 (1)	am	N83	ND	ND	-	ND	ND	-	ND	ND	-	N/A	N/A	N/A
	16/05/14 (2)	am	N89	ND	ND	-	ND	ND	-	ND	ND	-	N/A	N/A	N/A
Lincolnshire 2	14/05/2014	pm	N91	ND	ND	-	3.42	< LOD	-	ND	ND	-	N/A	N/A	N/A
	15/05/2014	am	N90	ND	ND	-	3.187	< LOQ	-	ND	ND	-	N/A	N/A	N/A
Lincolnshire 3	30/04/2014	pm	N25	ND	ND	-	ND	ND	-	ND	ND	-	N/A	N/A	N/A
	30/04/2014	pm	N94	ND	ND	-	ND	ND	-	ND	ND	-	N/A	N/A	N/A
	01/05/2014 (1)	am	N18	3.743	215.4053	215.4053	ND	ND	-	ND	ND	-	N/A	N/A	N/A
	01/05/2014 (2)	am	N19	ND	ND	-	ND	ND	-	ND	ND	-	N/A	N/A	N/A
	02/05/2014	am	N92	ND	ND	-	ND	ND	-	ND	ND	-	N/A	N/A	N/A
	15/05/2014	pm	N95	ND	ND	-	ND	ND	-	ND	ND	-	N/A	N/A	N/A
	13/06/2014	am	N93	ND	ND	-	3.222	< LOD	-	ND	ND	-	N/A	N/A	N/A
Lincolnshire 4	06/05/2014	pm	N82	ND	ND	-	3.33	< LOD	-	ND	ND	-	N/A	N/A	N/A
	17/05/14 (1)	am	N80	ND	ND	-	3.388	9.6686	9.6686*	ND	ND	-	N/A	N/A	N/A
	17/05/14 (2)	am	N81	3.625	< LOQ	-	3.31	8.1445	8.1445*	ND	ND	-	N/A	N/A	N/A
Denbinghshire	24/04/2014 (1)	am	N16	ND	ND	-	ND	ND	-	ND	ND	-	42.6 ± 5.59	0.62 ± 0.84	110.6 ± 1.95
	24/04/2014 (2)	am	N23	ND	ND	-	ND	ND	-	ND	ND	-	42.6 ± 5.59	0.62 ± 0.84	110.6 ± 1.95
	29/04/2014 (1)	am	N15	ND	ND	-	ND	ND	-	ND	ND	-	32.4 ± 6.43	N/A	128 ± 10.68
	29/04/2014 (2)	am	N22	ND	ND	-	ND	ND	-	ND	ND	-	32.4 ± 6.43	N/A	128 ± 10.68
	06/05/2014	pm	N14	ND	ND	-	ND	ND	-	ND	ND	-	N/A	0.32 ± 0.13	88.4 ± 10.36

Table 5.8: A summary of the neonicotinoid residues detected in nectar samples (n = 48) from various locations around the UK in 2014 as presented in Table 5.7. Mean value calculated assuming LOD values for each respective neonicotinoid where <LOD and 0 ppbv where ND.

		Number of samples (n = 48)			Residue concentration (ppbv)			
Neonicotinoid	**ND**	**< LOD**	**Detections (< LOQ)**	**Quantified**	**Low**	**High**	**Mean**	**S.D**
Imidacloprid	43	1	2	2	7.14	215.41	4.75	31.07
Clothianidin	40	3	2	3	6.89	9.67[3]	0.35	1.18
Thiamethoxam	47	-	-	1	4.17	4.17	0.09	0.60

No neonicotinoids were found below the above or below the LOD in the six collected pollen samples from 2014; consequently, this information has not been tabulated.

5.9 Discussion

Blacquière *et al.,* (2012) consider there to be a "…lack of reliable data as analyses are performed near to the detection limit". Indeed, this is true for the results presented here, as although a neonicotinoid may not have been detected, it is not possible to truly say that it is not present below the LOD.

5.9.1 Evaluation of sample collection

Nectar

The collection of nectar can be conducted in various ways, ranging from: the direct collection from the flower (Dively & Kamel, 2012; Pohorecka *et al*., 2012), from the honey stomach of a foraging honeybee (Pilling, Campbell, Coulson, Ruddle, & Tornier, 2013; Wallner, 2009) or from the hive (stored in comb) (Cutler & Scott-Dupree, 2007; Pilling *et al*., 2013). Direct collection from the flower offers an advantage over other methods, in that it represents what is available to foraging bees without enzymatic manipulation (in

[3] Peak was unusually broad and so the detected concentration may not be reliable.

honey stomach), dilution (mixture of non-contaminated nectar with contaminated nectar), water reduction (nectar maturation into honey) or contamination during storage (migration of chemicals from wax). However, hand-collection was found to be both time consuming and very labour intensive. In some instances it could take a number of hours to collect 100 µl of nectar. The nectar taken from each flower was typically between 0.1 – 2 µl, although some flowers were recorded to secrete as much as 10 µl. The low volumes collected meant it was necessary to pool the nectar in order to reach the 100 µl needed for sample analysis. Consequently, it is not possible to determine the actual concentration of neonicotinoid, if any, secreted by each flower, per se. The sampling procedure used in this study was aimed at collecting nectar from a diverse range of flowers, which included plants of various ages and height. However, it is not possible to eliminate unconscious sample bias in instances where more than one person was collecting nectar samples.

Analysis of collection sheet data and meteorological data

The small number of people collecting nectar from the flowers meant that the recording of various information, such as flower height and sugar concentration (see Appendix I), was sometimes neglected, as the main objective was to collect nectar for analysis. The data that has been recorded does not appear to offer any correlations between flower height or the levels sugars within the nectar and the levels of detected neonicotinoids. Nectar was collected both in the morning and afternoon on the same day, where possible. It was generally observed that nectar was more readily available in the morning than later in the day. The sugar content found within early and late collections differed in that a higher level of sugar was found in the latter instance; this finding appears consistent with those reasons considered in Section 5.2.4.1 (pp 189 - 191).

The collection of meteorological data using the iButton loggers was found to be unsuccessful, as some loggers were not recovered from a number of sites. Of those that were recovered it was found that the recording of data was continuous and, as a consequence, previous data was overwritten. The weather data provided from Keele University and the University of Oxford also fail to draw any correlations with the levels of neonicotinoids detected in nectar. However, this could be due to the relatively small amount of data collected.

Evaluation of micro-scale QuEChERS

QuEChERS is a multi-residue extraction technique which is applied to a number of matrices, including beeswax (Chapters 3 and 4). The volumes of solvents and amounts of extraction salts described in Chapter 3 (Section 3.6.3) were scaled down in to create a micro-scale version suitable for the extraction of 100 µl of nectar. An issue that was encountered came during the removal of the organic layer in the clean-up stage, as it was found that CUCARB could sometimes be transferred into the sample vial inserts. This was thought to be responsible for a number of blockages in the LC system. Unfortunately, the small volumes used during extraction, meant that this was often unavoidable as the organic layer was sometimes in direct contact with the clean-up powders. To filter out the CUCARB would mean the inclusion of an additional step which could result in a possible loss of extracted compounds. The later addition of an inline filter, before the LC column, helped to minimise system blockages. The mean percentage recoveries of the studied neonicotinoids are between 78 % and 102 %, which is comparable to recoveries seen in

the standard, larger scale QuEChERS technique (Tanner & Czerwenka, 2011). Based on this the overall performance of the micro-scale QuEChERS can be considered acceptable.

S*ample collection - pollen*

The original method of pollen collection considered was to capture foraging honeybees and to remove the pollen loads from their hind legs, as this would then ensure that the pollen removed from the bee had come from oilseed rape. However, as a minimum of 3 g of pollen is required for extraction using QuEChERS (Mullin, Frazier, Frazier, Ashcraft, & Simonds, 2010), a total of approximately 250 honeybees would therefore need to be captured[4]. This proved to be an extremely time consuming task with little reward. The collection of pollen was therefore later achieved by using pollen traps fixed to the front of a single hive, as this method is considered to be much easier than hand collection (Nicolson 2011). The use of a pollen trap, however, meant that the amount of food returning into the hive was reduced and, as previously mentioned, this can have an effect on the growth of a colony (Roman, 2006). This was a concern with participating beekeepers and although pollen traps were only fitted for 12 – 24 hours, their use was ultimately left to the discretion of each beekeeper. This meant that the number of pollen samples (n = 15) collected over the two seasons was significantly lower than for the nectar samples (n = 98). It also proved difficult to find beekeepers that were willing to transport their hives on or near to oilseed rape fields, as a lot of beekeepers deemed their colonies to be too weak to relocate (personal communication with beekeepers).

[4] Figure based on average weight of a pollen load (5.97 mg) collected during spring (Roman, 2006).

Given the immediate location of the pollen traps to the oilseed rape fields, it was felt that the identification of collected pollen by colour was sufficient enough for the scope of this thesis, although this practice may be considered somewhat subjective.

The levels of neonicotinoids in nectar

The results presented in this chapter are very much a single ‘snapshot’ in time. The sampling procedure used in this study was to ensure that flowers of varied height, age and location were sampled; the collected nectar would then be pooled. Each pooled sample would ultimately consist of a unique combination of these features, in addition to other factors which included temperature, soil water content and humidity. As previously mentioned, honeybees will rarely collect nectar with a sugar content lower than 20 % (Butler 1954). However, it was not possible to determine the levels of sugar from each flower without testing it during sampling; the small volumes collected meant that this would not be a viable option in the long-term. As such, it was assumed that the composition of nectar was similar across the whole of the sampled area. This assumption was also loosely based on the presence of various bee species seen on the flowers during collection, which was noted during each visit to an oilseed rape field. In all recorded instances, bees were seen foraging on the oilseed flowers; however, it was not determined whether these were pollen or nectar foraging bees.

Comparisons between samples collected from the same sites and on the same day revealed that detected concentrations were not always in agreement with the other sample; for example, the level of imidacloprid detected in N18 and N19 was 0 pbbv and 215 ppbv, respectively. This makes the comparison between other sites, and indeed the

same field, difficult or indeed impossible. Therefore each sample must be considered individually. Interestingly, very little was detected within those samples collected during the 2014 season, compared to 2013. The reasons for this have yet to be determined. Comparisons made between morning and afternoon collections were made; however, with the current sample size, no correlations could be established.

The recovery of a compound from a matrix can be low and in some cases was not corrected for in the published literature and therefore under estimated the true levels of contamination (Bonmatin, Giorio, Girolami, Goulson, & Kreutzweiser, 2015). The results presented here have been corrected according to their average recovery from a spiked matrix.

Thiamethoxam was recorded as having the smallest number of total detections, compared to clothianidin and imidacloprid (summarised in Tables 5.4 and 5.8). However, given that clothianidin is a metabolite of thiamethoxam, it is expected that thiamethoxam is metabolised and not detected. In a Polish study (Pohorecka *et al*., 2012), hand collected nectar showed no residual levels of thiamethoxam; however, comb collected nectar and honey revealed levels between 3.2 ppbv and 12.9 ppbv, whilst clothianidin was only detected in 17 % of combined honey and nectar samples. This does not agree with the findings made within this Chapter. The maximum level of clothianidin found in a Canadian study was 2.2 ppbv, which was lower than the levels detected in honey (0.9 ppbv) (Cutler & Scott-Dupree, 2007).

The detection of imidacloprid

The detection of imidacloprid has been highlighted due to the significantly high levels of this chemical found within nectar samples; as summarised in Table 5.4 and Table 5.8. The possible reasons for the detected levels will be explored; this includes the use of imidacloprid-d_4, the order of analysis as well as the presence of imidacloprid within soil.

The use of a deuterated internal standard

Isotopically labelled internal standards can determine the effectiveness of the extraction procedure (Lehotay, 2006), in addition to assisting in the identification of target analytes, as the retention times for both the labelled and non-labelled analytes should be similar (SANCO/12571/2013), but still distinguishable from each other (Gross, 2004). Given that imidacloprid was a target molecule, deuterated imidacloprid (imidacloprid-d_4) was selected as an internal standard; this would help indicate any possible losses of imidacloprid during extraction. However, the high levels imidacloprid (>13 ppbv), detected within 71 % of quantifiable samples over both seasons, in addition to positive matches for either or both thiamethoxam and clothianidin, would suggest that imidacloprid was somehow contaminating the analysed samples. However, it is unlikely that contamination played a role, but it cannot be excluded, as the high levels are too high to result from contamination.

The exchange between deuterium and hydrogen is used to study reaction mechanisms (Clayden, Greeves, Warren, & Wothers, 2005). The exchange between hydrogen and deuterium can occur gradually overtime and the reaction catalysed in the presence of an acid or base (Leis, Fauler, & Windschhofer, 1998). Consequently, it is recommended that

deuterated compounds should not be used, if the unlabelled compound is of analytical interest; as this can "lead to false positives and/or adversely influence quantitative results" (SANCO/12571/2013, 2013). Unfortunately, this was unknown at the time of analysis, as the use of deuterated compounds for ISTDs is recommended for use with the QuEChERS method (Lehotay, 2006). However, there is no mention of deuterium being lost from the ISTD during the extraction process - an extraction process which uses acetic acid to control the stability of basic-sensitive pesticides (Anastassiades *et al*., 2003).

In a closed system, it would be expected that the concentration of imidacloprid would be less than or equal to the initially added 30 ppbv of imidacloprid-d_4. This assumes complete deuterium-hydrogen exchange and 100 % recovery of the compound, which has been proven using the micro-scale QuEChERS technique. However, what is unclear is why concentrations as high as 474 ppbv have been detected. Compared to the concentrations of imidacloprid found in pollen and nectar, within the literature, it is expected to find detected levels between <1 ppbv and 10 ppbv (EFSA, 2012).

Nectar samples were assigned an identification (I.D.) number at random, in order to keep the analysis of the samples 'blind' and free of bias. These were then analysed on the Q-TOF LC/MS in order of N1 – N98. Looking at the data in this order it is observed that, out of the total 14 quantifiable imidacloprid detections (>LOQ), 9 out of 12 of the detections were found between samples N55 and N66, whilst only two quantifiable detections were made within the first 29 samples. N67 to N98 show no quantifiable results, although there is an increase in the number of detections <LOQ, compared to N1 – N29. This would disprove any significant deuterium-hydrogen exchange over time, as it would be expected that the later samples would demonstrate higher levels of imidacloprid. When arranged

into their respective locations (Table 5.3 and Table 5.7), the samples reveal the majority of quantifiable detections being located in samples from Lincolnshire and Wales for the 2013 season. No detections of imidacloprid were made in Wales in 2014, whilst only one sample contained a detectable level in Lincolnshire 2014, although it is difficult to compare between seasons, due to crop rotation cycles. The use of imidacloprid-d_4 has also been used to monitor the movement of soil-applied imidacloprid throughout squash plants (*Cucurbita pepo*); the use of the deuterated standard was not mentioned to have any effect on results of analysed nectar and pollen (Stoner & Eitzer, 2012).

Another possible reason for the high levels of imidacloprid may be a consequence of delays in the extraction and analysis of nectar samples, due to persistent instrument failures. Therefore, there may be some reduction in the levels of water from the nectar samples. This would be more dramatic in the 2013 samples compared to those from 2014. This cannot be fully determined as the volume of nectar collection was only estimated, which was based on filling half of a sample vial insert (total volume 300 µl), thus, collecting more nectar than was set by the 100 µl limit. Water evaporation was not anticipated from the nectar as the sample vials had not shown evaporation from previously made solutions, which were also kept refrigerated. However, this would only account for a slight increase in the detected concentrations.

The practice of no-til farming relies on chemicals remaining within uncultivated soils from previous crop applications (HGCA, 2012), which would support the hypothesis that the source of detected imidacloprid is from the soil. Similarly, it is proposed that neonicotinoids are persistent in soil and are capable of accumulating over time (Goulson, 2013). It is not known at what scale imidacloprid was previously applied to crops within

the same field in preceding years, if at all. However, imidacloprid was not found to be contained in the pollen or nectar of (untreated) sunflowers which were grown in soil containing imidacloprid from previous years (Schmuck, Schöning, Stork, & Schramel, 2001). Similar findings were also reported for thiamethoxam in oilseed rape (Pilling *et al.*, 2013), although conflicting data are available, showing that neonicotinoids can be detected in crops following on from previous treatments (EFSA, 2012) as well as flowers found on the edges of treated fields (Krupke, Hunt, Eitzer, Andino, & Given, 2012). Although the weather data available failed to draw any correlations to the levels of neonicotinoids, it would be interesting to see how much of an influence rainfall had on the levels detected. Collections made in Lincolnshire during the 2013 season were often made during or following heavy rainfall and this is where the majority of imidacloprid detections are found. Collections from other locations tended to be made in warm, sunny conditions.

The results of this chapter are based on the analysis of 100 µl of nectar, which reveal relatively varied levels of detected neonicotinoids, whilst other researchers often focus on the levels within honey collected from the comb, whereby 5 g is often analysed (Cutler & Scott-Dupree, 2007; Dively & Kamel, 2012; Pohorecka *et al.*, 2012). However, some studies fail to report the volumes used for analysis (Pilling *et al.*, 2013), making it difficult to assess the accuracy of their work. If the volumes of nectar have been pooled together, then the reported concentrations are representative of an average value.

The results presented here show that there is not a distinct concentration representative of neonicotinoids found within a single plant, which may be a consequence of a number of variables including, plant age, time of day, environmental conditions as well as the

amount of nectar collected. Studies appear to assume that nectar is contaminated with a single concentration. If the nectar was pooled for the four samples collected from Denbinghshire 1 (2013), before analysis, then the 'average' concentration for thiamethoxam would have been reported to be around 3.56 ppbv as oppose to 14.27 ppbv. A lower value would therefore be a result of dilution with samples containing levels below the LOD or absent from the sample. As it cannot be determined if there are any residue levels below the LOD then this figure would represent an average minimum concentration.

The levels of neonicotinoids in pollen

No quantifiable levels of clothianidin, thiamethoxam or imidacloprid were detected within any of the 15 pollen samples analysed; nor were any found within the single mixed pollen sample. However, thiamethoxam was determined to be below the limit of detection on two occasions from two separate locations (Table 5.5). With respect to the limit of detection, these results are similar to the maximum levels reported elsewhere (EFSA, 2012). Although the number of samples analysed over the two seasons is relatively small, it is not greatly dissimilar to the number of samples analysed by Bonmatin *et al*., (2003) whose findings have been used to represent 'field-realistic' doses in feeding trials (Whitehorn *et al*., 2012). Interestingly, a number of studies use concentrations deemed to be "worst-case scenarios" which are acknowledged to be rarely encountered by bees (Elston, Thompson, & Walters, 2013). This is thought to be due to a lack of sufficient or accurate data within the literature (Bonmatin *et al*., 2015). As no quantifiable levels were detected in this study, it does indicate that other studies are using values representative of worse-case scenarios.

Implications on honeybee health

It is known that honeybees from a single colony will not all visit the same floral source (von Frisch, 1954), meaning any trace levels of neonicotinoid could become diluted with uncontaminated nectar once stored in the hive (Cresswell, 2011). Consequently, the levels of neonicotinoids determined in sorted honey (and pollen) could underestimate the levels of a chemical from an individual source, but offer a more realistic average exposure range. However, should there be an exposure to a high level of neonicotinoid, it may result in a bee dying in the field (Marzaro *et al*., 2011), meaning that contaminated nectar would never return to the hive.

Rortais *et al*., (2005) discusses the different levels of sugar consumption required by honeybees, depending on their current role in the hive. Taking nectar sample N56 as an example (Table 5.3), which has an average sugar content of 36 %, a honeybee would need to ingest 2.78 mg of nectar in order to consume 1 mg of sugar. Using the density of a 50 % sugar solution (1.23 kg/l) (EFSA, 2012) this equates approximately to 2.18 µl of nectar. As the concentration of clothianidin was measured to be 40.02 ppbv (40.02 pg/µl) this would result in an intake of approximately 90.05 pg of active ingredient, which is below the LD_{50} oral dose for clothianidin of 0.0037 ug/bee (3700 pg/bee) (Tomlin, 2009).

As bees are thought to act like filters (Bogdanov, 2006), the amount of returned nectar will have a reduced level of neonicotinoid than was originally collected. This, in combination with the previously mentioned dilution of nectar with other none contaminated nectars, therefore resulting in lower concentrations in honey, as seen in the findings of Pohorecka *et al*., (2012).

There appears to be a relationship between the levels of neonicotinoids and the decline of insect pollinators; neonicotinoids affect the ability to forage for food, brain function and bee learning, thus adversely affecting colony expansion (Moffat *et al*., 2015). However, the loss of foraging honeybees can be compensated for by the large numbers within a colony; although other bee species may be more susceptible to the effects of losing foragers (Bryden, Gill, Mitton, Raine, & Jansen, 2013). In feeding trials, it is suggested that bees cannot taste neonicotinoids and were found to actually prefer solutions laced with these chemicals; although their consumption did reduce the overall amount of food consumed (Kessler *et al*., 2015).

The levels of nectar consumed by honeybees vary according to their role in the hive; it was found that the volumes of nectar ingested by bees is greater than the amounts pollen consumed, meaning exposure to pesticides is likely to result from the former. The ratio between the amount of contaminated and uncontaminated nectar ingested by bees cannot be precisely determined, meaning it is not possible to calculate the amount of pesticide consumed by a single bee (Rortais *et al*., 2005). LD_{50} values are considered to be unrealistic as they represent a single dose, which is unlikely to be encountered during foraging (Cresswell, 2011). Linking this to the nutritional values provided by a food source, it was found that honeybees shifted towards a high carbohydrate-filled diet with age (Paoli, Donley, Stabler, Simpson, & Wright, 2014); a similar finding was also shown for bumblebees (Stabler, Paoli, Nicolson, & Wright, 2015). So, depending on the levels of sugar available (carbohydrates), the amount of pesticide possibly ingested from a contaminated food source can vary (Rortais *et al*., 2005).

There are two main disputes that surround the neonicotinoid debate, which are: field vs laboratory studies and dose vs concentration/exposure. The imposed sanction on the use of neonicotinoids is to be reviewed in December (2015), but during the time since the imposed 'ban' no real monitoring schemes have been conducted, which means it will not be able to determine the benefits of not using neonicotinoids (Goulson, 2015). This chapter has not addressed either issue; however, it does give a perspective on the levels of neonicotinoids in UK fields – rather than extrapolating American or German findings to a UK situation. Cresswell (2011) suggests that although neonicotinoids are regarded as a stressor, they are not the sole cause of honeybee losses; it is felt that UK losses are more likely due to habitat loss (Wilson-Rich, Allin, Carreck, & Quigley, 2014). However, some alarmingly high imidacloprid values are presented in this chapter cannot be ignored and possibly require further study. Although a concentration for 474 ppbv was quoted for imidacloprid within a nectar sample, it does not necessarily mean that a bee will be exposed to the full amount.

As this was a field based study, there were a number of variables which could not be controlled. As there are no fixed concentrations, it would be useful to have a field realistic experiment in the laboratory which gives bees a choice of sugar solutions at various concentrations and volumes. It would be ideal to repeat this present study again, with a more refined protocol and access to an experimental plot. There would also need the correct infrastructure and man power, as the limited number of people helping within this project (often only a single person) may suggest that the study was perhaps over optimistic. The only realistic experimental plot which may be available is located near to

Keele University; however, this is not particularly large and any findings could be deemed site-specific.

What has not been considered by this study are the metabolites of the parent compounds, with the exception of clothianidin (primary metabolite of thiamethoxam), although this is recognised as a compound in its own right (Tomlin, 2009). The metabolism of thiamethoxam can occur in various ways, promoted by exposure to alkaline conditions (Tomlin, 2009). As 90 % of a seed coating enters the soils (Goulson, 2013) the pH of this media can determine the speed at which it is converted to clothianidin. Photodegradation (photolysis) is also another route of metabolism, which occurs following sunlight exposure. When exposed to light under laboratory conditions, imidacloprid was found to undergo photolysis and lose as much as 84 % of its original concentration after 6 hours (Soliman, 2012). As such, photodegradation is regarded as an important factor in the metabolism of agrochemicals (Martínez Vidal, Plaza-Bolaños, Romero-González, & Garrido Frenich, 2009). This means that neonicotinoids contained in nectar and exposed to sunlight will begin to metabolise. This may explain the small number of thiamethoxam detections, compared to clothianidin, although, as previously mentioned, clothianidin is also used as a seed treatment. Only farmers from Lincolnshire 1 and Oxfordshire 4 have confirmed the seed treatment used on their crops to be thiamethoxam based. No other replies were received.

Although not the main part of this thesis, the type of soil in each oilseed rape field may be a factor in the levels of neonicotinoids detected. The next paragraph will briefly consider this.

No soil type was determined at specific site during collection, as this was not considered to be of great influence throughout the planning of the investigation. However, through the use of NATMAP Soilscapes map (Appendix L) or online database, constructed by the Cranfield Soil and Agrofood Institute (www.landis.org.uk), it is possible, to some degree, to determine the soil type found at each site. A general trend for the soil variability across the UK, from West to East, is Loam to silty loam (Wales); clayey loam to sandy loam (Staffordshire); peat and sand to sandy loam (Lincolnshire). Unfortunately time restraints have not allowed for the comparison of the neonicotinoid results to the soil types found from around the UK. Although it is believed it would make for an interesting investigation, given the discussion of the half-lives of neonicotinoids in soil (Goulson, 2013). Appendix L does show part of Lincolnshire to contain soil of naturally high ground water, which may correlate to the high levels of detections within this region in 2013. Without further investigation this remains very speculative.

5.10 Conclusion

Presented within this chapter is a novel method of extraction, which has successfully applied to determine the levels of neonicotinoids within a small volume of nectar. The findings of this study suggest that bees are exposed to variable concentrations of neonicotinoids, originally applied as a seed treatment, during foraging.

Due to the large number of values that fall below the LOD and LOQ it was not possible to determine any meaningful data, such as the mean and standard deviation. Where these have been given it has only been calculated for those values which are above the LOQ. Therefore, the concentrations presented are to be considered on an individual basis due

to the reasons previously considered. Due to the large variability in the concentrations, it would be difficult to draw conclusions from these or to apply them to across the wider population.

Without good quality weather data it is difficult to precisely draw comparisons to the influence of weather on the levels of secreted nectar and the residues contained within nectar. Looking back objectively, it would have been better to focus on a smaller number of (local) fields, meaning more time could be spent collecting information as set out by the collection sheets. However, this would have its own limitations as it could be argued that any findings could be site specific and may not be applicable to the rest of the UK, as there is a large variation between sites suggested by the different soil types across the UK, for example.

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CHAPTER 6
The analysis of bumblebees exposed to thiamethoxam during feeding trials

6.1 Introduction

There are currently 25 native species of bumblebee (*Bombini*) in the UK (Free & Butler, 1959; Ollerton, 2012). Bumblebees are considered primitively-eusocial since all colonial duties are performed by a single queen until labour can be divided, following the emergence of daughter workers (Michener 2000, Free and Butler 1959), which are also sexually undeveloped. New queens, which are able to start new colonies, and males are not found within the nest until after several generations of workers have been reared (Free & Butler, 1959). Compared to the honeybee, bumblebees have much smaller colonies, containing around a few hundred workers. All bumble species will normally establish colonies in the disused nests of field-mice, shrews and voles; these tend to

contain old nesting materials, such as grass and moss, which can be used to line the floor of the new nest. Some species of bumblebee will also build nests above ground. The focus of this chapter is the buff-tailed bumbles (*Bombus terrestris*), which is considered to be fairly versatile during the selection of a nesting site. The schematic diagram, shown in Figure 6.1, displays the colour patterns of *B. terrestris* and size compassion of each caste; in addition to its distribution across the UK.

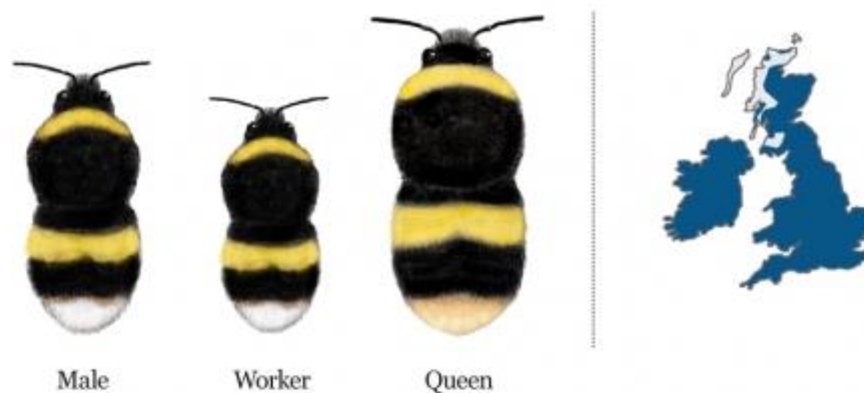

Figure 6.1: An image demonstrating the size and colour of *Bombus terrestris* for each caste (male, worker and queen). The distribution of this species thought the UK is also provided. Taken without permission from Bumblebee Conservation Trust (2015).

A nest will expand irregularly (upwards and outwards) as the colony grows. The structure of a typical bumblebee nest is a collection of old pupal cocoons and wax cells, the former often used to store honey (honey pots). Throughout the development of the colony, the queen will engage in a number of in-hive activities whilst foraging is left to the workers. Unlike a honeybee colony, a bumblebee colony does not overwinter; instead, the queen will hibernate. This means that extensive stores of pollen and honey are not required (Free & Butler, 1959).

6.2 The importance of bumblebee foraging

Bumblebees, such as *B. terrestris,* play an important role in the pollination of agricultural crops and wildflowers (Mommaerts *et al*., 2010). Both bumblebees and the honeybee are generalist pollinators (Goulson, Lye, & Darvill, 2008; Winter *et al*., 2006); however, the large variation in morphology, exhibited within the *Bombus* genus, means that bumblebees offer a greater efficiency during pollination than the honeybee (Winter *et al*., 2006). Despite this, the honeybee still provides effective pollination for a majority of crops (Breeze, Bailey, Balcombe, & Potts, 2011; Winter *et al*., 2006). Bumblebees are considered a hardy species, having gained a reputation for continuing to forage in conditions where the honeybee would remain in the hive (Free & Butler, 1959). The large body size of bumblebees enables them to forage over a temperature range of 10 – 32 °C, allowing for flower visits during most of the year; whilst honeybees are only found to be active at temperatures above 16 °C (Winter *et al*., 2006). Bumblebees are also found to forage over longer periods of time, including up to an hour after sunset. This activity will reduce to an hour before sunset around mid-August (Free & Butler, 1959). Although the work rate of each bumblebee species varies, it is nearly always found that bumblebees are two to three times quicker than honeybees when visiting the same flower type (Free & Butler, 1959), allowing bumblebees to visit more flowers over the course of a day.

Bumblebees are capable of sonication, also known as ‘buzz pollination’, which involves rapid contractions of their indirect flight muscles; when curled around a flowers androecium (group of stamen), this induces an ejection of pollen from the flower’s anthers (Winter *et al*., 2006). For example, buzz pollination of the tomato plant enables bumblebees to harvest ‘buzzed’ pollen 400 times faster than honeybees (Winter *et al*.,

2006), thus rendering the honeybee "ineffective" in tomato pollination (Breeze *et al*., 2011). The majority of bumblebees possess a longer proboscis than honeybees, which is essential for accessing deeper nectaries of certain flowers, for example, field beans (Breeze *et al*., 2011; Winter *et al*., 2006). The hardy nature of *B. terrestris* means it is often favoured for commercial pollination and as a result it has been extensively utilised since the late 1980s due to its adaptability to artificial conditions, well established and proven breading technologies and large colony production in comparison to other bumblebee species (Winter *et al*., 2006). Originally native to Europe, the species now exists in non-native terrains such as America, Japan and Australia (Winter *et al*., 2006).

6.3 Bumblebee decline

The decline of honeybees is regularly documented and often the main focus of researchers, whilst bumblebee decline has received limited attention (Elston, Thompson, & Walters, 2013; Mommaerts *et al*., 2010). As previously mentioned, eight native UK species of bumblebee have declined dramatically, while three species have become extinct (Goulson, 2010). Like the honeybee, there are multiple drivers behind bumblebee declines; they include: loss of habitat, parasites, disease (Potts *et al*., 2010) as well as competition with honeybees (Thompson, 2004). Pesticides are also one of the drivers which have received recent attention, particularly neonicotinoids (Blacquière, Smagghe, van Gestel, & Mommaerts, 2012). The implications of bumblebee losses are considered in further detail elsewhere (Goulson *et al*., 2008; Goulson, 2010).

6.3.1 The role of neonicotinoids

Those neonicotinoids, which are a concern to pollinator health, are: imidacloprid, thiamethoxam and clothianidin; as they were routinely applied as seed dressing to a number of crops (Tomlin, 2009), prior to December 2013. The effects of these chemicals on honeybees have been covered in Chapter 1 while the effect of imidacloprid on bumblebees is more extensive than for thiamethoxam and clothianidin. It is known that these chemicals can contaminate various floral resources (see Chapter 5). Bumblebees consume large volumes of nectar, so it has been suggested that this may be the main route of pesticide exposure (Goulson, 2010).

Using radio frequency identification (RFID) technology, Feltham *et al*., (2014) monitored bumblebee colonies following a 14 day exposure to imidacloprid spiked pollen (6 ppbv) and nectar (0.7 pbbv). Although there was no difference in lifespan between control and treated colonies, it was found that exposed colonies expressed a 31 % decrease in the amount of forage per hour (compared to control colonies). Pollen foraging trips were also reduced by 23 % (Feltham, Park, & Goulson, 2014). A reduction in the amount of pollen returning to the hive can limit colony success, as pollen is needed for brood development (Free & Butler, 1959; Rortais, Arnold, Halm, & Touffet-Briens, 2005). This can also be contributed to by workers being unable to navigate back to the colony; imidacloprid exposure of 10 ppbv was found (on average) to reduce worker return to the hive by 50 %, compared to controls (Gill, Ramos-Rodriguez, & Raine, 2012). Using the same spiking concentrations as Feltham *et al.,* (2014), an earlier study by Whitehorn *et al.,* (2012) demonstrated that imidacloprid reduced queen production in exposed colonies by 85 % as a result of reduced provisions of pollen (Whitehorn *et al*., 2012). The effect of

imidacloprid on worker performance (foraging) was found to continue up to four weeks after exposure (Feltham *et al.*, 2014). As previously mentioned, the toxicity of imidacloprid is higher than that of thiamethoxam and clothianidin (Iwasa, Motoyama, Ambrose, & Roe, 2004), resulting in a shift towards the use of the latter neonicotinoids in crop protection (García-Chao *et al*., 2010).

There are little data available on the effects of thiamethoxam on bumblebee colonies (Elston *et al*., 2013). Exposure to thiamethoxam at high concentrations was found to result in a number of lethal and sublethal effects, including an increase in mortality; unsurprisingly, mortality levels dropped with decreasing concentrations (Mommaerts *et al.*, 2010). Exposure to thiamethoxam at field-realistic levels in both pollen and nectar (1 – 10 pbbv) did not have an effect on the longevity of exposed colonies (Elston *et al.*, 2013) in line with findings of Thompson *et al* (2013). It was shown, however, that a 10 ppbv exposure resulted in a significant delay in nest building with only 20 % of the experimental colonies starting to build a nest. Fewer eggs were laid and of those that were, all failed to develop into larvae (Elston *et al.*, 2013). There were no major effects of thiamethoxam or its metabolite, clothianidin, on queen production (Thompson *et al.*, 2013).

Exposure to clothianidin at concentrations of 6 and 36 ppbv were found to have no adverse effects on colony health; as such, it is considered a safer alternative to imidacloprid (Franklin, Winston, & Morandin, 2004). No brood mortalities were witnessed by Scholer & Krischik (2014); however, queen mortalities were significantly higher at non-field realistic doses (50 – 100 ppbv) over an 11 week exposure. The speed at which workers moved at was 32 % slower at a 20 ppbv exposure compared to 0 ppbv. As with

thiamethoxam, it was also shown that the amount of constructed wax pots progressively decreased at higher concentrations of clothianidin (Scholer & Krischik, 2014).

6.4 Study by Elston, C., Thompson, H. M., & Walters, K. F. (2013)

The remainder of this chapter will focus on the analysis of bumblebee samples, which were kindly donated as part of a research project initiated by Professor Keith Walters during his time at Imperial College London. The bumblebees originate from feeding trials described in the published work of Elston *et al.,* (2013). The initial study aimed to contribute findings of the effect of thiamethoxam and a fungicide on nest building and brood production in *B. terrestris* colonies, as there is very little information available concerning this in the literature.

6.5 Aims and objectives

The aim of this chapter is to quantify, if possible, the levels of thiamethoxam and its metabolite clothianidin in bumblebee specimens following exposure to thiamethoxam at 'field-realistic' doses in a laboratory setting.

6.6 Materials and Methods

6.6.1 Feeding trials

Feeding trials were conducted at Imperial College London, the experimental design used by Elston *et al.,* (2013) focused on two treatments, thiamethoxam and propiconazole (fungicide). This chapter will solely focus on the former, which is briefly described below.

Queenless micro-colonies, consisting of three workers, were kept within the laboratory at 27 °C and 70 % relative humidity, with an 8:16 hour light/dark photoperiod. At 2-day intervals (mixed) pollen (1-2 g) was made accessible to each colony, in addition to sugar solution (ad libitum). In order to monitor the weight of each bee they were anaesthetised using CO_2 carbon dioxide (1 minute exposure). Those which failed to recover were replaced (before the experiment); however, those which did die, once the experiment had begun, were not replaced. An artificial nectar solution was prepared (pure honey and water at 60% *w/v*) and offered ad libitum. A pollen paste (dried pollen soaked in sugar solution) was also placed within the colonies on a weekly basis. During the feeding trials, two different doses of thiamethoxam (contained within artificial nectar and pollen) were made available: a “high-dose” and “low-dose”. Each dose was replicated ten times. The high-dose (HD) is considered a field-realistic maximum (10 ug/kg = 10 ppbv), while the low-dose (MD) (mean dose) is considered a field-realistic mean dose (1 ug/kg = 1 ppbv). Solvent control micro-colonies were also offered both artificial nectar and pollen, both containing 2,000 ug/kg (=2 ppmv) of acetone; whilst untreated colonies were given untreated artificial nectar and pollen. The experiment was started once signs of nest building (wax production) were observed. After 28 days the workers were weighed and frozen.

6.6.2 Sample collection

A total of 168 bumblebees (*Bombus terrestris*) were transported, packed in dry-ice, to Keele University. The bumblebees arrived as 91 pre-divided samples, each containing a single bee, two bees or three bees. These 91 samples were further sub-divided into four

boxes, as determined by Professor Keith Walters. All samples remained stored at -43 °C until analysis. The information regarding the feeding trials was agreed not to be released until the samples had been analysed.

6.6.3 Reagents and standards

All solvents and chemicals used in the study were of HPLC grade. Pesticide standards Thiamethoxam PESTANAL® and Clothianidin PESTANAL® (Fluka Analytical, Germany). Tri (2,3-dichloropropyl) phosphate OEKANAL® (TDCPP), (Fluka Analytical, Germany). Ammonium formate (NH_4HCO_2) ≥99 %, (Fluka Analytical, Germany). Acetonitrile (Fisher Scientific, Loughborough, England). Dichloromethane CHROMASOLV® (DCM) (Sigma-Aldrich, Germany). Distilled water was purified at 18.2 MΩ with a Purelab Option-Q DV25 purification system.

6.6.4 Calibration using standards

A 'semi-matrix-matched' calibration was conducted. This was achieved by diluting a stock solution to various concentrations (40, 20, 10, 5, 2.5, 1.25, 0.625) of thiamethoxam or clothianidin in water along with TDCPP at a constant concentration of 30 ppbv (20 µl at 300 ppbv) in a final volume of 200 µl. Dichloromethane (800 µl) was then added to the water and the two solvents then shaken (1 minute). The aqueous (top) layer was removed and discarded, whilst the remaining organic layer was then dried under a stream of nitrogen, which was then dissolved in 200 µl water/acetonitrile (95:5 v/v) and transferred into a glass insert ready for analysis. Each concentration was injected onto the LC column five times (injection volume: 15 µl). The correlation coefficient (R^2) values of both

thiamethoxam and clothianidin were 0.9963 and 0.9933, respectively (see Appendix M). Both the LOD and LOQ values were estimated from calibration levels, corresponding to a signal-to-noise ratio of about 3 and 10, respectively; in addition to the presence of the $[M+H]^+$ ion (within a mass accuracy of 5 ppm) for each compound. The LOD and LOQ of clothianidin were determined to be 1.25 ppbv and 2.5 ppbv. The LOD and LOQ were both found to be below 0.625 ppbv for thiamethoxam and therefore any value below this will be referred to as < LOQ.

6.6.5 Sample preparation

Each bumblebee sample (containing one, two or three bees) was placed within a 5 ml Eppendorf tube® (Eppendorf AG, Hamburg, Germany) and homogenised using Castroviejo spring scissors (Electron Microscopy Sciences, Pennsylvania, USA) and 2 ml extraction solution (dichloromethane/water, 4:1 v/v) was then added along with 6 to 10, 1.0 mm zirconia/silica beads (Thistle Scientific, Glasgow, Scotland) and vortexed (2 min). The extraction was then carried out within an ultrasonic bath at room temperature (23 °C). Each sample was then centrifuged (1 min/ 10,000 rpm) and stored at -40 °C until the aqueous layer had frozen. The organic layer was then removed and filtered through a Acrodisc® CR PTFE (0.45 µm, 13 mm) syringe filter (PALL Life Sciences, USA), before being dried under a stream of nitrogen. Each sample was then dissolved in 50 µl of water/acetonitrile (95:5 v/v) and was ready for Q-TOF LC/MS analysis.

6.6.6 Instrumentation

Samples were injected into an Agilent 1260 Infinity LC system (injection volume: 20 µl) equipped with an autosampler, thermostatted column compartment (set to 35 °C) with

an Agilent Poroshell 120 EC-C18 (3 x 50 mm, 2.7 µm) column connected to an Agilent 6530 Accurate-Mass-Q-TOF LC/MS. The LC mobile phase was water (5 mmol ammonium formate) (A), acetonitrile (B). The elution gradient, at a flow rate of 1 ml/min, was as follows: 0 – 0.5 min (95 % A/ 5 % B), 0.5 – 9 min (0 % A/ 100 % B), 9 – 9.5 min (0 % A/ 100 % B), 9.5 – 10 min (95 % A/ 5 % B). The Q-TOF settings were as follows: acquisition mode MS; with MS range 100 – 1000 *m/z*; MS scan rate 1 spectrum/s; electrospray ionization (ESI) source – gas temperature: 300 °C; gas flow: 11 L/min; nebulizer: 50 psig, positive ion polarity; scan source parameters: Vcap 4000 V; fragmentor, 125 V; skimmer, 65 V; OCT RF Vpp, 750 V.

6.7 Results

The data for the bumblebee analysis can be found Tables 6.1 to 6.4. This data shows three concentration thresholds that could suggest to which concentration group (10 ppbv or 1 ppbv) each bumblebee sample was potentially exposed. Each threshold is based on the detected levels of the thiamethoxam metabolite clothianidin and are as follows: *(1)* no clothianidin detected (control group); *(2)* detections made <LOD – 30 ppbv (low-dose, MD); and *(3)* detections made >30 ppbv (high-dose, HD). There may, however, be some overlap at each threshold boundary, but this cannot be determined without first acquiring the feeding data. A summary for each box is given in Table 6.5.

Table 6.1: Analysis results for 28 bumblebees contained in BOX 1. Results were obtained using the extraction procedure as described in Section 6.6.5 and analysed using Q-TOF LC/MS. Concentration values were calculated using semi-matrix matched calibration curves, as given in Appendix M.

BOX 1			Thiamethoxam				Clothianidin			
Sample ID	Frozen weight (g)	Number of bees	RT (min)	Conc (ppbv)	Conc per bee (ppbv)	ng/g	RT (min)	Conc (ppbv)	Conc per bee (ppbv)	ng/g
BOX1_35	0.7731	3	ND	ND	-	-	3.124	< LOD	-	-
BOX1_34	0.3335	2	ND	ND	-	-	3.09	< LOD	-	-
BOX1_34(1)	0.1841	1	ND	ND	-	-	ND	ND	-	-
BOX1_33	0.6204	3	ND	ND	-	-	3.128	< LOD	-	-
BOX1_32	0.1815	1	2.77	1.582	1.582	0.158	3.102	< LOQ	-	-
BOX1_32(2)	0.3489	2	ND	ND	-	-	ND	ND	-	-
BOX1_31(3)	0.433	3	ND	ND	-	-	ND	ND	-	-
BOX1_30	0.2962	2	ND	ND	-	-	3.003	< LOD	-	-
BOX1_30(1)	0.1973	1	2.707	< LOQ	-	-	3.039	2.5535	2.5535	0.25535
BOX1_29(2)	0.4672	2	ND	ND	-	-	3.136	< LOD	-	-
BOX1_29	0.1537	1	2.706	< LOQ	-	-	3.038	< LOD	-	-
BOX1_28	0.5019	3	2.711	6.612	2.204	0.661	3.043	3.1606	1.0535	0.3161
BOX1_27	0.6074	3	ND	ND	-	-	3.111	< LOD	-	-
BOX1_26	0.4661	3	ND	ND	-	-	3.054	< LOQ	-	-
BOX1_24	0.4016	3	ND	ND	-	-	ND	ND	-	-
BOX1_22	0.4819	2	ND	ND	-	-	3.056	< LOD	-	-
BOX1_20	0.7184	3	ND	ND	-	-	ND	ND	-	-
BOX1_19	0.5857	3	2.706	< LOQ	-	-	3.154	< LOD	-	-
BOX1_18	0.6021	3	2.725	< LOQ	-	-	3.04	< LOD	-	-
BOX1_17	0.1552	1	ND	ND	-	-	ND	ND	-	-
BOX1_15	0.6165	3	2.709	< LOD	-	-	ND	ND	-	-
BOX1_14	0.5508	3	2.715	< LOD	-	-	3.047	< LOD	-	-
BOX1_13	0.6544	3	ND	ND	-	-	3.028	< LOD	-	-
BOX1_12	0.3569	2	ND	ND	-	-	3.032	< LOQ	-	-
BOX1_4	0.4409	3	ND	ND	-	-	ND	ND	-	-
BOX1_3	0.3595	2	ND	ND	-	-	ND	ND	-	-
BOX1_2	0.4571	3	ND	ND	-	-	3.048	2.8544	0.9515	0.2854
BOX1_1	0.6362	3	ND	ND	-	-	3.032	< LOD	-	-

Table 6.2: Analysis results for 13 bumblebees contained in BOX 2. Results were obtained using the extraction procedure as described in Section 6.6.5 and analysed using Q-TOF LC/MS. Concentration values were calculated using semi-matrix matched calibration curves, as given in Appendix M.

BOX 2			Thiamethoxam				Clothianidin			
Sample ID	Frozen weight (g)	Number of bees	RT (min)	Conc (ppbv)	Conc per bee (ppbv)	ng/g	RT (min)	Conc (ppbv)	Conc per bee (ppbv)	ng/g
BOX2_11	0.6093	3	ND	-	-	-	3.032	< LOD	-	-
BOX2_23	0.667	3	ND	-	-	-	3.029	< LOD	-	-
BOX2_25	0.5567	3	ND	-	-	-	3.019	< LOD	-	-
BOX2_50(1)	0.1896	1	2.7	<	-	-	3.032	< LOQ	-	-
BOX2_52(1)	0.1013	1	ND	-	-	-	3.032	< LOQ	-	-
BOX2_54(1)	0.1223	1	ND	-	-	-	3.145	< LOD	-	-
BOX2_57(1)	0.272	1	ND	-	-	-	3.134	< LOQ	-	-
BOX2_21	0.615	3	ND	-	-	-	3.163	< LOD	-	-
BOX2_16	0.5918	3	ND	-	-	-	3.133	< LOD	-	-
BOX2_5	0.7889	3	ND	-	-	-	ND	-	-	-
BOX2_56(2)	0.6539	2	ND	-	-	-	3.104	13.7273	6.8637	1.3727
BOX2_37(2)	0.5837	2	ND	-	-	-	3.058	38.9716	19.4858	3.8971
BOX2_44(1)	0.1273	1	ND	-	-	-	3.105	17.4925	17.4921	1.7492

Table 6.3: Analysis results for 22 bumblebees contained in BOX 3. Results were obtained using the extraction procedure as described in section 6.6.5 and analysed using Q-TOF LC/MS. Concentration values were calculated using semi-matrix matched calibration curves, as given in Appendix M.

BOX 3			Thiamethoxam				Clothianidin			
Sample ID	Frozen weight (g)	Number of bees	RT (min)	Conc (ppbv)	Conc per bee (ppbv)	ng/g	RT (min)	Conc (ppbv)	Conc per bee (ppbv)	ng/g
8(1)_23_11_12	0.0978	1	ND	-	-	-	3.055	3.4722	3.4722	0.3472
37(1)_23_11_12	0.2756	1	ND	-	-	-	3.056	4.354	4.3540	0.4354
49(1)	0.1363	1	ND	-	-	-	3.056	< LOQ	-	-
60(1)	0.1136	1	ND	-	-	-	3.054	< LOQ	-	-
59(2)	0.2601	2	2.717	< LOQ	-	-	ND	-	-	-
58(2)	0.4616	2	2.718	3.0569	1.52845	0.3056	3.05	43.0618	21.5309	2.1531
57(2)	0.5463	2	2.722	1.6342	0.8171	0.1634	3.054	126.6617	63.3309	6.3309
55(3)	N/A	3	2.717	< LOQ	-	-	3.065	69.3247	23.1082	2.3108
54(2)	0.3411	2	ND	-	-	-	3.068	3.5874	1.7937	0.1794
51(2)	0.3239	2	ND	-	-	-	3.062	19.8346	9.9173	0.9917
48(2)	0.424	2	ND	-	-	-	-	-	-	-
45(1)	0.2534	1	ND	-	-	-	3.063	< LOQ	-	-
43(1)	0.1852	1	ND	-	-	-	3.054	45.8952	45.8952	4.5895
41(1)	0.263	1	ND	-	-	-	3.044	15.7568	15.7568	1.5757
39(2)	0.494	2	ND	-	-	-	3.056	2.7975	1.3988	0.1399
38(1)	0.2146	1	ND	-	-	-	ND	-	-	-
36(1)	0.1661	1	ND	-	-	-	ND	-	-	-
10(1)	0.173	1	ND	-	-	-	ND	-	-	-
9(2)	0.2928	2	ND	-	-	-	3.048	19.2167	9.60835	
8(1)	0.2256	1	ND	-	-	-	ND	-	-	-
52(1)	0.1244	1	ND	-	-	-	3.045	16.727	16.727	1.6727
50(1)	0.1913	1	2.704	14.9915	-	1.4991	3.052	5.6738	5.6738	0.5674

Table 6.4: Analysis results for 28 bumblebees contained in BOX 4. Results were obtained using the extraction procedure as described in section 6.6.5 and analysed using Q-TOF LC/MS. Concentration values were calculated using semi-matrix matched calibration curves, as given in Appendix M.

BOX 4			Thiamethoxam				Clothianidin			
Sample I.D	Frozen weight (g)	Number of bees	RT (min)	Conc (ppbv)	Conc per bee (ppbv)	ng/g	RT (min)	Conc (ppbv)	Conc per bee (ppbv)	ng/g
56	0.227	1	2.713	1.9837	1.9837	0.87387	3.045	26.825	26.8250	11.8172
53(1)	0.3075	1	-	-	-	-	3.038	23.9443	23.9443	7.7868
8(1)	0.1273	1	-	-	-	-	3.048	109.7592	109.7592	86.2209
53	0.5074	2	2.707	2.6067	1.30335	0.51373	3.056	52.2179	26.1090	10.2913
45(2)	0.3375	2	-	-	-	-	3.048	80.6061	40.3031	23.8833
40	0.5643	3	-	-	-	-	3.046	21.6881	7.2294	3.8433
48	0.164	1	2.71	0.8875	0.8875	0.54115	3.042	101.0749	101.0749	61.6310
41	0.3876	2	-	-	-	-	3.05	76.8703	38.4352	19.8324
60	0.4964	2	2.72	7.4422	3.7211	1.49923	3.052	75.556	37.778	15.2208
36	0.348	2	2.708	< LOQ	-	-	3.04	60.9338	30.4669	17.5097
49	0.2742	1	2.711	< LOQ	-	-	3.043	41.8517	41.8517	15.2632
58	0.2502	1	2.714	7.8016	7.8016	3.11814	3.046	29.8983	29.8983	11.9498
59	0.1528	1	2.711	5.6604	5.6604	3.70445	3.043	43.8076	43.8076	28.6699
46	0.432	2	2.716	2.6038	1.3019	0.60273	3.048	99.0673	49.5337	22.93225
47	0.2672	2	2.692	< LOQ	-	-	3.04	437.6601	218.8301	163.7949
43	0.0862	1	-	-	-	-	3.044	5.8549	5.8549	6.7922
44	0.1472	1	-	-	-	-	3.04	23.3357	23.3357	15.8531
52(1)	0.0124	1	2.702	0.8335	0.8335	6.72177	3.034	91.0197	91.0197	734.0298
51(1)	0.0913	1	-	-	-	-	3.033	11.6249	11.6249	12.7326
46(1)	0.1421	1	-	-	-	-	3.04	13.7914	13.7914	9.7054
47(1)	0.1965	1	2.715	< LOQ	-	-	3.047	178.6127	178.6127	90.897
9	0.1537	1	-	-	-	-	3.038	28.7531	28.7531	18.7073
10	0.2867	2	-	-	-	-	3.032	86.8934	43.4467	30.30813
7	0.646	3	-	-	-	-	3.035	44.1483	14.7161	6.8341
6	0.4113	3	-	-	-	-	3.037	4.7494	1.5831	1.1547
44(1)	0.2244	1	-	-	-	-	3.043	66.5917	66.5917	29.6755
43(1)	0.1327	1	-	-	-	-	3.031	8.1008	8.1008	6.1046
39(1)	0.1919	1	-	-	-	-	3.037	2.9082	2.9082	1.5155

Table 6.5: A summary of raw data contained in Table 6.1 to 6.4 for boxes 1 – 4. Each individual bee was considered to be part of a control (no clothianidin detected); low-dose (<LOD – 30 ppbv); or high dose (>30 ppbv) feeding trial. The limit of detection for clothianidin was 1.25 ppbv.

Box	Number of samples	Control	Low dose (1 ppbv)	High dose (10 ppbv)
1	28	9	19	0
2	13	1	11	1
3	22	6	12	4
4	28	0	12	16

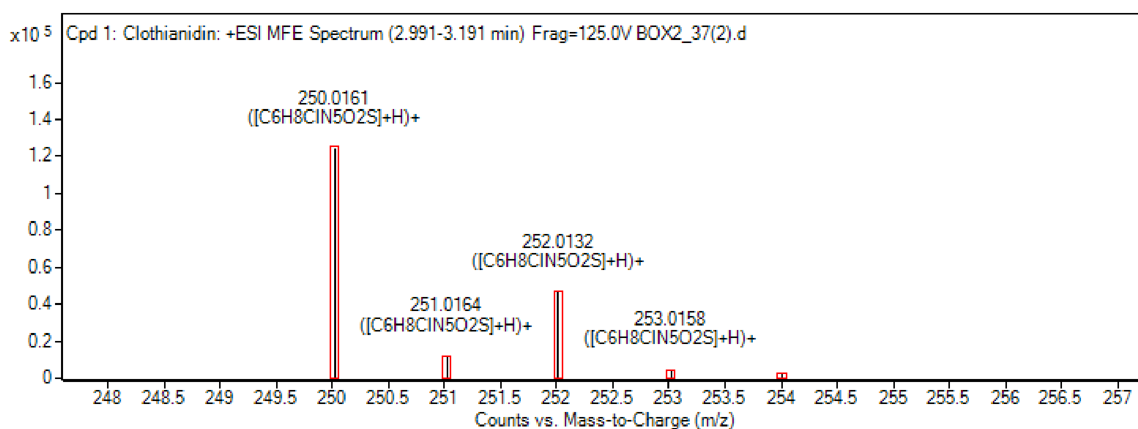

Figure 6.2: An overlay comparison between clothianidin, detected in sample 37(2) from Box 2, to its theoretical isotopic pattern (red boxes).

The presence of clothianidin (Figure 6.2) and thiamethoxam were confirmed using their respective theoretical isotopic pattern, in addition to comparing RT values with previously analysed standards.

6.8 Discussion

6.8.1 Extraction technique

The extraction technique used for the experiments described in this chapter was developed as an amalgamation of two studies which extracted various pesticides from honeybees. The first was a relatively simple ultrasonic extraction method, which was used to isolate three chlorinated pesticides. Here three honeybees were homogenised in dichloromethane and then placed in an ultrasonic bath for 30 minutes. This showed good recoveries for DDT (79 %) and methoxychlor (DMDT) (86 %), although a lower recovery was found for lindane (γ-HCH) (55 %) (Bańka, Buszewicz, Listos, & Madro, 2010). The principle of using immiscible solvents (water and dichloromethane) was adopted from a second study, which used a modified QuEChERS method, with the addition of hexane (13 % v/v) to the standard extraction solution (water and acetonitrile) - with the aim of

eliminating any co-extracted beeswax. The inclusion of hexane was also found to improve the recovery of some pesticides, such as thiamethoxam, while recoveries of other pesticides decreased. However, the overall trend appeared to be an increase in recovery, with a range for all pesticides tested to be between 70.1 % to 110.6 % (Bargańska, Ślebioda, & Namieśnik, 2014). However, due to the miscibility of dichloromethane and hexane it would mean that fatty deposits within the hexane would also be contained within the dichloromethane fraction. Consequently, hexane was not used in the extraction solution. Instead, the extractions were stored at -40 °C, leaving the dichloromethane to be decanted away from the frozen water and lipid layer. Homogenisation of the bumblebee sample was achieved using Castroviejo spring scissors to cut the bee into smaller fragments, allowing for the solvent to access the internal of the bee. Zicronia/silicon beads were then added to the Eppendorf tube to further homogenise the sample whilst being vortexed. The use of beads is normally applied to genomic studies in order to extract DNA from cells (Roberts, 2007).

6.8.2 The levels of thiamethoxam and clothianidin

Clothianidin is a known metabolite of thiamethoxam and therefore was expected to be detected within the bumblebee samples. The data given in Tables 6.1 to 6.4 show a concentration of each neonicotinoid at the time of analysis; however, in order to determine an understanding of the true exposure to thiamethoxam, the detected levels of clothianidin and thiamethoxam must be pooled together. It must be noted that some information (quantity) is lost where values fall below the LOD and thus the true limit of exposure cannot be determined.

Although it is known that there were two possible concentrations on which the bees could have been feeding, in addition to a blank control group, it has not been possible to find any data which enable the comparison between the levels of thiamethoxam fed to bees and the amounts recovered, therefore, making it difficult to set a threshold to determine which sampled bees belong to which group. From the observed concentrations presented in Tables 6.1 to 6.4 all that can be assumed is whether the bees may have been part of the high-dose, low-dose or control group. It was assumed that bees containing clothianidin below the LOQ were part of the MD group; an upper limit for this group was set at 30 ppbv based on visual observations from the data. There were found to be strong visual correlations between detectable levels of thiamethoxam and high levels of clothianidin, suggesting that the bee had died, or was frozen - as defined by the experimental procedure of Elston *et al.,* (2013), before thiamethoxam was able to completely metabolise. However, high levels of clothianidin did not necessarily mean that thiamethoxam would be detected, as information on the actual rate of metabolism, energy expenditure or size of the each bee is either unknown or was not recorded prior to analysis. In the study by Elston *et al.,* (2013) it was not possible to determine the consumption of pollen within each micro-colony due to the storage of pollen in the nest by the bees. Consequently, even if the feeding data were present, it would be difficult to determine the exact contribution that each of the food sources would have on the overall detected concentration.

Honeybees are thought to act as 'filters', resulting in low levels of environmental contaminants in honey (Bogdanov, 2006); this presumably arises from the metabolism and extraction of ingested chemicals from the body. If so, then the same principle can

surely be applied to bumblebees. Although it was not possible to find any data on the metabolism of thiamethoxam in bees, concentrations of 20 and 50 ppbv of imidacloprid were reported to be completely eliminated from the honeybee after six and twenty four hours exposure, respectively (Suchail *et al.,* 2004, as cited in FERA (2013).

The dietary requirements of the bumblebee were found to alter depending on age as well as the amount of amino acids present in their food source (Stabler, Paoli, Nicolson, & Wright, 2015). It is not clear if this is acknowledged by feeding trials investigating pesticide exposure, as this may have some influence on the levels of sugar solution consumed, thus increasing/ decreasing the ingested amount of pesticide contained within their feed. Indeed, it is acknowledged that artificial feeds may affect bees differently to natural foods (Godfray *et al.*, 2014). It was reported that bees demonstrated a preference to sugar solutions containing neonicotinoids, although consumption of these agrochemicals reduced food intake (Kessler *et al.*, 2015). This is consistent with findings of Elston *et al.,* (2013) who reported that the amount of sugar solution consumed was lowest for the High dose treatment than for those from the Low dose group, compared to the control. The presence of neonicotinoids at low concentrations (0.7 ppbv nectar and 6 ppbv) in food sources was found to reduce the efficiency of pollen foraging (Feltham *et al.*, 2014) as well as a reducing in queen production by 85 % (Whitehorn *et al.*, 2012). However, the findings of Whitehorn *et al.,* (2012) have been questioned, as exposure was conducted over a two week period, meaning bumblebees would have to solely feed on treated crops (EFSA, 2012). Further to this, it was found that bumblebees do not feed exclusively on one particular crop, despite being located next to an oilseed rape field (treated with thiamethoxam); only 35 % of the pollen analysed was from this crop

(Thompson *et al.*, 2013). As previously considered in Chapter 5, field and semi-field studies have found that there are unfavourable effects to both honeybee and bumblebees feeding on crops treated with neonicotinoid-based seed treatments (Cutler, Scott-Dupree, Sultan, Mcfarlane, & Brewer, 2014; Cutler & Scott-Dupree, 2007, 2014; Pilling, Campbell, Coulson, Ruddle, & Tornier, 2013; Schmuck, Schöning, Stork, & Schramel, 2001; Thompson *et al.*, 2013). It remains difficult to control or standardise procedures within studies of this nature (Goulson, 2015). However, laboratory studies are perhaps guilty of using doses higher than those found within the field or use of unrealistic time frames (EFSA, 2012), while the well-defined protocols of laboratory studies are able to allow for better observations that are not influenced by other stressors found within the field (Godfray *et al.*, 2014).

6.9 Conclusion

Without the related feeding data for all of the samples analysed, it is not possible to make any direct comparisons between the amounts of neonicotinoids consumed and the levels detected within the bee bodies. The unavailability of this material is due to the agreement of it only becoming accessible after the samples had been analysed. This was delayed due to setbacks relating to machine errors and a lack of confidence in the analytical equipment available at the time, which has since been replaced.

If the feeding information was available prior to analysis, then it would be preferable to have analysed the bees found in Box 4 individually, as oppose to samples of two or three; as this box contained the highest quantifiable levels of neonicotinoids. This would provide a better indication of the levels consumed per bee. However, as this information was not

known, the bees were analysed according to the pre-determined groups, as the analysis of greater number of bees would increase the likelihood of detection, a strategy that is also applied elsewhere (Bańka *et al.*, 2010; Bargańska *et al.*, 2014). This was also matched with an injection volume of 20 µl, which allows for more ions to be introduced into the Q-TOF LC/MS.

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CHAPTER 7
Discussion

The aims of this thesis were to determine the presence of pesticides within UK honeybee comb wax and to monitor the accumulation of such chemicals over a two-year time frame, as well as determining the levels of neonicotinoids, which may be contained with pollen and nectar samples of oilseed rape, resulting from their application as a seed-treatment. The aims of a later, additional study, was to quantify the levels of thiamethoxam and its metabolite in bumblebee bodies, following exposure to the parent compound in a feeding trial conducted by researchers at Imperial College London.

The aim of Chapter 3 was met through the analysis of 152 comb wax samples, obtained through various advertising campaigns and appeals to beekeeping associations. Analysis of all the samples was conducted using GC-MS; however, due to the detection limit of the

instrument, it was not possible to detect any chemical present within the wax unless found above 1 ppmv. Later analysis of 15 samples, using a much more sensitive Q-TOF LC/MS, revealed that tau-fluvalinate was present within 11 of these. This was not considered surprising as this chemical was once a popular anti-*Varroa* treatment. Questionnaires received from beekeepers reported that this chemical was more popular in Scotland than the rest of the UK. Although no Scottish samples were analysed using Q-TOF LC/MS, it is assumed that it would represent a large number of detections within that country. Butylated hydroxtoluene (BHT) was also detected by GC-MS and this was originally thought to be due to possible wax adulteration during recycling; however, it is also used in chemical treatments as a stabiliser, which may be the probably source of its detection, although this cannot be confirmed.

It was not possible to find information on a study in the literature that had attempted to monitor the accumulation of pesticides within comb wax using new 'clear' or 'pesticide free' foundation as a starting point. So here is presented a procedure which could be further utilised for observing potential chemical accumulations in this medium. However, it was concluded that the aim of Chapter 4 was not met, due to the small number of samples used, thus making it difficult to draw any definite conclusions on accumulation. From the analysis of foundation wax submitted by beekeepers, it has been possible to learn that tau-fluvalinate was found to be persistent in wax, which could lead to continuous exposure to the developing brood. If this study was to be conducted again, it is suggested that a bigger sample set should be used. It would also be useful to be able to quantify the levels of pesticides.

Through surveying the levels of neonicotinoids found in oilseed rape nectar, it has also been possible to identify varied concentrations of these chemicals. No quantifiable levels were identified in pollen. The use of 100 µl of nectar within Chapter 5 is considered to be novel, as the small volumes used have not been quoted elsewhere described in the literature. Although the process can be time consuming, collection directly from the flower avoids possible metabolisation of the target chemical though prior collection by a pollinator. However, other sources of compound break-down cannot be avoided i.e. exposure to sunlight. In order to match the sample volumes, a micro-scale QuEChERS technique was also developed, which showed good recovery rates (78 – 102 %). It is felt that the extraction methodology and small sample volumes used in this study can be applied successfully to future research. However, due to the variability of results, further information including: soil type, ground moisture and metrological data may be required in order to aide interpretation.

The aim of Chapter 6, which was to determine the levels of thiamethoxam in bumblebee bodies, was successful. However, it is felt that further interpretation of the results is needed. This would be in the form of comparing the values obtained to the feeding trial information in order to confirm the results were obtained successfully. Yet, despite this, an extraction technique has been developed and found to give three sets of results - which could relate to the three feeding groups.

Recommendations for future work

The introduction of 'random comb wax screening', as part of the already existing colony inspections, performed by the National Bee Unit (NBU) would help to generate a clearer

picture of the contamination of UK comb wax. Relaying the resulting information back to beekeepers may help to reduce contamination over the long-term, as it might make beekeepers more concious of what may be present in their hives. Likewise, a smaller project analysing the various sheets of foundation wax available to UK beekeepers in the market may highlight the issue of contaminated foundation. It would alo be interesting to try and monitor the accumulation of other environmental contaminants within the hive.

Dissemination of work

Publications

Wisniewski, K. D., Kirk W. D. J., Drijfhout, F. (2014). What levels of neonicotinoids are found in UK oilseed rape fields? *British Bee Journal*, Sept 2014.

Other publications

Pringle, J. K., Jervis, J. R., Roberts, D., Dick, H. C., Wisniewski, K. D., Cassidy, N. J., Cassella, J. P. (2016). Long-term geophysical monitoring of simulated clandestine graves using electrical and Ground Penetrating Radar methods: 4-6 years after burial. *Journal of Forensic Sciences,* 61, 309-321. doi:10.1111/1566-4029.13009.

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Presentations

IUSSI14 Conference, Cairns Australia, 13th – 18th July 2014. *What are the levels of neonicotinoids in UK oilseed rape fields?*

NSBKA Meeting, Quaker Meeting House, 6th February 2013. *Surveying the levels of pesticides in UK apiaries.*

Posters

BBKA Spring Convention 2014, Harper Adams University, 5th April 2014. Presented a poster - Surveying the levels of pesticides in oilseed rape.

Joint BES, BS and SEB Meeting, Charles Darwin House 22nd – 24th January 2014. Presented a poster – Surveying the levels of pesticides in oilseed rape.

Eurbee 5, Martin-Luther Universität Germany, 3rd – 7th September 2012. *Surveying the levels of pesticides in UK apiaries.*

EPSAM Fest 2012, Keele University, 4th July 2012. *Surveying the levels of pesticides in UK apiaries.*

BBKA Spring Convention 2012, Harper Adams University, 21st April 2012. *Surveying the levels of pesticides in UK apiaries.*

ENTO11, University of Greenwich, 7th – 9th September 2011, *Pesticide residues within apiary samples.*

Midlands Hub Poster Competition, Nottingham Conference Centre, 11th July 2011. *Pesticides: Something worth buzzing about?*

EPSAM FEST, Keele University, 11th May 2011. *Pesticides: Something worth buzzing about?*

BBKA Spring Convention 2011, Stoneleigh Park, 16th April 2011. *What are the levels of pesticide residues in beeswax - can you help?*

Other posters

NSGG Conference, Geological Society of London, 3rd December 2014. *Quantifying geophysical response of graves burial age: electrical resistivity magnetic susceptibility and GPR methods.*

Can you help Keele University researchers survey the levels of pesticide residues in apiary sources?

Honey bee decline has been highlighted as a key concern over the last 20 years or so and many investigations have been carried out to explore the reasons behind this.

In reference to *BBKA News*, p14, August 2010, pesticides or their residues within beehives could have a considerable negative effect on honey bee health, thus making them more susceptible to disease. In addition bee behaviour is thought to be adversely affected, for example an increased number of bees are prone to disorientation and can therefore lose their way back to the hive. With regard to pesticides, various investigations have been conducted across numerous European countries and in the United States. However, as far as we are aware, no work to date has been conducted within the UK to determine pesticide levels in apiary sources, with the exception of honey.

As part of a PhD research project at Keele University it is our aim to survey wax, pollen and bee samples from across the UK using highly sensitive chromatographic techniques to determine which pesticides exist within UK apiaries. We are currently investigating pesticide extraction techniques and optimising our analysis methods, but to achieve success in this project, Keele University needs willing and enthusiastic beekeepers to volunteer themselves and donate a section of comb from one hive so that it can be subjected to analysis. Participation is anonymous and each participating individual will receive a copy of their sample results. A maximum of 500 samples are to be screened and analysed so this can only be offered as a 'first-come, first-served' basis.

We will have a stand at the 2011 Spring Convention on 16 April for those who are interested to come along and collect a 'sample collection pack'. which will contain necessary instructions and equipment needed for you to help in this project. We cannot do this without your help so please come along and support us.

Kristopher Wisniewski, Keele University,
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Find out what pesticides are present in your hives

Researchers are optimising their methods for measuring pesticides and want to analyse samples from hives in apiaries in the UK. You can help by sending the researchers a sample of comb from your hive. In return they will tell you what pesticides they find in your wax, pollen and bees. Only you will know your hive's results as all data will be anonymised.

Get your sample collection pack from the Keele University stand at the Spring Convention and contribute to the UK survey for pesticide residues

Appendix A: BBKA news article (April 2011, No: 188) requesting for volunteer beekeepers to participate in the beeswax study described in chapter 3.(Article accessible online at: www.bbka.org.uk/files/library/bbka_april_2011_1305796240.pdf) [Accessed 22/01/2016]

Instructions

Thank you for agreeing to take part in this study.
This study requires wax, capped brood and pollen samples. A suggested way of obtaining such samples is to remove a section of comb wax from the hive. Ideally this comb should contain both capped brood and pollen samples (figure 1). If this is not possible then wax and capped brood samples are preferred.

- Please collect the sample between 27 June 2011 and 6 July 2011 and state the exact day on both the provided questionnaire and label within the pack.

- Please clean any hive or cutting equipment used to cut the comb wax with soapy water and then the provided alcohol wipes prior to collection; this is to avoid/limit contamination from other hives on which you use the same tools.

- Likewise a pair of disposable gloves has been provided. These are to place over the top of your existing gloves and are again to avoid any secondary contamination.

- Once the wax has been cut, please place the comb within the provided container and seal securely.

- In order to eliminate the brood from further developing...we ask that you put the cut comb container in the freezer for around and hour or so. This is will kill the brood without causing any pain.

- Please write your name and address plus the date of collection on the provided label and stick it to the cut comb container.

- Once collected please return the sample as soon as possible within the provided self- addressed envelope along with the completed questionnaire (see other side). Any additional information you wish to include is also welcome.

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Questionnaire

Appendix B: Instruction sheet provided to beekeepers detailing a protocol on how to collect beeswax samples needed for the study described in chapter 3.

Questionnaire

When completing this questionnaire please answer the questions honestly and provide as much detail as possible (where applicable) in order to assist with sample processing in the laboratory.

Please state the city/town and the county in which your hive is based:

City/town...

County............................

....

Please tick what best describes your local environment:

[ ] Rural [ ] Semi-rural [ ] Urban

Please estimate the age of the comb from which you will provide a sample: (YEARS/MONTHS)

Do you currently use any kind in or around your hive? (Tick all that are applicable)

[ ] Apistan [ ] Bayverol [ ] Fumidil B [ ]

Thymol [ ] Paradichlorobenzene (PDB) [ ] Other

If other, please specify...

....

Are you aware of any recent pesticide applications at the time of sample collection?

If possible, please give details on the local flora including agricultural crops that currently surround your hive.

Please briefly describe the condition of your colony:

If used, please state the origin of the foundation wax used for the collected sample?

Do you recycle comb wax? If so, how?

Please give the exact date of the sample collection: (DD/MM/YYYY)

Would you be willing to participate in later studies? I.e. provide further samples if needed?

[ ] Yes [ ] No

Thank you for your time. You will receive your results as soon as all of the samples have been processed.

KEELE UNIVERSITY

Appendix C: Questionnaire provided during 2011 – 2012 beekeeping season. Questionnaire was to be completed and returned with every beeswax sample, as instructed in Appendix B.

(Quantitative) Questionnaire results

England (n = 98)

Please tick what best describes your local environment:

Environment	Response	%
Rural	50	51.02
Semi-rural	28	28.57
Urban	17	17.35
Not specified	3	3.06

Do you currently use any kind of treatment in or around your hive?

Treatment	Response	%
Apistan (tau-fluvalinate)	12	7.55
Apivar (amitraz)	1	0.63
Bayverol	3	1.89
Flumidil B	16	10.06
Thymol	59	37.11
Oxalic acid	32	20.13
Formic acid	5	3.14
Not Specified	6	3.77
PDB	2	1.26
Other	12	7.55
No	11	6.92

Are you aware of any recent pesticide applications at the time of sample collection?

Aware	Response	%
Yes	5	5.10
No	80	81.63
Not Specified	13	13.27

(a) Do you recycle comb wax?[5] *(b)* If so, how?

Recycling	Response	%
Solar	27	26.21
Steam	13	12.62
Other	12	11.65
No	23	22.33
Not Specified	28	27.18

Trade	Response	%
Trade In	38	38.78
Not Traded	32	32.65
Own Foundation	7	7.14
Not Specified	21	21.43

Estimate the age of the comb from which you provide a sample.

Comb age (months)	Response	%
0-6	13	13.27
7-12	20	20.41
13-18	15	15.31
19-24	11	11.22
25-30	7	7.14
31-36	8	8.16
37-42	4	4.08
43-54	1	1.02
54+	1	1.02
N/S	19	19.39

Scotland (n = 46)

Please tick what best describes your local environment:

Location	Response	%
Rural	28	60.87
Semi-rural	13	28.26
Urban	5	10.87
Not specified	0	0.00

[5] Multiple methods of recycling have been recorded (e.g. candle making, wax trading), where applicable.

Appendix D: Quantitative questionnaire results from relating to the questionnaires (Appendix C) returned with each beeswax sample. Results have been separated into each respective country: England, Scotland and Wales.

Do you currently use any kind of treatment in or around your hive?

Treatment	Response	%
Apistan (tau-fluvalinate)	19	22.62
Apivar (amitraz)	2	2.38
Bayverol	2	2.38
Flumidil B	3	3.57
Thymol	15	17.86
Oxalic acid	22	26.19
Formic acid	5	5.95
para-dichlorobenzene	1	1.19
Other	7	8.33
No	4	4.76
Not Specified	4	4.76

Are you aware of any recent pesticide applications at the time of sample collection?

Aware	Response	%
Yes	3	6.52
No	43	93.48
Not Specified	0	0

(a) Do you recycle comb wax? *(b)* If so, how?

Recycling	Response	%
Solar	5	10.87
Steam	2	4.35
Other	3	6.52
No	24	52.17
Not Specified	12	26.09

Trade	Response	%
Trade In	11	23.91
Not Traded	28	60.87
Own Foundation	2	4.35
Not Specified	5	10.87

Estimate the age of the comb from which you provide a sample.

Comb age (months)	Response	%
0-6	10	21.74
7-12	5	10.87
13-18	2	4.35
19-24	7	15.22
25-30	6	13.04
31-36	6	13.04
37-42	2	4.35
43-54	2	4.35
54+	2	4.35
N/S	4	8.70

Wales (n = 8)

Please tick what best describes your local environment:

Location	Response	%
Rural	3	37.50
Semi-rural	4	50.00
Urban	1	12.50
Not specified	0	0

Do you currently use any kind of treatment in or around your hive?

Treatment	Response	%
Apistan (tau-fluvalinate)	0	0
Apivar (amitraz)	0	0
Bayverol	0	0
Flumidil B	0	0
Thymol	6	46.15
Oxalic acid	6	46.15
Formic acid	0	0
para-dichlorobenzene	0	0
Other	0	0
No	0	0
Not Specified	1	7.69

Appendix D: Quantitative questionnaire results from relating to the questionnaires (Appendix C) returned with each beeswax sample. Results have been separated into each respective country: England, Scotland and Wales.

Are you aware of any recent pesticide applications at the time of sample collection?

Aware	Number	%
Yes	0	0
No	6	75.00
Not Specified	2	25.00

(a) Do you recycle comb wax? *(b)* If so, how?

Recycling	Number	%
Solar	2	25.00
Steam	0	0
Other	0	0
No	4	50.00
Not Specified	2	25.00

Trade	Number	%
Trade In	2	25.00
Not Traded	0	0
Own Foundation	0	0
Not specified	6	75.00

Estimate the age of the comb from which you provide a sample.

Comb age (months)	Response	%
0-6	1	12.50
7-12	3	37.50
13-18	0	0.00
19-24	1	12.50
25-30	1	12.50
31-36	1	12.50
37-42	0	0.00
43-54	0	0.00
54+	0	0.00
N/S	1	12.50

Appendix D: Quantitative questionnaire results from relating to the questionnaires (Appendix C) returned with each beeswax sample. Results have been separated into each respective country: England, Scotland and Wales.

Only thymol reported here, as no other pesticide was detected. BHT also reported.

Scotland

Sample ID	Thymol	Thymol used?	BHT
ARL_1_5/7/11		✔	
ARL_2_5/7/11		✘	
ARL_3_5/7/11		✘	✔
ARL_4_8/7/11	✔	✔	✔
ARL_5_8/7/11		✘	
ARL_6_18/7/11		✘	✔
AYR_1_4/7/11		✘	✔
AYR_2_5/7/11		✘	
AYR_3_8/7/11		✔	✔
AYR_4_18/7/11		✘	
BEW_1_5/7/11		✘	✔
BEW_2_6/7/11		✘	✔
BOR_1_4/7/11		?	✔
BOR_2_6/7/11		✘	
BOR_3_8/7/11	✔	✔	✔
DGY_1_6/7/11	✔	✔	✔
DNB_1_5/7/11		✘	✔
DNB_2_15/7/11		✘	✔
FIF_1_1/7/11	✔	✔	✔
FIF_2_4/7/11	✔	✔	✔
FIF_3_5/7/11		✘	✔
FIF_4_6/7/11	✔	✘	
FIF_5_8/7/11	✔	✔	✔
FIF_6_12/7/11	✔	?	✔
FIF_7_12/7/11	✔	✔	✔
HLD_1_5/7/11		✘	✔
MLN_1_30/6/11	✔	✔	✔
MLN_2_11/8/11	✔	✔	✔
PEE_1_5/7/11		?	✔
PER_1_5/7/11	✔	✘	
PER_2_5/7/11		✘	✔
PER_3_9/8/11		✘	✔
RFW_1_4/7/11	✔	✘	✔
RFW_2_5/7/11	✔	?	✔
RFW_3_5/7/11	✔	✔	✔
RFW_4_31/8/11		✘	
ROC_1_4/7/11		?	
ROX_1_30/6/11		✘	
SEL_1_8/7/11	✔	✔	
STI_1_5/7/11	✔	✔	✔
STI_2_6/7/11		✘	✔
STI_3_8/7/11		✘	✔
STI_4_16/8/11		✘	✔
STI_5_16/8/11		✘	✔
TAY_1_9/8/11		✘	✔
WIS_1_14/7/11		✘	✔

Wales

Sample ID	Thymol	Thymol used?	BHT
CMN_1_8/7/11		✔	✔
GNT_1_1/7/11	✔	✔	✔
MON_1_5/7/11	✔	?	✔
WGM_1_5/7/11	✔	✔	
WGM_2_6/7/11	✔	✔	
WGM_3_1/7/11		✔	
WGM_4_11/7/11	✔	✔	
WGM_5_12/7/11		✘	✔

England

Sample ID	Thymol	Thymol used?	BHT
CAM_1_6/7/11		✔	
CAM_2_6/7/11	✔	✔	
CAM_3_6/7/11		✔	✔
CAM_4_8/7/11	✔	✔	
DBY_1_12/7/11	✔	✔	
DEV_1_30/6/11		✘	
DEV_2_6/7/11	✔	✔	✔
DEV_3_6/7/11		✔	
DEV_4_6/7/11	✔	✔	✔
DEV_5_6/7/11	✔	✔	✔
DEV_6_6/7/11		✔	
DEV_7_8/7/11	✔	?	
DEV_8_12/7/11	✔	✔	
DEV_9_18/7/11	✔	✘	
DEV_10_18/7/11	✔	✘	
DOR_1_30/6/11		✘	

Appendix E: GC-MS raw data for beeswax samples analysed in Chapter 3. Only thymol and butylated hydroxytoluene (BHT) are reported, in addition to whether thymol was reportedly used by the beekeeper.

Appendix E

Sample ID	Thymol	Thymol used?	BHT
DOR_2_5/7/11	✔	✘	✔
DOR_3_12/7/11		✔	
DUR_1_8/7/11		✘	
ERY_1_15/7/11	✔	✘	
ERY_2_15/7/11	✔	✘	
ESS_1_4/7/11		✘	✔
ESS_2_6/7/11		✔	
GLS_1_6/7/11		✔	
GLS_2_15/7/11		✘	
HAM_1_3/6/11		✔	
HAM_2_1/7/11		✔	
HAM_3_6/7/11		✘	✔
HAM_4_7/7/11		✘	
HAM_5_8/7/11		✘	
HAM_6_8/7/11	✔	✔	
HAM_7_8/7/11		✔	
HAM_8_8/7/11		✔	
HAM_9_8/7/11	✔	✔	✔
HRT_1_30/7/11	✔	✔	✔
HRT_2_5/7/11		✔	
HRT_3_12/7/11	✔	✔	✔
HRT_4_12/7/11	✔	✔	
KEN_1_4/7/11		✔	
KEN_2_5/7/11	✔	✘	
KEN_3_5/7/11	✔	✔	✔
KEN_4_5/7/11		✘	✔
KEN_5_8/7/11	✔	✔	
KEN_6_8/7/11		✔	
KEN_7_12/7/11		✔	
LAN_1_30/6/11		✘	✔
LAN_2_8/7/11		✔	
LIN_1_1/8/11		✘	✔
LON_1_6/7/11		✔	
MSX_1_7/7/11	✔	✔	✔
NBL_1_6/7/11		✔	
NBL_2_6/7/11	✔	✔	✔
NFK_1_4/7/11	✔	✔	✔
NFK_2_5/7/11	✔	✔	
NFK_3_6/7/11	✔	✔	
NFK_4_6/7/11		✔	✔
NFK_5_7/7/11	✔	✔	
NFK_6_8/7/11	✔	✔	
NFK_7_25/7/11	✔	✔	✔
NFK_8_25/7/11	✔	✔	✔
NFK_9_25/7/11	✔	✔	✔
NFK_10_25/7/11	✔	✔	✔
NFK_11_27/7/11	✔	✔	
SAL_1_5/7/11	✔	✔	
SFK_1_12/7/11	✔	✔	
SOM_1_30/6/11	✔	✘	
SOM_2_5/7/11		✘	
SOM_3_5/7/11	✔	?	
SOM_4_6/7/11		✘	
SOM_5_8/7/11		✔	
SOM_6_8/7/11	✔	✔	
SOM_7_12/7/11	✔	✔	
SSX_1_12/1/12	✔	?	
STS_1_18/8/11		✘	✔
STS_2_18/8/11		✘	
SXE_1_1/7/11		✘	
SXE_2_1/7/11	✔	✘	
SXE_3_1/7/11		✘	✔
SXE_4_6/7/11		✘	✔
SXW_1_1/7/11	✔	✔	
SXW_2_6/7/11	✔	✔	
SYR_1_8/7/11		✔	
WAR_1_6/7/11	✔	✔	✔
WIL_1_1/7/11		✘	✔
WIL_2_8/8/11		✔	✔
WIL_3_8/7/11		✘	
WIL_4_8/7/11		✘	
WIL_5_12/7/11		✔	✔
WOR_1_12/7/11	✔	✘	
WYK_1_30/6/11		?	
WYK_2_4/7/11	✔	✘	
WYK_3_6/7/11	✔	✔	
WYK_4_6/7/11	✔	✔	
WYK_5_6/7/11	✔	✔	
YKS_1_5/7/11		✘	✔
YKS_2_8/7/11	✔	✔	
YKS_3_8/7/11	✔	✔	
IOM_1_30/4/11		✘	
IOM_2_5/7/11		✘	✔
IOM_3_5/7/11		✘	✔

Appendix E: GC-MS raw data for beeswax samples analysed in chapter 3. Only thymol and butylated hydroxytoluene (BHT) are reported, in addition to whether thymol was reportedly used by the beekeeper.

Surveying the possible accumulation of pesticides in wax samples over time

Dear beekeeper, thank you for agreeing to take part in this study.

The aim of this study is to be able to monitor the possible accumulation of pesticides in wax samples over time. Last year 'organic' foundation was distributed to a number of beekeepers in one of five key regions (North Staffordshire & South Cheshire, South West, Eastern, South East and Yorkshire and the Humber). We hope to use the distributed foundation wax as a background reading from which any possible accumulation of pesticides in the wax can be determined. There is also interest in monitoring any levels of pesticides in larvae contained within the wax as well as collected pollen.

For those who have not participated in any previous studies a suggested way of obtaining samples which would be useful to this study is to remove a section of comb wax from the hive; ideally containing both capped brood and pollen samples. If this is not possible then wax and capped brood samples are preferred.

Please avoid including nectar/honey within the section of cut comb, as this is difficult to extract prior to analysis.

- Please collect the sample between the remainder of June and July and state the exact day on both the returned questionnaire and wax sample(s).

- Once the wax has been cut, please place the comb within the provided container and seal securely.

- In order to eliminate the brood from further developing we ask that you put the cut comb container in the freezer for around and hour or so. This is will kill the brood without causing any pain.

- Please write your name and address plus the date of collection on the provided label and stick it to the cut comb container.

- Once collected please return the sample as soon as possible within the provided self-addressed envelope along with the completed questionnaire (see other side). Any additional information you wish to include is also welcome.

It is understood that not all of those participating in the study use National bee hives, however it was only possible to obtain organic foundation for National hives only. If you do not use a national and have perhaps used the foundation in your hive as a starter strip or some other way please mention this when returning the questionnaire. Alternatively, if you have used your own foundation wax we ask you to please kindly provide a sample of this (3 g minimum) so that a background reading may be established.

When completing this questionnaire provide as much detail as possible (where applicable) in order to assist with sample processing in the laboratory. Please feel free to include any additional information that you may feel is relevant to this study.

Appendix F: Instruction sheet provided to beekeepers detailing a protocol on how to collect beeswax samples needed for the study described in Chapter 4.

Questionnaire

Please state the city/town and the county in which your hive is based:

City/town.. County....................................

Please tick what best describes your local environment:

[ ] Rural [ ] Semi-rural [ ] Urban

Type of hive used:

[ ] National [ ] Smith [ ] Langstroth [ ] WBC

[ ] Dadant [ ] Commercial [ ] Top-bar [ ] Other

What is the source of you foundation wax used in this study?

[ ] Provided by Keele [ ] Naturally drawn (No foundation) [ ] *Home recycled

[ ] *Bought from distributer [ ] *Other (please specify)...

*(Please provide approximately a separate 3 g sample of blank foundation also when returning this questionnaire)

Approximately when did you place the provided foundation wax into the hive? : (YEARS/MONTHS)

If using own foundation or newly drawn comb what is the approximate age? ( )

Do you currently use any of the following in or around your hive? (Tick all that are applicable)

[ ] Apistan [ ] Bayverol [ ] Fumidil B [ ] Thymol [ ] Paradichlorobenzene (PDB)

[ ] Other (Please specify)...

Are you aware of any recent pesticide applications at the time of sample collection?

If possible, please give details on the local flora including agricultural crops that currently surround your hive.

Please briefly describe the condition of your colony:

How many colony mortalities have you experienced since last winter (2012)?

Please give the date of the sample collection: (DD/MM/YYYY)

Thank you for your time. You will receive your results as soon as all of the samples have been processed.

Appendix G: Questionnaire provided during 2012 – 2013 and 2013 - 2014 beekeeping seasons. Questionnaire was to be completed and returned with every beeswax sample, as instructed in Appendix F.

(Quantitative) Questionnaire results

1st year (n = 16)

Please tick what best describes your local environment:

Environment	Response	%
Rural	9	56.25
Semi-rural	7	43.75
Urban	0	0
Not specified	0	0

Type of hive used:

Hive type	Response	%
National	13	81.25
Smith	0	0
Langstroth	0	0
WBC	0	0
Dadant	1	6.25
Commercial	0	0
Top-bar	0	0
Other	2	12.5

What is the source of you foundation wax used in this study?

Foundation Source	Response	%
Keele	13	81.25
Natural	0	0
Home made	0	0
Distributer	3	18.75
Other	0	0

Do you currently use any of the following in or around your hive?

Treatment	Response	%
Apistan (tau-fluvalinate)	2	12.5
Apivar (amitraz)	0	0
Bayverol	0	0
Flumidil B	0	0
Thymol	12	75
Oxalic acid	8	50
Formic acid	0	0
para-dichlorobenzene	0	0
Other	2	12.5
No	3	18.75
Not Specified	0	0

Are you aware of any recent pesticide applications at the time of sample collection?

Aware	Response	%
Yes	3	18.75
No	13	81.25
N/S	0	0

How many colony mortalities have you experienced since last winter (2012)?

Colonies lost	Response	%
0	6	37.5
1 - 2	5	31.25
3 - 4	2	12.5
5 - 10	1	6.25
11 - 20	0	0
25 - 50	2	12.5

Appendix H: First year questionnaire (Appendix G) results, returned with the second collected beeswax sample. Only quantitative results have been tabulated.

(Quantitative) Questionnaire results

2nd year (n = 2)

Please tick what best describes your local environment:

Environment	Response	%
Rural	9	56.25
Semi-rural	7	43.75
Urban	0	0
Not specified	0	0

Type of hive used:

Hive type	Response	%
National	13	81.25
Smith	0	0
Langstroth	0	0
WBC	0	0
Dadant	1	6.25
Commercial	0	0
Top-bar	0	0
Other	2	12.5

What is the source of you foundation wax used in this study?

Foundation Source	Response	%
Keele	13	81.25
Natural	0	0
Home made	0	0
Distributer	3	18.75
Other	0	0

Do you currently use any of the following in or around your hive?

Treatment	Response	%
Apistan (tau-fluvalinate)	2	12.5
Apivar (amitraz)	0	0
Bayverol	0	0
Flumidil B	0	0
Thymol	12	75
Oxalic acid	8	50
Formic acid	0	0
para-dichlorobenzene	0	0
Other	2	12.5
No	3	18.75
Not Specified	0	0

Are you aware of any recent pesticide applications at the time of sample collection?

Aware	Response	%
Yes	0	0
No	1	50
N/S	1	50

How many colony losses have you experienced since last year (2012)?

Colonies lost	Response	%
0	1	50
1 – 2	0	0
3 – 4	0	0
5 -10	0	0
11 – 20	0	0
25 – 50	0	0
Not specified	1	50

Appendix H: Second year questionnaire (Appendix G) results, returned with the second collected beeswax sample. Only quantitative results have been tabulated.

OSR Study - Collection Sheet

Name: Date: DD / MM / YY Time (on): Time (off):

Location: Field: Bee coverage:

Weather: Temp: °C Bloom quality:

Nectar

% Sugar		
1	2	3
4	5	

Av. Vol of nectar from each flower (µl)

1	5	9
2	6	10
3	7	Average:
4	8	Error:

Total volume collected (µl):

Height of rape (cm)		
1	5	9
2	6	10
3	7	Average:
4	8	Error:

Vial Label:

Pollen Trapped

Hive:

Amount collected (g):

Predominant colour:

Vial Label:

Pollen from Bees

Amount collected: (g)

Number of bees:

Vial label:

Bees

Pollen foragers caught on rape:

Vial label:

Nectar foragers caught on rape:

Vial label:

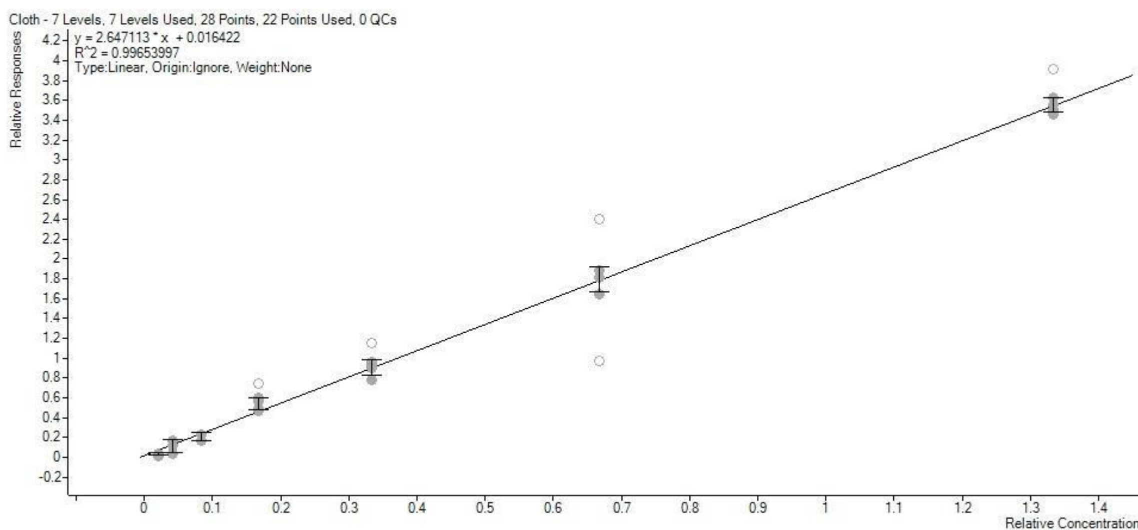
Pollen foragers caught at hive:

Vial label:

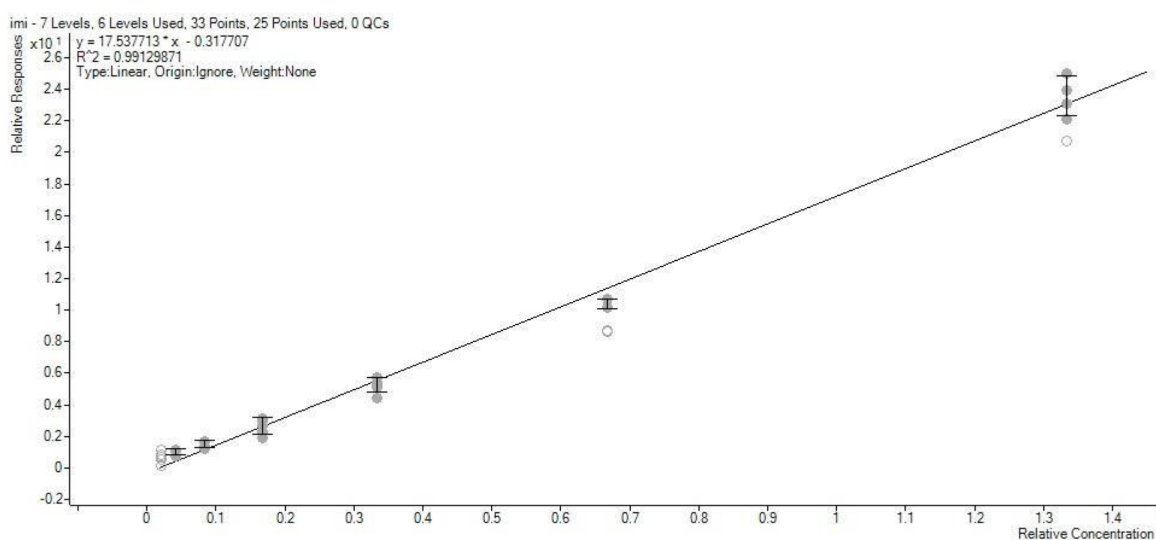
Nectar foragers caught at hive:

Vial label:

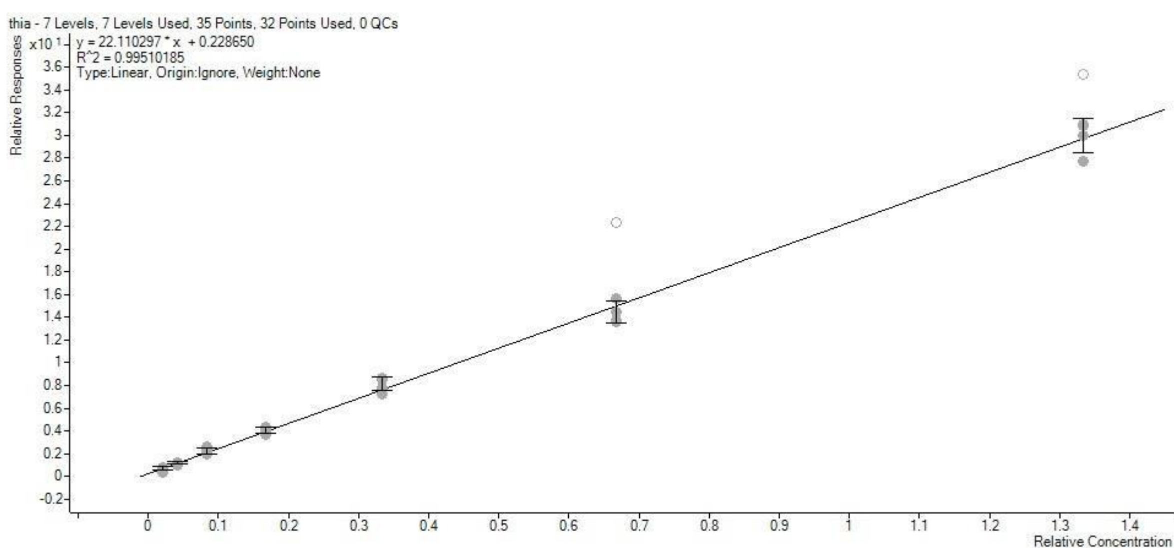
Appendix I: A blank collection sheet typically completed during every oilseed rape sample collection date. The information gathered is then to be used during sample analysis.



Above: Clothianidin calibration curve (R^2: 0.9965)



Above: Imidacloprid calibration curve (R^2: 0.9913)



Above: Thiamethoxam calibration curve (R^2: 0.9951)

Appendix J: Q-TOF-LC/MS matrix matched calibration curves for three neonicotinoids, from top to bottom: clothianidin, imidacloprid and thiamethoxam. Calibrations were matrix matched, using diluted honey to give an overall sugar content of 50%.

Thiamethoxam recoveries using micro-QuEChERS extraction method.

Name	Expected concentration	Detected concentration
Thia 1	25	23.17
Thia 2	25	17.47
Thia 3	25	17.52
Thia 4	25	21.73
Thia 5	25	17.63

Mean	Standard Dev	% Recovery	% RSD
19.50498	2.73713	78.02	14.03298

Clothianidin recoveries using micro-QuEChERS extraction method.

Name	Expected concentration	Detected concentration
Imi 1	25	23.17
Imi 2	25	17.47
Imi 3	25	17.52
Imi 4	25	21.73
Imi 5	25	17.63

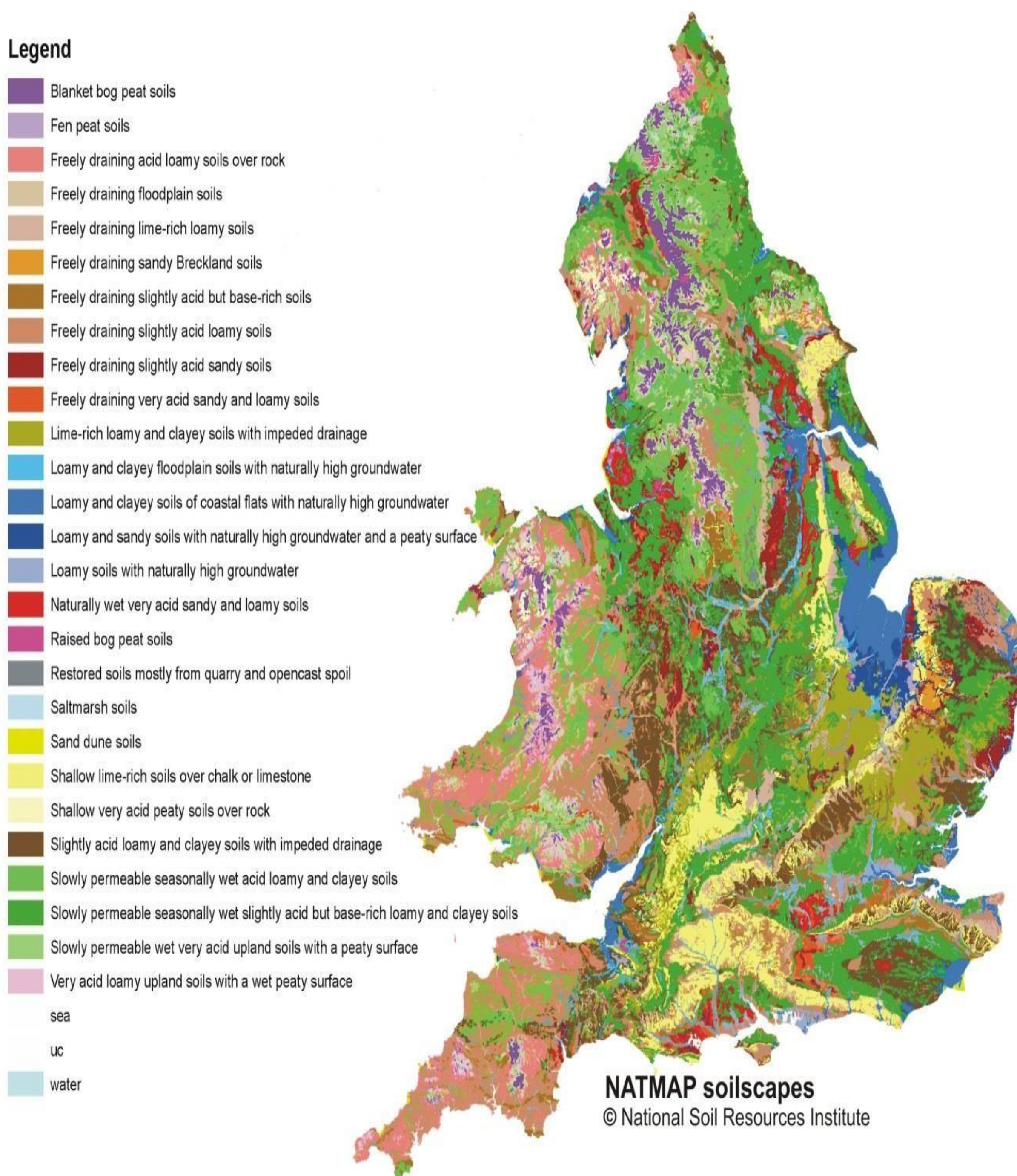
Mean	Standard Dev	% Recovery	% RSD
25.59058	2.73713	102.36	10.6958

Imidacloprid recoveries using micro-QuEChERS extraction method.

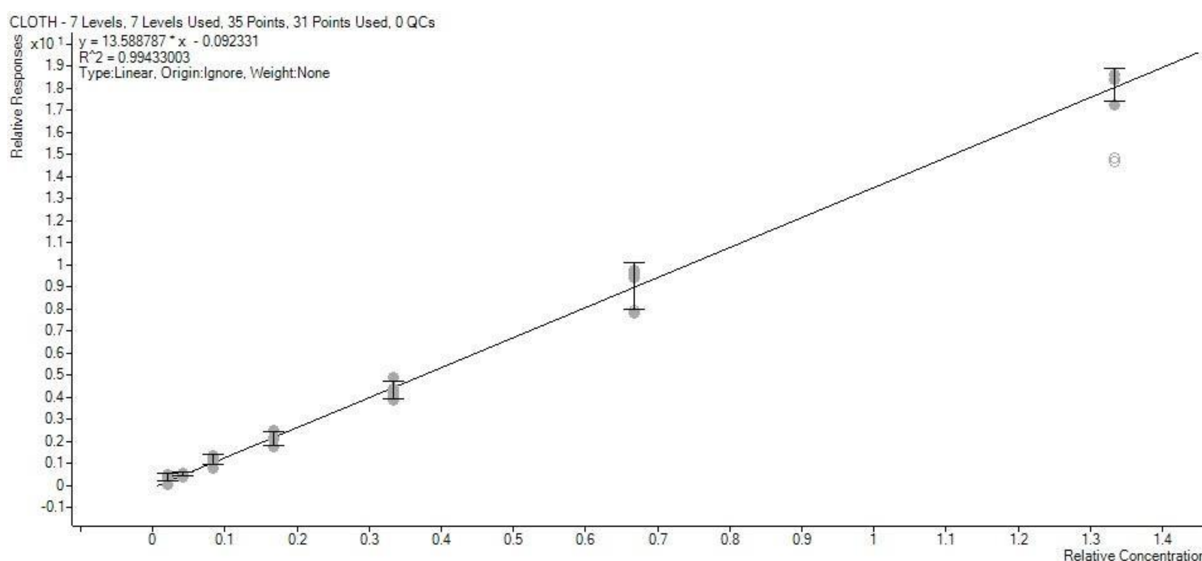
Name	Expected concentration	Detected concentration
Cloth1	25	21.449
Cloth2	25	22.4988
Cloth3	25	22.0082
Cloth4	25	21.7445
Cloth5	25	22.3818

Mean	Standard Dev	% Recovery	% RSD
22.01646	0.45064	88.07	2.0468

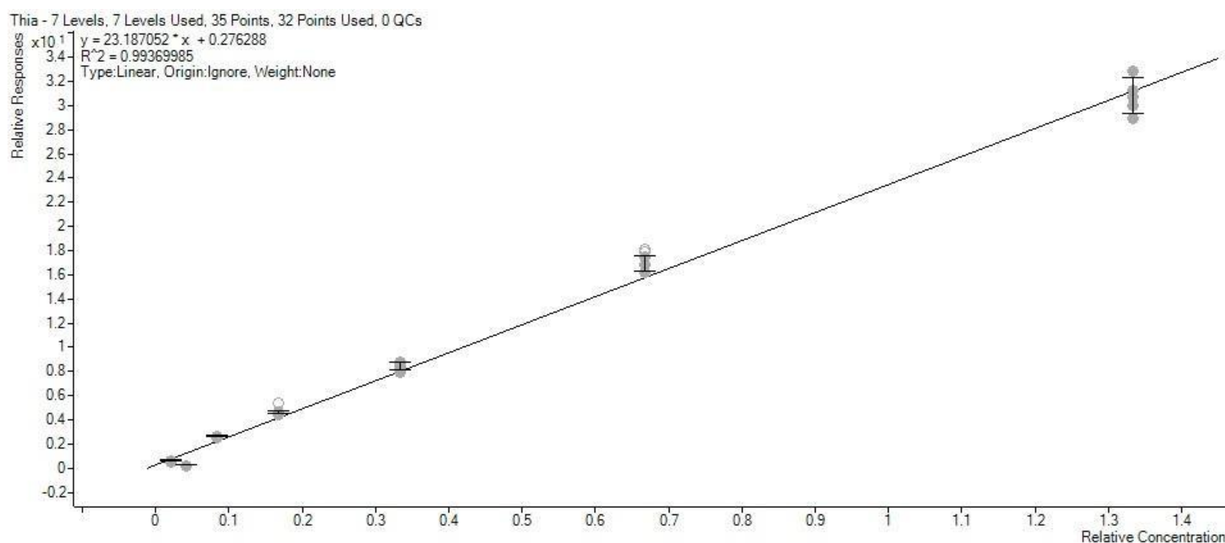
Appendix K: Raw data for the recovery of three neonicotinoids using a novel micro-QuEChERS extraction technique. Recovery values generated using 5 extraction replications.



Appendix L: NATMAP Soilscape © map, constructed by the Cranfield Soil and Agrofood Institute, showing various soil types from around England and Wales. Taken without permission from http://www.landis.org.uk [Accessed 11/06/2015].



Above: Clothianidin calibration graph (R^2: 0.9943).



Above: Thiamethoxam calibration graph (R^2: 0.9937).

Appendix M: Q-TOF-LC/MS semi-matrix matched calibration curves for two neonicotinoids, from top to bottom: clothianidin and thiamethoxam. Calibrations were semi-matrix matched, by adding each neonicotinoid to water, representing the aqueous phase of each bumblebee, before extracting with dichloromethane.