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PESTICIDE DETECTION IN MANAGED HONEY BEE COLONY RESOURCES THROUGHOUT THE ISLAND OF IRELAND

by
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Thesis presented to *Escola Superior de Biotecnologia* of the
Universidade Católica Portuguesa to fulfill the requirements of Master of Science degree in
Applied Microbiology

by
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December, 2021

Dedication

To my family.
To my talented and passionate mentors who guide my path towards beekeeping and research.
To the bees.

Resumo geral

Vários fatores, incluindo a aplicação de pesticidas na agricultura e produtos veterinários para colmeias, desempenham um papel no declínio de polinizadores selvagens e abelhas manejadas. A exposição a pesticidas foi relatada como um fator-chave nas perdas de colônias, em particular de abelhas manejadas. Os pesticidas podem se acumular no pólen e néctar de flores tratadas e não tratadas, solo e cursos d'água, e podem ser posteriormente transportados de volta para as colônias por abelhas forrageiras e acabar em recursos e indivíduos da colônia, como cera, suprimentos de alimento, abelhas e cria. Tem sido demonstrado que pesticidas sistêmicos, como os neonicotinóides, são responsáveis por danos fisiológicos, funcionais e imunológicos em abelhas. No entanto, o nível de pesticidas aos quais as abelhas estão expostas no meio ambiente e nos recursos dentro das colônias não tem sido caracterizado em várias regiões importantes para a apicultura, incluindo a ilha da Irlanda, que é o lar de mais de 50 associações de apicultores. Portanto, esta tese visa ajudar a preencher essa lacuna, identificando os níveis de pesticidas aos quais as abelhas manejadas estão expostas nos recursos apícolas na ilha da Irlanda. Amostras de mel e pólen foram coletadas por apicultores nas suas colônias em toda a ilha, e os níveis de pesticidas nessas amostras foram determinados usando espectrometria de massa em tandem por cromatografia líquida e gasosa. Os pesticidas foram extraídos das amostras usando o método de extração Dutch mini-Luke. É importante ressaltar que aqui o método Dutch mini-Luke foi otimizado para pequenas amostras (5 gramas de amostra e 10ml de solventes), que é um tamanho comum para as amostras de pólen em particular, e o método foi validado para a detecção de pesticidas em amostras de mel e pólen (Capítulo 1). Em uma segunda etapa, a técnica mini-Luke otimizada foi utilizada para investigar os níveis de pesticidas aos quais as abelhas melíferas estão expostas nas colônias nos diferentes apiários na ilha da Irlanda (Capítulo 2). Possíveis fontes de contaminação e os Níveis Máximos de Resíduos (MRLs) para os compostos detectados são determinados e discutidos. Quinze por cento de todas as amostras de toda a ilha foram consideradas positivas para pesticidas. Três inseticidas-acaricidas foram detectados na maioria das amostras positivas (85%). O inseticida Propargite foi detectado no mel e pólen, e os acaricidas para controle de Varroa, Coumaphos e Fluvalinato-Tau, foram encontrados no pólen. O método de extração de pesticidas otimizado aqui, em combinação com os métodos de análise, fornece uma ferramenta chave para detectar pesticidas nos recursos apícolas e para detectar o nível de agroquímicos aos quais as abelhas estão expostas dentro da colmeia e no meio ambiente.

Palavras chave: Detecção de pesticida, método miniaturizado, mel, pólen, inseticida.

General abstract

Multiple factors, including the application of pesticides in agriculture and veterinary in-hive products, play a role in the decline of wild pollinators and managed bees. Pesticide exposure has been reported as a key factor in managed honey bee colony losses in particular. Pesticides can accumulate in pollen and nectar of treated and untreated flowers, soil, and waterways, and can subsequently be transported back to colonies by foraging honey bee workers and end up in colony resources and individuals, such as wax, food supplies, bees, and brood. It has been shown that systemic pesticides such as neonicotinoids, are responsible for physiological, functional, and immunological damage in bees. However, the level of pesticides that bees are exposed to in the environment and in the resources inside their colonies has not been characterised in several key beekeeping regions, including the island of Ireland which is home to more than 50 beekeeping associations. Therefore, this thesis aims to help fill this gap by identifying the level of pesticides that managed honey bees are exposed to in their colony resources throughout the island of Ireland. Honey and pollen samples were collected by beekeepers from their colonies throughout the island, and pesticide levels in these samples were determined using liquid and gas chromatography-tandem mass spectrometry. Pesticides were extracted from samples using the Dutch mini-Luke extraction method. Importantly, here the Dutch mini-Luke method was optimised for small samples (5 grams of sample and 10ml of solvents), which is a common sample size for pollen samples in particular, and validated for the detection of pesticides in honey and pollen samples (Chapter 1). In a second step, the optimised mini-Luke technique was utilised to investigate the levels of pesticides honey bees are exposed to in their colonies in different apiaries throughout the island of Ireland (Chapter 2). Possible sources of contamination and the Maximum Residue Levels (MRLs) for the compounds detected are determined and discussed. Fifteen percent of all samples from throughout the island were found to be positive for pesticides. Three insecticides-acaricides were detected in most of the positive samples (85%). The insecticide Propargite was detected in honey and pollen, and the acaricides for *Varroa* control, Coumaphos and Fluvalinate-Tau, were found in pollen. The pesticide extraction method optimised here, in combination with the analysis methods, provides a key tool for detecting pesticides in beekeeping resources, and for detecting the level of agrochemicals honey bees are exposed to inside the hive and in the greater environment.

Key words: Pesticide detection, miniaturisation method, honey, pollen, insecticide.

Acknowledgments

To my parents and sisters for having always provided an insuperable amount of love and support, especially during my professional development and my international experience. To my new friends and colleagues who made from my international experience something memorable and unique. To my old friends for inspiring me, listening to me and making me smile. To all those angels I have found in my way.

To my supervisor at UCD, Dr Julia Jones, and my tutors at DAFM, Dr Jim Garvey, and Dr Paul Martins, who have spent a lot of effort and time towards my job. To my colleagues at UCD and DAFM for its patience and support over the past year. In particular, to Catriona O'Connor and Darren O'Connell, who provided essential feedback and guidance for the development of this research. A special thanks to Seana Jordan, who taught me the lab techniques utilised in this thesis.

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General introduction

Pesticides are chemical products essential for agriculture that pose a potential risk to living beings and the environment (Özkara, Akyil and Konuk, 2016). They are used to combat pests and diseases and to protect crops to ensure production. Without pesticides, an average of 34% of field production of essential crops, such as cottonseeds, maize, rice, tomatoes, and wheat, could be lost due to pests (Popp, Pető and Nagy, 2013). In the long term, food production, food security and sustainability may be problematic without the use of pesticides (OERKE, 2006; Popp, Pető and Nagy, 2013; Özkara, Akyil and Konuk, 2016). However, it has been shown that the continuous use of agrochemicals can have devastating and irreversible effects on human and ecosystem health (Bernardes *et al.*, 2015; Nicolopoulou-Stamati *et al.*, 2016; Kumar, Kumar and Singh, 2019; Rani *et al.*, 2021). For instance, a combination of recent studies shows that pesticides have contributed to the decline of populations of non-target insect species (Chamberlain *et al.*, 2000; Brühl and Zaller, 2019; Kumar, Kumar and Singh, 2019; Moe *et al.*, 2019), particularly wild pollinators and bees (Whitehorn *et al.*, 2012; Ndakidemi, Mtei and Ndakidemi, 2016; Sánchez-Bayo *et al.*, 2016).

Bees are highly efficient pollinators, predominantly because they can transport large amounts of pollen on their hairy bodies and corbiculae (Thorp, 1979, 2000). For example, more than 90% of over 100 main crops (e.g., cotton seeds, potatoes, tomatoes, and cereals) worldwide produced depend on bees' pollination service (Patel *et al.*, 2021). Wild and managed bees play a key role in food production and environmental wellness (Patel *et al.*, 2021). Overall, the ecological and social benefits provided by the diversity of bees species may likely be affected if bees and other pollinator populations continue to decline (Dangles and Casas, 2019).

Pesticides have been suggested to play a role in Colony Collapse Disorder (CCD) a phenomenon describing the loss of honey bee colonies in particular (Rortais *et al.*, 2017; Steinhauer *et al.*, 2018). CCD is characterized by the loss of adult worker bees from healthy hives and is likely an effect of the interaction of multiple factors (vanEngelsdorp *et al.*, 2009). Some of these factors include the presence of pathogens and parasites, the reduction in available nutritional food sources, the decline in native flora, and the use of pesticides and other agrochemicals in farmlands and beehives (vanEngelsdorp *et al.*, 2009; Williams *et al.*, 2010; Uhl and Brühl, 2019). Synthetic agrochemicals require particular consideration because some can persist in the environment for extended periods and they are systemic (e.g. neonicotinoids and phenyl-pyrazoles (Johnson *et al.*, 2010)), meaning these substances can accumulate in pollen and nectar of treated and untreated flowers, as well as in the soil and waterways (Botías *et al.*, 2015). Consequently, these substances can be collected by foraging worker bees, transported back to the colony and can subsequently contaminate colony resources such as wax and food supplies, as well as bees and the developing brood (Johnson *et al.*, 2010; Whitford and Steeger, 2017). The fact that most pesticides are lipophilic suggests that these compounds can accumulate in wax and persist in the honeybee hive for decades (Ravoet, Reybroeck and de Graaf, 2015), enfeebling the hive and driving colony collapse (Mommaerts *et al.*, 2010).

The contribution of pesticides to CCD is still undefined but the effects of pesticides on honey bees have been studied intensively in recent years (Rossi *et al.*, 2020). As an example, imidacloprid, a neonicotinoid insecticide used worldwide in agriculture and apiculture for *Varroa* control (Johnson *et al.*, 2010) has been shown to have an important impact on bees. Imidacloprid has been implicated in reduced queen production (Feltham, Park and Goulson, 2014), disparity in foraging behaviour (by the reduction in pollen collection and consumption) (Decourtye *et al.*, 2004; Mommaerts *et al.*, 2010; Woodcock *et al.*, 2017), decreases in immune responses (Woodcock *et al.*, 2017), impairment of homing behaviour (Henry *et al.*, 2012), reduction of learning abilities (Decourtye *et al.*, 2004), and changes in the expression of key genes such as abaecin (ABA) and hymenoptaecin (HYM) encoding for antimicrobial peptides involved in immune responses (Boncristiani *et al.*, 2012; Garrido *et al.*, 2013). Herbicides and fungicides have also been found to be associated with similar physiological, functional and immunological damage in bees (Rossi *et al.*, 2020). In addition, these agrochemicals have been shown to be associated with disorders in the gut microbiome of honey bees (Motta, Raymann and Moran, 2018), and suppression of the beneficial fungi in the colony(Yoder *et al.*, 2013).

Pesticides have been found in beekeeping commodities such as honey, pollen and bee wax (Ravoet, Reybroeck and de Graaf, 2015; Rossi *et al.*, 2020). The presence of Organochlorine, Carbamate, Organophosphorus, Pyrethroid and Neonicotinoid pesticides has been widely reported in beekeeping (Bogdanov, 2006; Laaniste *et al.*, 2016). As an example, after monitoring 45 apiaries in Spain, the presence of two organophosphorus substances (Coumaphos, and Chlorfenvinphos) was reported, as well as two pyrethroids (Fluvalinate and Acrinathrin), among other pesticides. More than 75% of the samples (133) in this study contained pesticides and pollen was found to be the most contaminated resource (Calatayud-Vernich *et al.*, 2018). Determining the level of pesticides in honey bee resource matrices enables the identification of the levels of chemicals bees are exposed to in the hive and the environment. Likewise, it helps to determine whether bee products are safe for consumption by other insects and humans (SANTE/11956/2016, 2016). In this context, the EU has set up the MRLs (Maximum Residue Levels) in Regulation (EC) No 396/2005 and Regulation (EU) No 37/2010 for plant protection products and veterinary products, respectively, as the highest concentration of pesticide that can be legally present in a commodity when good agriculture practices are applied (European Commission, 2021). The MRLs are set for each pesticide/commodity combination and must be considered for the chemical analysis (SANTE/11956/2016, 2016).

There are several extraction methods used in the analysis of pesticides but the mini-Luke technique and QuEChERS (quick, easy, cheap, efficient, rugged and safe) are the most popular for honey and pollen matrices (David *et al.*, 2015; Kiljanek *et al.*, 2016). There are some differences between both methods. Both are very effective general-purpose extractions, however, the QuEChERS method tends to give dirtier extracts that are more prone to interference (Rejczak and Tuzimski, 2015). Also, because it is buffered, pH-sensitive pesticides can give low recoveries and sometimes are completely lost (i.e. Chlorothalonil) (Rejczak and Tuzimski, 2015). The main drawback with the mini-Luke method is its use of chlorinated solvents (David *et al.*, 2015). After samples have been extracted and cleaned up, if necessary, the analytes are determined by a combination of Gas or Liquid Chromatography coupled to a Mass Spectrometric detector. Gas or Liquid chromatography in combination with mass spectrometry are high sensitivity techniques used for the detection of pesticides including neonicotinoids and fungicides (David *et al.*, 2015). These are the determination methods of choice for the multi residues analysis of pesticides in a wide variety of matrices including beekeeping samples (Kiljanek *et al.*, 2016).

The aim of this project was to detect levels of pesticides in honey and pollen samples collected throughout the island of Ireland using the mini-Luke method of extraction combined with liquid and gas chromatography-tandem mass spectrometry. Here, the extraction method was optimized according to the volume of the sample collected. A complete description of the method, the suggested adaptation, and the validation are described in the first chapter of this thesis. In the second chapter, the analysis of real samples (honey and pollen) is detailed, where the new validated method of extraction was utilised.

Chapter 1 Optimisation of the method for detecting pesticides in honey and pollen

Abstract

Sample preparation and extraction are key processes in pesticides detection. The mini-Luke extraction method is considered a useful method because it gives clean extracts and prevents losses of pH-sensitive compounds. Several analytical techniques have been tested for pesticide detection in beekeeping resources but the GC-QQQ (triple quadrupole) and HPLC-QQQ have been widely used because they give good selectivity and sensitivity, and facilitate multi-residue methods suitable for the detection of hundreds of analytes. Pollen is a vital source of protein for honey bees and other pollinators and is essential for monitoring the levels of pesticides that bees are exposed to in the environment and the hive. However, the availability of this matrix is limited. The availability of pollen varies with the season and it can be scarce during autumn and winter which is also when colony losses can occur. Therefore, here we optimised (miniaturised) the Dutch mini-Luke extraction method for small samples of pollen. This method was optimised for 5 grams of sample and 30ml of solvent. The miniaturised method was validated following the SANTE guidance document suitable for laboratories in Europe responsible for the official control of pesticides. It was found that the optimised extraction method combined with liquid and gas chromatography-tandem mass spectrometry presented consistent results in terms of accuracy and precision measured in terms of linearity, recovery, repeatability and matrix effect. The linear response fit to 278 out of 346 compounds and the coefficient of variation (R^2) was between 0.960 and 0.999. Repeatability and recovery experiments were performed at 10 and 100 µg/kg. A total of 281 compounds were validated at 10 µg/kg, and 303 at 100 µg/kg, where recovery in the range of 60 to 140% and relative standard deviation (RSD%) lower than 30% was considered valid. In addition, we found that there is a more significant matrix effect in pollen than in honey. Our optimisation and miniaturisation of the extraction method facilitates an operator-friendly and less pollutant process, compared to other related methods. This optimised process can be used in routine analyses of food samples and is suitable for the detection of multiple compounds including a variety of polar and non-polar analytes. We suggest that this method is a useful tool for monitoring the presence of pesticides in beekeeping resources and for evaluating pesticide levels in the environment, and will therefore allow us to determine the potential effects of pesticides on untargeted pollinators, and help preventing colony losses.

Key words: Pesticide detection, miniaturisation method, chromatography, honey, pollen

Introduction

Bees are exposed to pesticides used in agriculture and apiculture. These pesticides contaminate beekeeping commodities, reduce the quality of consumable products (Kasiotis *et al.*, 2014) and threaten the wellbeing of bees and humans (Botías *et al.*, 2015). Therefore, accurate detection of pesticides in bee resources and other products is essential (SANTE/11956/2016, 2016). Pesticide detection can be defined as an analytical procedure used to screen and quantify levels of chemicals present in food from different sources and is key for certifying that the contaminants are under the Maximum Residue Levels (MRLs) (SANTE/11956/2016, 2016).

Figure 1.1. General steps for residue analysis.

Residue analysis in general involves five possible steps (figure 1.1). Pre-treatment (sample preparation and homogenization) and extraction are systematic steps that remove potential contaminants from a sample leaving a solvent extract containing the compounds of interest (Narenderan, Meyyanathan and Babu, 2020). There are three main extraction methods for pesticide detection in fruits and vegetables, as well as honey (Sulaiman, Rovina and Joseph, 2019; Narenderan, Meyyanathan and Babu, 2020): QuEChERS (acetonitrile extraction), SweEt (ethyl acetate extraction), and Dutch mini-Luke (acetone extraction). The QuEChERS method is fast and easy (Narenderan, Meyyanathan and Babu, 2020). It is used to detect multiple pesticides and veterinary products in fruits and vegetables, and is flexible and effective in low volume of solvent (Narenderan, Meyyanathan and Babu, 2020). Unlike the mini-Luke method, the QuEChERS require a clean-up procedure and can lose pH-sensitive compounds (Rejczak and Tuzimski, 2015). Some researchers consider the mini-Luke as the least time-consuming extraction method (Lozano *et al.*, 2016) and the most suitable for obtaining clean extracts (Narenderan, Meyyanathan and Babu, 2020). Accordingly, the mini-Luke method is used to determine a wide range of pesticides such as organochlorines, organophosphates, organonitrogen, hydrocarbons and neonicotinoids, and has been largely used for the detection of pesticides in a variety of matrices such as fruits and vegetables, honey and milk (Lozano *et al.*, 2016). The Swedish ethyl acetate method (SweEt) is an elemental method used for routine analyses and the detection of basic pesticides in fruits and vegetables (Pihlström *et al.*, 2007).

It has been reported that the QueEChERS method has good performance in environmental samples (fresh pollen from flowers) and pollen from traps (Zioga *et al.*, 2020). However, the pH of the matrix and a small volume of pollen sample can affect the recovery of some compounds (Aparna and Rajalakshmi, 1999; David *et al.*, 2015; Narenderan, Meyyanathan and Babu, 2020). On the other hand, the liquid-liquid (mini-Luke) method has been widely used for removing contaminants from honey bee colony resources because it gives good recoveries in the majority of compounds in multi-residues analysis, including polar, non-polar and pH-sensitive compounds (Souza Tette *et al.*, 2016; Narenderan, Meyyanathan and Babu, 2020).

There are several analytical methods available for pesticide detection including High-performance Liquid Chromatography (HPLC), ELISA (immunosorbent assay) and capillary electrophoresis (CE) (Sulaiman, Rovina and Joseph, 2019). Such methods can be highly sensitive but also, very expensive (Sulaiman, Rovina and Joseph, 2019). Chromatography is a conventional approach that gives a precise and valid response (Stan, 2005). Chromatography can be coupled with selective detectors such as mass spectrometers to enhance selectivity and sensitivity, and to reduce bias, but mainly, to detect multiple residues (Kujawski and Namieśnik, 2008; Hadian, Samira and Yazdanpanah, 2019). GC-QQQ (triple quadrupole) and HPLC-QQQ have been the workhorses in this area for the last 15-20 years because they give good selectivity and sensitivity, and they facilitate multi-residue methods containing hundreds of analytes (Hernández *et al.*, 2013; Garvey *et al.*, 2020).

Matrix composition plays a role in pesticide detection (Kujawski and Namieśnik, 2008). In analytical chemistry, honey is considered a complex matrix, highly hygroscopic, and sugar concentrated (Kujawski and Namieśnik, 2008). Honey is mainly composed of glucose and fructose (30 and 37%, respectively (Kujawski and Namieśnik, 2008)), enzymes (originated by biochemical decomposition of sucrose by microbial activity (Aparna and Rajalakshmi, 1999)) and water (< 20% (Aparna and Rajalakshmi, 1999), 17-18% in ripe honey (Kujawski and Namieśnik, 2008)). Honey is a viscous and acid commodity, and likely the most analysed matrix for pesticides residues because it is the main beekeeping product for human consumption (Aparna and Rajalakshmi, 1999; Kujawski and Namieśnik, 2008). However, pollen (stored/bee pollen) and bee bread are likely more important for the bee's diet (Schulte *et al.*, 2008; Komosinska-Vassev *et al.*, 2015) and the environment (van der Steen, 2016, 2020). In general, the chemical composition of fresh stored pollen and bee-bread is very similar (Herbert Jr. and Shimanuki, 1978; Anderson *et al.*, 2014). The difference is that bee-bread has been stabilized by microbial fermentation and the addition of nectar and glandular secretions (Herbert Jr. and Shimanuki, 1978).

Liquid and Gas chromatography-tandem mass spectrometry (LC-MS/MS and GC-MS/MS) have been used for many years to detect pesticide levels in complex matrices like honey, honey bees and bee wax (Ravoet, Reybroeck and de Graaf, 2015), as well as in environmental samples (e.g., fresh pollen from flowers) (Zioga *et al.*, 2020). However, methods for the analysis of pesticide residues in stored pollen are less well defined

(Lozano *et al.*, 2016). In this study, we optimise the mini-Luke extraction method for the detection of pesticides in beekeeping matrices and adapt it to a small sample volume such as the volumes that tend to be available for pollen samples. In honey bee colonies, stored pollen can be scarce and availability varies with the season, but pollen is key for monitoring the levels of pesticides that bees are exposed to in the environment and the hive (van der Steen, 2016, 2020). In this chapter, a quantitative method adapted and validated for the detection of pesticides in honey and pollen samples is described, as well as the miniaturisation of the mini-Luke extraction method adapted for 5 grams of sample and 10ml of solvents.

Materials and methods

Samples

Honey and pollen samples from the National Apiculture Program (NAP) collected by 25 beekeepers throughout the Island of Ireland were used as a model for developing this method. Pollen samples included both fresh stored pollen and bee-bread. The analysis of fresh stored pollen, characterized by bright colour and matte appearance (Traynor *et al.*, 2021), was prioritised over bee-bread that is a fungal fermentation of stored pollen (David *et al.*, 2016). When the volume of fresh stored pollen was low a combination of stored pollen and bee-bread was used. Honey samples were necessarily collected in Autumn which also coincides with when bees are fed sugar syrup in preparation for winter. Consequently, some of the honey samples were found to contain a large amount of sugar and wax. The presence of wax is caused by the fact that honey is stored in the combs, from where the samples were collected but also because no separation method was applied.

Chemicals and reagents

HPLC (high-performance liquid chromatography) grade solvents such as Acetate, Dichloromethane, Petroleum ether, Ethyl Acetate, Methanol, Sodium Sulphate and Ammonium formate, (Fisher Scientific, Ireland) were used for removing the potential contaminants from the matrix (honey and pollen) into a solvent extract, to make up standards and to prepare the liquid chromatography (LC) mobile phase. Centrifuge tubes (250 ml), 50 ml conical centrifuge plastic tube, 10 ml disposable syringes and single-use filters (0.2 and 0.45 µm) from Fisher Scientific, Ireland, were used for solvents extraction. All glassware used in this experiment was grade A.

Standards and calibration solutions

To determine the presence and concentration of specific target compounds (analytes) of interest in this study, gas chromatography (GC-MS/MS) and liquid chromatography (LC-MS/MS) coupled with mass spectrometry was used. The samples were processed using the Agilent MassHunter software (Agilent Technologies, no date) and the data collected after every analytical run was analysed under two different processing methods: screening and quantitation. The screening method was executed to determine whether any of ~470 pesticides were present, and quantitation was used to determine the concentration of 346 analytes (180 pesticides for GC-MS/MS, and 166 for the LC-MS/MS). A list of analytes quantified in this study is outlined in Annexe 1.

Before determining the presence and concentration of target compounds, pure standards for all analytes tested and the spike solutions were made up according to the specifications presented by Garvey *et al.*, 2020 (Garvey *et al.*, 2020). The spike solutions, here called **standards**, were prepared at ~ 1 mg/l and used to spike blank samples for quality control. The recovery of the pesticides from the blank samples was used to monitor the extraction efficiency. In addition, a set of serial dilutions of the standards in methanol and ethyl-acetate were prepared. The dilutions, here called **calibration solutions**, were used for quantitation. The software Agilent MassHunter uses the response of the analytes from each of the calibration solutions to create an analytical curve that is then used to determine the response factor of each analyte (Agilent Technologies, 2017). The response factor is a key factor for establishing the concentration of the targeted analyte in the sample (Agilent Technologies, 2017). The points of calibration were set at 10, 20, 50, 100 and 250 µg/kg for the compounds tested by gas chromatography (figure 1.3), and at 10, 20, 30, 40 and 50 µg/kg for the analytes tested by liquid chromatography.

In addition, a **blank sample** (clear aliquot or matrix blank (SANTE/12682/2019, 2019)) was used to get the calibration solutions for the GC analysis. A blank sample does not contain pesticides or, if they are present, the concentration does not exceed 30% of the detectable levels (SANTE/12682/2019, 2019). Here, blank honey samples, previously analysed and reported as clear from pesticides were matrix-matched with the calibration solutions. This ensures accurate quantification which compensates for matrix effects and improves the analytical sensitivity of the method (Agilent Technologies, 2017). In the LC-MS/MS analysis, the samples were diluted at 1:20 which minimises the matrix effect. In each run, the batch of samples was bracketed by two sets of calibration solutions that were used to generate the calibration curve for quantitation and to ensure that the chromatography is stable throughout the run (Garvey *et al.*, 2020).

Solvent extraction

Solvent extraction is the process of transferring or diluting substances from the matrix (honey and pollen) to a liquid phase (solvent) (IUPAC, 2019). Here, the extraction method used is based on a variation of the mini-Luke extraction method modified to use sodium sulphate (Lozano *et al.*, 2016; Garvey *et al.*, 2020) to remove water from the matrix and enhance the recovery of certain polar compounds (Garvey *et al.*, 2020).

In each solvent extraction, 15 g of homogenised honey sample were weighed in a 250 ml PTFE centrifuge tube and hydrated with 10 ml of deionized water for 20 minutes. Two samples were spiked separately. The first one was spiked with 1.5 ml of the GC compounds (Mix AB) for a target recovery of 100 µg/kg, and the second one, with 0.75 ml of each LC mixture (ESI+ and ESI-) for 50 µg/kg of recovery. Next, 30 ml of Acetone was added and the samples were blended using an ultra-Turrax blender. Then, Dichloromethane (30 ml), Petroleum ether (30 ml) and Sodium sulphate (30 g) were added to the samples, and the samples were re-blended. The mixture was centrifuged for 5 minutes at 3500 rpm. After spinning, 2/3 of the supernatant (60 ml) were transferred to a rounded flask and the solvents were evaporated to ~ 2 ml using a BÜCHI rotary evaporator. Following this, excess ethyl-acetate (15 ml) was added and the extract was reduced again to circa 2 ml. The extract was transferred to a 10 ml volumetric flask, the rounded flask was washed twice with ethyl-acetate and the washings were transferred to the same volumetric flask. The aliquot was made up to volume with ethyl acetate. Due to the nature of the matrix, whenever the aliquot was cloudy, it was transferred to a 50 ml conical centrifuge plastic tube and centrifuge for 5 minutes at 4000 rpm. Then, the supernatant was filtered using a 0.2 µm disposal filter. A portion of this fraction (0.5 ml) was transferred to a new 10 ml volumetric flask and made up to volume with methanol. When required, this fraction was also filtered through a 0.20 µm filter before the analysis. The aliquot made up in ethyl-acetate was analysed by GC-MS/MS and the fraction diluted in methanol was analysed with the LC-MS/MS system. This gives a sample matrix concentration of 1g/l.

Blank samples were prepared using the method described above but using 30 g of honey. For the spiking process, clear samples were spiked with 1.5 ml or 0.75 ml of the spiking standards for the GC and LC analysis respectively. The target concentration reached in the GC and LC spiked samples, here called **LC/GC spikes**, was between 50 and 100 µg/kg for most of the pesticides. Blank samples were fortified with the spiking solutions pre-extraction.

Miniaturisation method

Due to the fact that any collection of pollen samples tends to result in individual samples of less than 15 g, the Dutch mini-Luke method had to be optimised for 5 grams of sample. Here, solvents were reduced to one-third of their initial quantity. Following the primary extraction method, the sample was blended with solvents and centrifuge at 3500 rpm for 5 min. As a modification, the full extract (supernatant), approx. ~25 ml, was collected. Then, solvents' reduction was performed in the same way as in the original protocol including the addition of excess ethyl acetate. When the sample was reduced to circa 2 ml and the solvents were evaporated, the extract was transferred to a 5 ml volumetric flask to make up to volume with ethyl acetate. Then, the sample was transferred to a 50 ml conical centrifuge plastic tube and cooled down to -20 °C for 15 min. Cooling down the sample was essential to remove a high quantity of contaminants, collect the maximum volume of sample and avoid double filtration of each fraction. Excess filtration steps can be problematic because they can remove some analytes and affect the recovery outcome (Majors, 2013). Following this, the sample was centrifuged at 4000 rpm for 5 minutes and the supernatant was filtered using a disposable plastic syringe fitted with a 0.2 µm filter. The sample concentration at this point was 0.83 g/ml and the aliquot was used for the GC analysis. To get the LC fraction, 0.5 ml of the GC fraction was collected in a 10 ml volumetric flask and made up to volume with methanol. The aliquot resulted in a 1 to 20 dilution.

Blank samples for the miniaturisation method weighted 10 g. The samples were processed in the same way as outlined above. Two blank samples were spiked, one with 0.75 ml of the GC standard spike and the other with 0.25 ml of the LC standard spike. The target or expected concentration of the spikes added at the beginning of the extraction was 50 and 100 µg/kg after running in the instrument.

Instrumental conditions

GC method

GC detection uses an Agilent 7000 Triple Quadrupole (QQQ) system and operates in pulsed split-less injection mode at a constant flow rate of 1.4 mL/min. The system works with a fused silica capillary Agilent J&W GC Column (15 m x 0.250 mm x 0.25 μm) suitable for working from 60 to 325 °C (350 °C). The injection was performed at 80 °C and helium was used as a carrier. Retention time locked at 24 ± 0.01 min. with ppDDE.

Table 1.1. Agilent 7000 Triple Quadrupole (QQQ) GC/MS operation system.

	Oven temperature			
	Rate °C/min	**°C**	**Hold time (min)**	**Run time (min)**
Initial	-	80	2	2
Ramp 1	25	150	0	4.8
Ramp 2	3	200	0	21.467
Ramp 3	8	280	10	41.467

Column temperature:	80 °C
Injection volume:	2μL
Transfer line temperature:	250 °C
Ion source:	Electron Ionization Mode (EI 70 eV)
Ion source temperature:	280°C
Run time:	41.867 min
Collision cell:	Helium (2.25 mL/min), Nitrogen (1.5 mL/min)

LC method

LC detection uses an Agilent 6490 LC-Triple Quadrupole (QQQ) in MS/MS with a binary pump. The system is equipped with a Phenomenex Kinetex C18 coreshell Column (50 x 4.6 mm) and with an electrospray ionisation source for multianalyte monitoring operating in positive (ESI+) and negative (ESI-) mode. The separation was carried out with two mobile phases, A and B, that were prepared with Ammonium phosphate (0.315 g), deionised water and methanol. Buffer A contained 990 ml of water and 10 ml of methanol, and buffer B had 10 ml of water and 990 ml. The flow rate was 0.5 μl/min, and the separation was performed in 45 minutes cycle. The initial flow rate was conditioned to get 95 % of buffer A and 5% of buffer B.

Mass spectrometer method

The MS/MS used in the GC system operates in electron ionization mode. Electrospray Ionization (ESI+ and ESI-) mode for the LC analysis. MS parameters are described in Garvey *et al.*, (2020) (Garvey *et al.*, 2020) and the functioning mechanism is described in chapter 2.

Validation method

Validation of an analytical technique is a procedure that tells us whether the technique is fit for its purpose and can give reliable results in terms of accuracy and precision (Lister, 2005).

Precision is assessed at three levels: repeatability, internal repeatability (when the target experiment is performed by a different analyst) and reproducibility (when the analytical method is executed in different laboratories) (Lister, 2005). Precision explains variations of the chromatographic outcome related to the mean value and is statistically explained by the standard deviation and the relative standard deviation (%RSD) (Lister, 2005). In this experiment, precision was characterised by repeatability which measures the ability of the method to produce similar results for serial preparations of the same sample (Lister, 2005). Here, two repeatability experiments were carried out in honey by the same analyst. The first one was performed with 6 repetitions at 100 µg/kg, and the second one was done with 7 repetitions at 100 µg/kg.

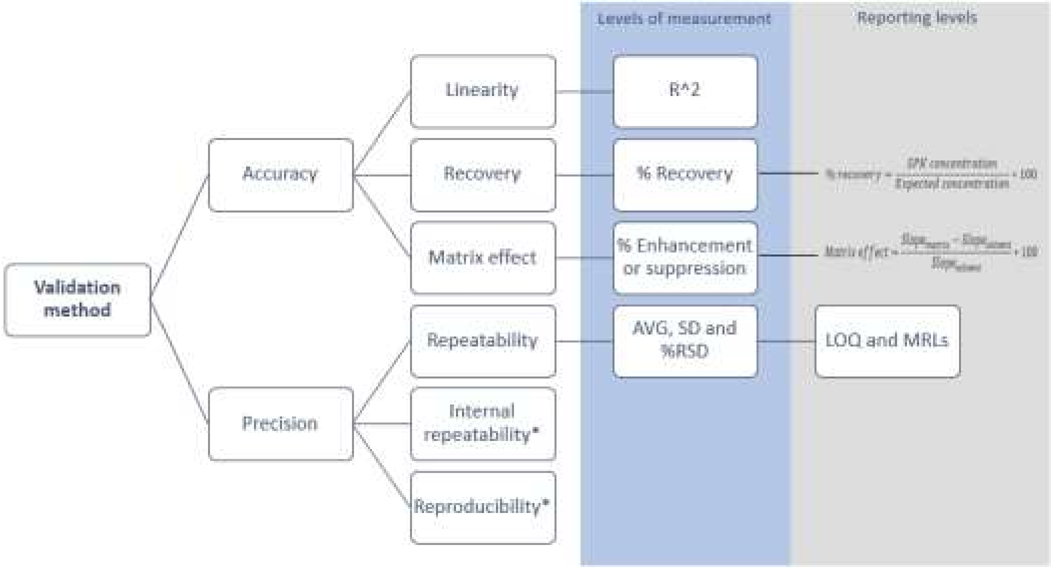


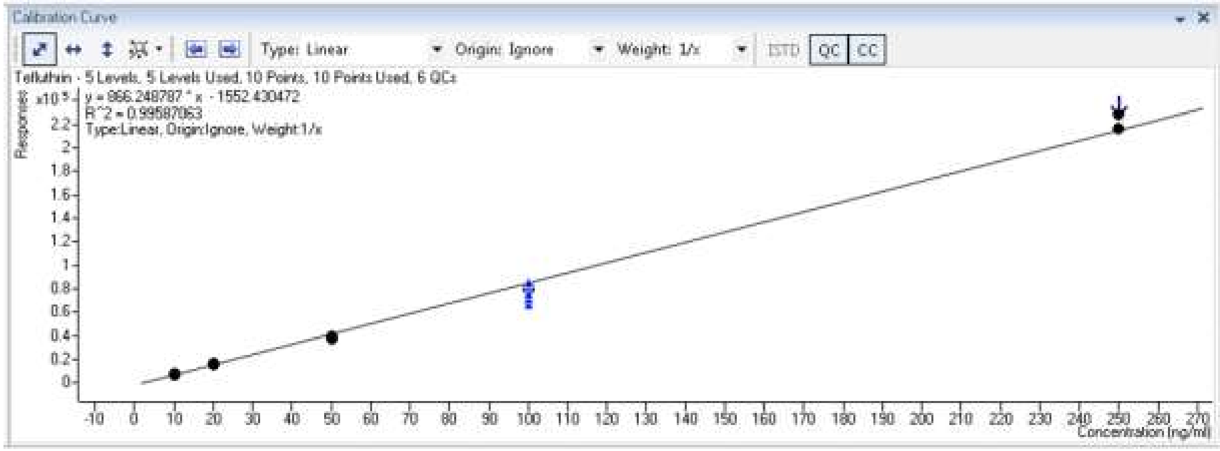
Figure 1.2. Scheme of the validation method for analytical procedures.
(*), parameters not measured in this experiment. R^2, coefficient of variation. AVG, average. SD, Standard deviation. %RSD, relative standard deviation. LOQ, Limit of Quantitation. MRLs, Maximum residue levels.

The recovery of a pesticide from a sample extract is used to give an estimate of the accuracy of the method (Lister, 2005). It is determined by the relation between the concentration of the analyte found in a spiked sample (SPK), and the introduced concentration of the analyte in a solvent spiked (expected concentration) as it is shown in equation 1.1 (Lister, 2005).

Equation 1.1. Percentage of recovery.

$$\% recovery = \frac{SPK\ concentration}{Expected\ concentration} * 100$$

Linearity is a visual and numerical outcome represented by a fitted calibration curve and measured by the coefficient of variation (R^2). It gives us a response signal that is proportional to the analyte to be determined (Lister, 2005). Here, linearity was measured for all the compounds (346) over a range of 5 different concentration levels (from 10 to 250 µg/kg). Bracketing sets of standards are used which are injected before and after the samples in the analytical run. The calibration curve is constructed using both sets of standards with a total of 10 points. As an example, figure 1.3 shows the response of the compound Tefluthrin and its relation to the concentration in the sample. The compound has good linearity, R^2 > 0.95, which is considered an acceptable value for validation purposes (SANTE/12682/2019, 2019; Garvey *et al.*, 2020).


Type: Linear
Origin: Ignore
Weight: 1/x

Tefluthrin - 5 Levels, 5 Levels Used, 10 Points, 10 Points Used, 6 QCs

y = 866.248787 * x - 1552.430472
R^2 = 0.99587063
Type:Linear, Origin:Ignore, Weight:1/x

Figure 1.3. Signal response of Tefluthrin matrix matched with honey.
Screenshot taken from the software Agilent MassHunter and generated by the quantitation method. Here, two sets of calibration solutions (represented by the black dots) at 5 different levels (from 10 to 250 µg/kg) are injected during the run. The calibration points are overlapping at each level, except at level 5 (250 µg/kg) where a short distance between them can be seen.

The matrix effect is an interaction between the analyte and components of the matrix which can cause an increase or decrease in the measured signal in comparison to the signal obtained from solvent standards (Souza Tette *et al.*, 2016). This influences the signal response and therefore, can have a significant effect on the quantitation of the pesticides in the sample (Lehotay *et al.*, 2010). To determine matrix effect (ME), the response from solvent standards is compared with the response of the matrix-matched standards (Steiner *et al.*, 2020). The difference between the slopes determines whether the signal increases or decreases the signal (Steiner *et al.*, 2020). The result was expressed in percentage, as it is shown in the formula (Lehotay *et al.*, 2010; Garvey *et al.*, 2020):

Equation 1.2. Matrix effect (ME)

$$ME = \frac{Slope_{matrix} - Slope_{solvent}}{Slope_{solvent}} * 100$$

Values ± 20% indicate a minimal matrix effect, positive values greater than 20% leads to an increase in the signal and negative values (< 20%) lead to a decrease in the signal (Lozano *et al.*, 2016; SANTE/12682/2019, 2019). Matrix effect is commonly found in gas and liquid chromatography (Lehotay *et al.*, 2010). However, here, it was considered for GC compounds only since the LC fraction was diluted (1:20) which dilutes the matrix sufficiently to minimise the matrix effect in liquid chromatography.

Results

In order to show that the miniaturisation method of extraction is appropriate for detecting pesticides in honey and pollen, validation parameters such as linearity, repeatability, and matrix effects were assessed. Validation was performed mostly in honey but pollen was also used to assess matrix effects. Validation was based on the SANTE document (SANTE/12682/2019) available on the EURL for pesticides website (www.eurl-pesticides.eu) (SANTE/12682/2019, 2019). In general, matrix-matched and spiked blanks were bracketed by a set of calibration standards, conditioning of the GC and LC systems was performed before the injection of the first series of calibration standards, and three or more calibration points were used to assess the outcome. The lowest calibration level (LCL) was set up at 10 µg/kg for gas and liquid chromatography. The limit of quantitation (LOQ), also known as reporting limit (RL) was defined at the lowest spiked level (LCL) measurable at acceptable recovery (70 to 120 %) and precision (reliable standard deviation). The validated method was applied for the detection of pesticides in real samples and the results of those samples are presented in Chapter 2 of this thesis.

Linearity

Calibration curves were generated with 5 calibration points for gas and liquid chromatography. The calibration points for the GC-MS/MS were 10, 20, 50 and 250 µg/kg with matrix-matched standards, and 10, 20, 30, 40 and 50 µg/kg for the LC-MS/MS. The calibration curves are not forced through the origin. The coefficient of variation (R^2) was used to measure linearity. Values above 0.95 were considered as acceptable (Garvey *et al.*, 2020). The coefficient of variation was higher than 0.95 for almost all the compounds. Only 44 out of 346 analytes had poor linearity. Compounds with bad linearity are summarized in table 1.2. Figure 1.4 summarises the linearity results by correlation coefficient.

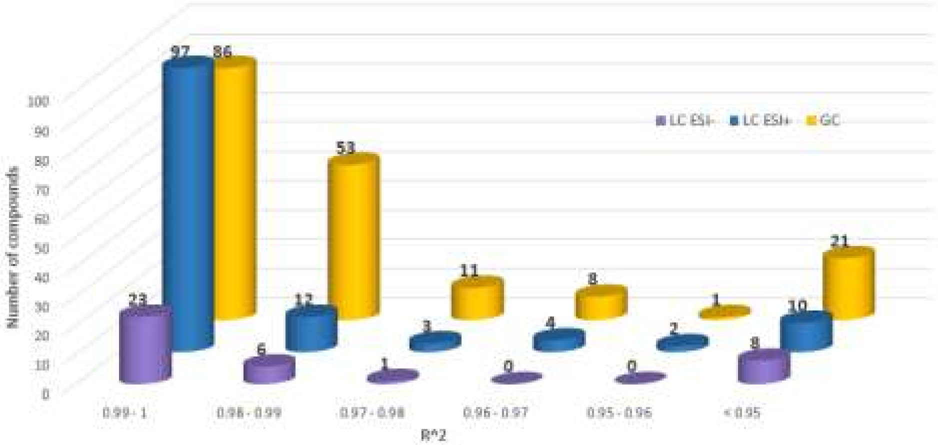


Figure 1.4. Linearity expressed by values of coefficient of variation (R^2).
All pesticides tested by liquid and gas chromatography are shown in the chart.

Table 1.2. Compounds with linearity under the acceptable limit (< 0.95)

GC compound	R^2	LC compound	R^2
Azinphos-methyl	0.917	*BAC14*	0.906
Azoxystrobin	0.904	*BAC16*	0.949
Binapacryl	0.527	*Cyproconazole II*	0.924
Captafol	0.176	*Difenoconazole*	0.827
Captan	0.515	*Dodine*	0.179
Cyfluthrin	0.801	*Methiocarb Sulfone*	0.762
Cypermethrin	0.599	*Spirotetramat*	0.950
Dicofol	0.475	*Thiabendazole*	0.916
Etoxazole	0.933	*Thiophanate-Ethyl*	0.334
Etridazole	0.898	*Thiophanate-Methyl*	0.364
Fenvalerate-II	0.828	**2,4,5-T**	0.938
Fluvalinate-tau-I	0.748	**Bixafen**	0.896
Fluvalinate-tau-II	0.895	**Chlorfluazuron**	0.923
Folpet	0.539	**Cycloxydim**	0.889
HCH-alpha	0.883	**Diflubenzuron**	0.811
Heptachlor	0.944	**Fluazifop**	0.851
Methoxychlor	0.796	**Flubendiamide**	0.765
Permethrin-I	0.764	**Haloxyfop**	0.903
Phorate	0.792		
ppDDT	0.688		
Propargite	0.902		
Azinphos-methyl	0.917		

In italic, pesticides analysed in positive Electrospray Ionisation mode (ESI+). In bold, pesticides analysed in negative Electrospray Ionisation mode (ESI-).

Recovery and repeatability

According to the quality control and method validation SANTE/12682/2019, an acceptable recovery is defined by the range of 70 to 120% (Lozano *et al.*, 2016; SANTE/12682/2019, 2019). The document explains that recoveries outside of this range can also be accepted if there is a well-defined reason (SANTE/12682/2019, 2019). Recoveries as low as 30% can also be allowed in some cases (SANTE/12682/2019, 2019). The range of 60 to 140% is considered practical and acceptable in routinary analysis (SANTE/12682/2019, 2019; Garvey *et al.*, 2020). Here, over 83% of the compounds tested by gas and liquid chromatography at 10 to 100 µg/kg presented suitable recovery (60 to 140%). The recovery data is summarized in figures 1.5 and 1.6 for GC and LC analytes. Recovery was calculated using the formula given here and explained in the methodology for validation (Equation 1.1).

Dodine, Ethiofencarb Sulfoxide, Fenpyroximate, Fluxapyroxad, Methiocarb Sulfone were not detected by the LC-MS/MS instrument at 10 or 100 µg/kg. Cypermethrin, Dicofol, Etoxazole and Folpet were not detected by GC-MS/MS analysis at any concentration.

Repeatability was assessed in 7 blank honey samples at 10 µg/kg and 6 blank honey samples at 100 µg/kg. The replicates were used to calculate average recovery, standard deviation and relative standard deviation (RSD%) for all the analytes tested in each validation batch. The relative standard deviation (RSD%) determines whether the average results are statistically equivalent (Lister, 2005). The RSD% was considered acceptable at ± 20% (Lozano *et al.*, 2016; SANTE/12682/2019, 2019; Garvey *et al.*, 2020). Values close to 30% were considered adequate when the recovery results were consistent (SANTE/12682/2019, 2019; Garvey *et al.*, 2020).

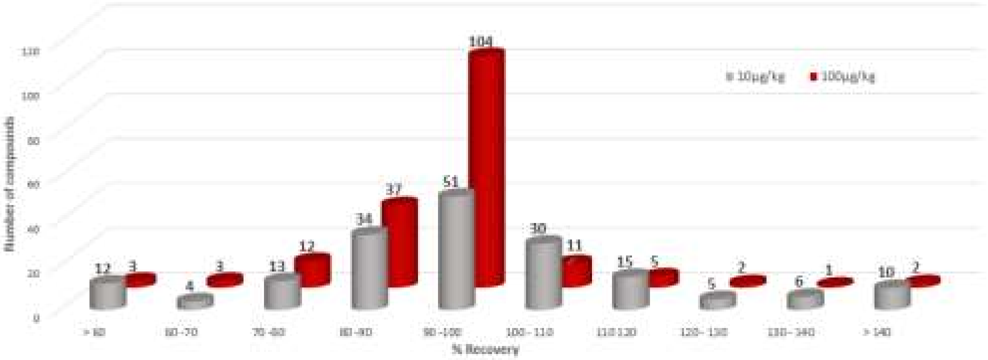


Figure 1.5. Percentage of recovery in pesticides analysed by gas chromatography (GC-MS/MS) at 10 and 100 µg/kg.

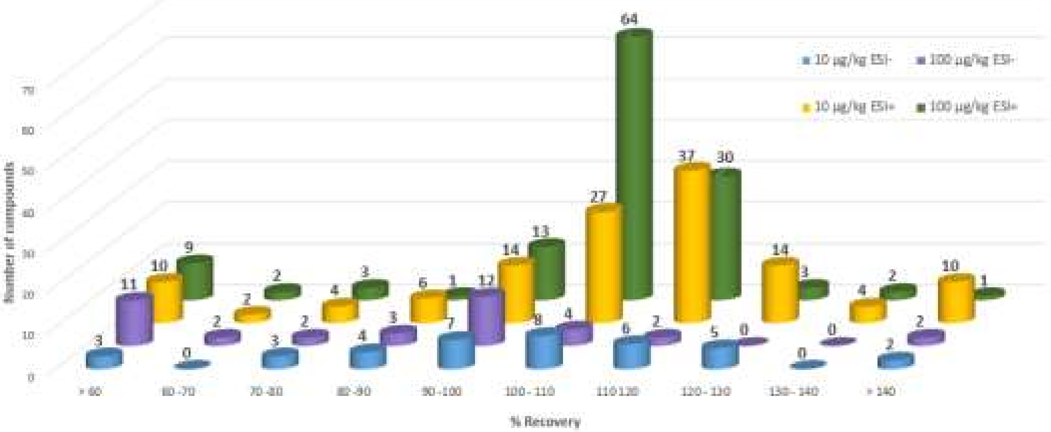


Figure 1.6. Percentage of recovery in pesticides analysed by liquid chromatography (LC-MS/MS) in positive (ESI+) and negative (ESI-) electrospray ionisation.

Matrix effect (ME)

To determine the matrix effects of honey and pollen samples, five clear samples of each matrix were spiked at 5 different levels between 10 to 250 µg/kg. The run was bracketed by two sets of calibration standards matched with solvent (ethyl-acetate). The matrix effect was determined by comparing the slope of the solvent calibration standards vs the slope generated by the matrix-matched samples and was expressed in percentage. A matrix effect between -20 to 20% is not considered significant. The results show that there is a more significant matrix effect in pollen than in honey. Here, 54% of pesticides determined in pollen were influenced by the matrix and 35% of the pesticides determined in honey were affected by the matrix.

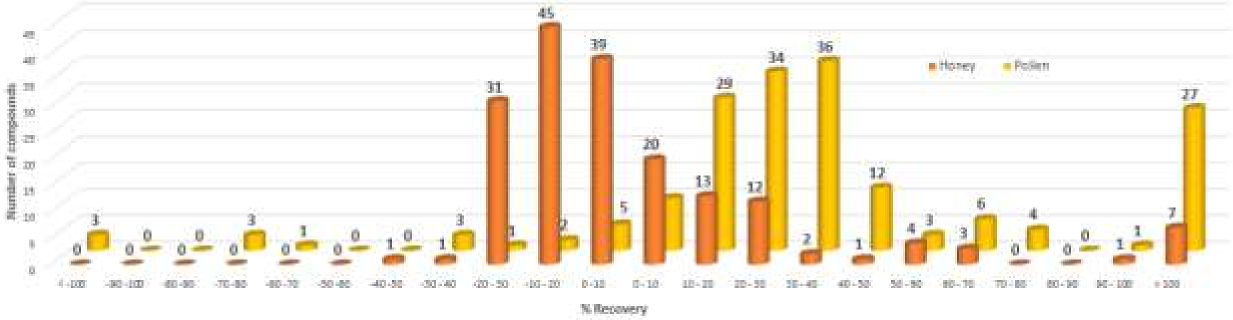


Figure 1.7. Matrix effect of honey and pollen in the signal response of all the compounds analyzed by gas chromatography (GC-MS/MS).

Pesticide detection in real samples

The miniaturisation method was validated in honey by using a sample previously analysed at DAFM (No. 804-194) that was positive for Boscalid at 0.018 µg/kg. Under the miniaturisation method, 0.015 µg/kg of Boscalid were detected in the same sample. The percentage of recovery was acceptable (between 70 and 120%), and the linearity was above 0.95 (Table 1.3). In addition, 5 pollen samples from unknown origins were analysed by the miniaturisation method, two herbicides (MCPA and Mecoprop) and the fungicide Boscalid were detected. Good linearity (R^2 = 0.992) and low recovery was detected (Table 1.3). Samples from the NAP analysed with the new method are presented in Chapter 2.

Table 1.3. Samples analysed by the miniaturisation method.

Sample ID	Pesticide	Sample volume (g)	Final Concentration (mg/kg)	R^2	MRL (mg/kg)	LCL (mg/kg)	% Recovery in batch
Honey (804-194)	Boscalid (F)	4.99	0.012	0.991	0.15	0.009	83.50
Pollen (JLD12)	MCPA (H)	5.01	0.028	0.996	0.05	0.010	48.60
	Mecoprop (H)		0.015	0.996	0.05	0.009	56.50
Pollen (SC212)	Boscalid (F)	4.45	0.018	0.986	0.15	0.010	83.50

(F), Fungicide. (H), Herbicide Boscalid was determined by GC-MS/MS. Mecoprop and MCPA were determined by LC-MS/MS in negative Electrospray Ionisation mode (ESI-). The origin of the pollen samples is unknown.

The percentage of recovery in the batch for the herbicides was lower than 60%. MCPA and Mecoprop are from a class of compounds called phenoxy-acetic acids that are formulated as esters, but when they are applied in the field, they break down into free acids (Paszko *et al.*, 2016). In the free acid form, these compounds can easily bind to matrices with hydroxyl groups (-OH) and thus are removed from the extract leading to poor recoveries (Zhang *et al.*, 2014).

Discussion

This chapter aims to optimise and validate a method of extraction for detecting pesticides in samples where a limited quantity of the sample is available, in particular in pollen that is a very important food resource for honey bees and other pollinators. The accurate detection of pesticides in pollen is essential for determining the level of pesticides that honey bees and other pollinators are exposed to in the environment and their colonies. Here, the Dutch mini-Luke method, one of the most suitable methods for detecting pesticides in beekeeping resources, was adapted to a small sample size - 5 grams of sample and 10 ml of solvents. The extraction method was combined with liquid and gas chromatography-tandem mass spectrometry (GC-MS/MS and LC-MS/MS) and validated for honey and pollen. All validation parameters were evaluated according to the guidelines from the SANTE document (SANTE/12682/2019) suitable for laboratories in Europe responsible for the official control of pesticides in foodstuff.

Extraction method

The Dutch mini-Luke is a liquid-liquid extraction method that uses acetone, among other solvents (i.e., dichloromethane and petroleum ether), to remove analytes from the sample (Narenderan, Meyyanathan and Babu, 2020). According to Baša Česnik *et al.,* 2019, acetone extraction has a big advantage when compared to acetonitrile, a compound used for the QuEchERS method, this is because acetone is much more volatile and easier to remove and concentrate. Such properties are essential for reducing additional cleaning steps (Baša Česnik, Kmecl and Velikonja Bolta, 2019). Baša Česnik *et al.,* 2019 also suggest that acetone is suitable for the extraction of sugary samples such as honey because it prevents interference with this type of matrix, which can occur when acetonitrile is used for extraction. Moreover, the addition of solvents such as dichloromethane and petroleum ether have an advantage in the liquid-liquid method since it enables the extraction of polar and non-polar compounds. This permits the detection of multiple compounds in a single run (Narenderan, Meyyanathan and Babu, 2020) and increases the possibility of detecting important residues involved in apiculture such as Cyhalothrin-lambda (non-polar), Coumaphos (polar) and Chlorfenvinphos (polar) in a single run (Baša Česnik, Kmecl and Velikonja Bolta, 2019).

The fact that liquid-liquid extraction methods utilize a variety of solvents has been documented as a pitfall because of the toxicity of the chemicals (Baša Česnik, Kmecl and Velikonja Bolta, 2019). However, by reducing the volume of solvents used in the process, we turn it into a less pollutant method that can also be used in routine analyses of food samples. The work by Baša Česnik *et al.,* 2019, and Lozano *et al.,* 2016, also confirms that a reduction in the volume of solvents utilised during extraction is convenient, operator and environmental-friendly, and less expensive.

Optimisation of pesticide detection in small samples

Accuracy was measured in terms of linearity (R^2), recovery (expressed in percentage) and matrix effect (tested in honey and pollen). Precision, on the other hand, was measured in terms of repeatability and was determined by the relative standard deviation (RSD%). Recovery and repeatability were used to establish the Limit of Quantitation (LOQ) for all the compounds, as well as to assess the risk of exposure when it was compared to the minimum residue levels (MRLs).

In general, the miniaturised method developed and utilised here provided consistent results in terms of accuracy and precision. The linear response fit to 278 out of 346 compounds and the coefficient of variation (R^2) ranged from 0.960 to 0.999. The LOQ for all the compounds was 10 µg/kg and is equal to or lower than the minimum residue levels (MRLs) set in Regulation (EC) No 396/2005 and Regulation (EU) No 37/ for plant protection products and veterinary products, respectively. The validation of GC-MS and LC-MS/MS methods for the determination of pesticides in organic and commercial honey samples in Slovenia provided similar results, where the linearity ranged from 0.960 to 0.988 for GC and from 0.991 to 0.999 for LC (Baša Česnik, Kmecl and Velikonja Bolta, 2019). In our experiment, linearity was demonstrated for 80% of the analytes evaluated.

Repeatability and recovery experiments were performed at 10 and 100 µg/kg. A total of 281 (83.6 %) compounds were validated at 10 µg/kg, and 303 (90 %) compounds were validated at 100 µg/kg for liquid and gas chromatography (GC-MS/MS and LC-MS/MS) when a percentage of recovery from 60 to 140% with RSD% lower than 30% was considered. Accordingly, 55 and 33 analytes, at 10 and 100 µg/kg respectively, could not be validated as they were not in line with the validation criteria outlined in the SANTE document (SANTE/12682/2019) for recovery and repeatability (SANTE/12682/2019, 2019). Additionally, 9 compounds were not detected at 10 µg/kg by GC (Binapacryl, Captafol, Captan, Cypermethrin, Dicofol, Etoxazole, Fenvalerate-II, Folpet and Spirodiclofen). Dodine and Cycloxydim were not detected by LC at any concentration. The LC detectors did not find Methomyl at 10 µg/kg and Bromoxynil at 100 µg/kg. Getting a better response at 100 µg/kg was expected due to the fact that at higher concentrations the pesticides are more easily detected.

Lozano *et al.*, 2016, used the same extraction method for the detection of pesticides in vegetable samples weighing 15 grams. There, the method was miniaturised to reduce the volume of solvents but the amount of sample was not modified. The experiment performed by Lozano *et al.*, 2016, showed that by reducing the solvents to 10ml, as done in the current study, the majority of the pesticides (from 167 to 171, out of 175 analytes) get a good recovery (70-120%) with RSD% under 20% in GC-MS/MS and LC-MS/MS. Likewise, in our experiment, good recovery (from 70 to 120% with RSD% under 20%) was reached by 95% of the pesticides analysed by GC-MS/MS and by 87% of the compounds analysed by LC-MS/MS (figures. 1.5 and 1.6). Importantly, the parameters used to validate the precision of the technique (recovery and repeatability) measured by LC-MS/MS had better results at 10 µg/kg than at 100 µg/kg under negative Electrospray Ionisation mode (ESI-).

In comparison to the experiment presented by Lozano *et al.*, 2016, our method shows that apart from reducing the volume of solvents, the sample volume can also be reduced without affecting the recovery percentage. In general, the miniaturised method combined with liquid and gas chromatography was found to perform well in terms of recovery and repeatability.

Matrix effect (ME)

The matrix effect was assessed in honey and pollen in gas chromatography. The matrix effect was not considered in LC since LC aliquots were diluted (1:20) to minimise the effect. First, clear (pesticides-free) honey and pollen samples were matrix-matched with calibration standards at 10, 20, 50 and 250 µg/kg, and 346 compounds were tested. Next, the slopes from the calibration standards were compared to those from the matrix-matched samples to calculate the matrix effect. An effect outside the range (-20 to 20%) was considered significant according to the SANTE guidance document (SANTE/12682/2019) (SANTE/12682/2019, 2019). A negative effect (decreased signal) was considered when ME% was under 20%. In contrast, when the signal was higher than 20%, the effect was considered positive (increased signal). In general, an increase in the signal has been more common in gas chromatography for fruits and vegetables (orange and lettuce), as was noted by Lozano *et al*., 2016.

We found that 54% of pesticides detected in pollen were influenced by the matrix and 35% were affected by the honey matrix. Some compounds were not affected by any kind of matrices, as was the case for Deltamethrin and Permethrin. However, other compounds were found to have opposite signal responses in honey versus pollen. For example, the response of Propargite and Coumaphos decreased in the honey matrix, but increased in pollen. Similarly, the signal from Cyhalothrin-lambda and Fludioxonil was positive in pollen, but not significantly influenced in the honey matrix. Boscalid, Fluvalinate-Tau, and Prochloraz were negligibly affected by the pollen matrix, but negatively affected by honey. The signal of Dimethoate and Methamidophos increased in both matrices. The same response was obtained by Gawel *et al*. 2019, who suggest that the signal of Cyhalothrin-lambda, Fludioxonil, Deltamethrin and Permethrin was not influenced by the honey matrix (Gaweł *et al.*, 2019). Similarly, Parilla Vázquez *et al.,* 2015, found that Chlorpyrifos, Chlorfenvinphos, Chlorothalonil, Coumaphos, Fludioxonil and Fluvalinate-Tau was unaltered in pollen (Parrilla Vázquez *et al.*, 2015). According to Parilla Vázquez *et al.,* 2015, changes in response can be attributed to the type of pollen and the composition of the matrix which can also be attributed to the geographical distribution of the samples (Parrilla Vázquez *et al.*, 2015).

Pesticide detection

The miniaturised method of extraction, optimised for 5 grams of sample and combined with liquid and gas chromatography for pesticide detection, enabled rigorous results in terms of recovery, repeatability and linearity. Therefore, it was deemed reasonable to apply the validated method for pesticide detection in real honey and pollen samples. As an illustration, we tested honey sample No. 804-109 previously analysed by technicians at DAFM under the standard method (suitable for 15 grams of sample and 30 ml of solvents). In the previous analyses, the sample was found to be positive for Boscalid at 0.018 µg/kg. By using the new method, we detected Boscalid, as expected, but in a lower concentration (0.012 µg/kg) which is considered acceptable under the measurement uncertainty of +/- 50% (SANTE/12682/2019, 2019). The difference in concentration can be seen because of the presence of contaminants in the column, and the accuracy in preparing the calibration standards. The signal response can improve if both parameters are adjusted. The MRLs for pesticides from the environment and veterinary applications in beekeeping associated matrices are, in general, set at 0.05mg/kg. The LOQ for 90% of the compounds, here analysed, was found at 10 µg/kg. Boscalid in the honey sample No.804-194 was below the MRLs set by the EU.

The miniaturised extraction method has been used in honey samples for the detection of organic compounds such as thymol in combination with gas chromatography (Gaweł *et al.*, 2019). In addition, it can be adapted to techniques for single residues detection such as the detection of Amitraz in gas chromatography by HPLC (High-Performance Liquid Chromatography) (Gaweł *et al.*, 2019). Moreover, it can be used for the analysis of different commodities such as fruits and vegetables and may be applicable for routine analysis of this type of samples with an eco-friendly and low-cost focus (Lozano *et al.*, 2016). We suggest that the miniaturised method is also a useful monitoring tool for determining the presence of pesticides in beekeeping resources, and for evaluating pesticide levels in the environment and therefore potential effects on managed and wild pollinators.

Chapter 2 Detection of agrochemicals in honey bee colony resources across the island of Ireland

Abstract

Pesticides are essential resources for agriculture, but are also associated with honey bee colony losses. Monitoring the pesticide levels that honey bees are exposed to is important for determining which compounds may be involved in colony losses. In this context, we tested honey and pollen samples from managed honey bee colonies across the island of Ireland for the presence of pesticides. Twenty-five honey samples and twenty pollen samples, collected from the same apiaries in Autumn 2020, were tested for the presence of 33 target compounds. Liquid and gas chromatography methods, coupled with mass spectrometry was used, as well as the validated miniaturisation method presented in Chapter 1, to determine the level of the target pesticides in the samples. The most commonly utilised plant protection products across the island of Ireland were targeted, as well as five neonicotinoid insecticides commonly applied in agriculture globally, and two veterinary products used worldwide for *Varroa* control. It was found that 84.4% of the samples were clean (free of pesticides) and 15.6% contained pesticides. Among the positive samples, 85.7% contained Propargite (insecticide) including 5 honey and one pollen sample. In addition, 50% of the positive pollen samples were positive for three compounds: Propargite, Coumaphos and Fluvalinate-Tau (acaricides), and the remining 50% was positive for Fludioxonil and Cyprodinil (fungicides). Three compounds were found to be above the Maximum Residue Levels (MRLs). The LOQ for 90% of the compounds here analysed was found at 10 µg/kg. Positive samples were detected on the East coast of the island specifically in Dublin, Kildare, Down, and Louth apparently attracted by large regions of crops and grassland where large volume of pesticides is applied.

The annual European questionnaire for beekeepers, the *COLOSS survey*, was completed by most of the beekeepers that supplied samples for this study. This survey was used to collect information about colony losses and the factors that may have been involved in colony losses, such as the application of pesticides for *Varroa* control. However, we could not establish a correlation between the survey's results and the chemical analysis, likely due to limitations on the information supplied in the surveys. Nonetheless, we suggest that the chemical analysis can be considered as a monitoring tool for 1) detecting pesticide levels in honey bee colony resources, 2) determining the effects of the control methods that beekeepers are applying to combat bee pests, particularly *Varroa*, and 3) discovering contaminants present in the landscape where bees are foraging. Overall, this study enables a better understanding of the key factors that may contribute to honey bee colony losses, as well as the decline in other pollinators throughout the island of Ireland.

Key words: Insecticide, Acaricide, Propargite, Coumaphos, Fluvalinate-Tau

Introduction

Pesticides are used worldwide in farming and agriculture; however, they can also have detrimental effects on honey bees and other non-target insects (Whitehorn *et al.*, 2012; Ndakidemi, Mtei and Ndakidemi, 2016; Sánchez-Bayo *et al.*, 2016). In 2020 it was estimated that 3.5 million tonnes of pesticides are used every year in agriculture worldwide (Sharma *et al.*, 2019). China and the USA are the lead consumers but Europe utilizes around 45% of the pesticides globally applied (Sharma *et al.*, 2019; Rani *et al.*, 2021). Herbicides are the most utilised products in agriculture followed by fungicides and insecticides (Pariona, 2017; Sharma *et al.*, 2019; López-Ballesteros *et al.*, 2021). Herbicides represent more than 40% of the plant protectants applied throughout the world (Sharma *et al.*, 2019). Based on the Grassland & Fodder Crops Survey Report presented by the Pesticide Control Division at the Department of Agriculture, Food and Marine (DAFM) in Ireland, in 2017, 97% of the arable area in Ireland applied pesticides for crop protection. Herbicides are the leading group (67%) dominated by compounds such as Glyphosate, MCPA and the formulation Fluroxypyr/Triclopyr. Fungicides (21%) commonly found were Chlorthalonil and Azoxystrobin, and the formulations: Boscalid/Pyraclostrobin and Epoxyconazole/Pyraclostrobin. The small group of insecticides (4%) was represented by Cyhalothrin-lambda, Pirimicarb and Deltamethrin (DAFM, 2017). Constant precipitation in the island throughout the year suggest that fungi-like diseases are more relevant in agriculture in Ireland, therefore fungicides are used more than insecticides (Lennon, 2015).

Similarly, the Survey Report 293 about pesticide usage in Northern Ireland presented by the Agri-food and Biosciences Institute (AFBI) and the Department of Agriculture and Rural Affairs in 2019 reports that the spray area is dominated by herbicides (40%). Fungicides (24%), insecticides (24%) and seed treatment (12%) are also prevalent (Lavery *et al.*, 2019). Some of the most common agrochemicals used are the insecticides: Lambda-cyhalothrin (pyrethroid acaricide, nematicide and miticide), Oxamyl (carbamate acaricide nematicide and miticide); the herbicides Glyphosate (systemic organophosphorus) and Bromoxynil (nitrile), and the fungicides: Difenoconazole and Prothioconazole (systemic) (Lavery *et al.*, 2019). The pesticides usage report suggests that in Northern Ireland seeds are commonly treated against fungi with the formulation Cymoxanil/Fludioxonil/Metalaxyl-m or with single compounds such as Tefluthrin, Thiram, Metalaxyl-M and Fludioxonil (broad-spectrum fungicide). Seed treatments represent 12% of the pesticides applied in the country (Lavery *et al.*, 2019). In Ireland, seed treatments represent 10% of the pesticides applied in farmlands (DAFM, 2017). Some of the most important seed dressing pesticides include Methiocarb (carbamate repellent insecticide), Tefluthrin (pyrethroid insecticide), Thiram (organic fungicide), Prochloraz (imidazole fungicide) and the neonicotinoid insecticides Thiamethoxam and Imidacloprid.

It is clear that honey bees are exposed to different types of pesticides applied in agriculture, and insecticides are considered to be of particular concern because they are used for in-hive pest control (Johnson *et al.*, 2013). Biological and chemical control methods have been applied throughout the world against *Varroa*, one of the most problematic pathogens for beekeepers (Bogdanov, 2006; Ravoet, Reybroeck and de Graaf, 2015). The mite (*Varroa destructor*), weaken bees and is a vector of viruses such as the Deformed Wing Virus (DWV) (Whitford and Steeger, 2017). There are many veterinary products worldwide applied to control this pathogen that include some organic compounds such as oxalic acid, thymol and formic acid, and chemical solutions such as Lambda-Cyhalothrin, Coumaphos, Amitraz and Tau-fluvalinate (synthetic organic) (Bogdanov, 2006; Johnson *et al.*, 2010). For instance, 45% of beekeepers in Northern Ireland who utilise a control method for *Varroa* applied Amitraz (Forsythe and Murchie, 2021). Amitraz and Flumethrin are also used and authorized in Ireland (DAFM, 2020). Monitoring the pesticide levels that honey bees are exposed to is important for determining which factors may be involved in colony losses. This chapter investigates the levels of pesticides that honey bees are exposed to in their colonies in different apiaries throughout the island of Ireland. The main sources of contamination and the Maximum Residue Levels (MRLs) for the compounds detected are determined and discussed. The analytical method for pesticides detection used here, an adaptation of the mini-Luke technique, is described in Chapter 1.

Materials and methods

Sample collection

Sampling of pollen and honey from individual honey bee hives was completed by beekeepers throughout the island of Ireland according to the instructions provided by the National Apiculture Program of Ireland (Annex 2). Briefly, each beekeeper sampled up to five of their hives, whereas those with fewer colonies sampled less. Sample collection from each hive involved cutting a section of comb from a frame that contained stored pollen (approx. 10 x 10 cm) and storing it in a cardboard box. Honey was collected in 50ml plastic centrifuge tubes directly from the comb. All pollen and honey samples were stored at -20 °C on the same day of collection. The samples were subsequently transported on ice to the School of Biology and Environmental Sciences at University College Dublin (UCD). Once at UCD, the samples were stored at -80 °C for analysis. Before analysis, samples from each apiary (beekeeper) were pooled into a single sample. All pesticide analyses were conducted at the Pesticide Residues Laboratory in the Food Chemistry Division at the Department of Agriculture Food and the Marine (DAFM), Dublin, Ireland.

A total of 25 samples of honey and 20 samples of pollen were analysed from throughout the island of Ireland. Seven samples were obtained from apiaries located in County Kildare, two samples from each of Dublin, Down, Waterford, Galway and Clare counties, and one sample from apiaries in the counties of Wicklow, Louth, Cavan, Limerick, Kerry, Cork and Tipperary (see map in figure 2.7). All samples were collected in Autumn 2019, and the COLOSS survey was completed by the participating beekeepers that collected samples. For this survey, beekeepers summarise their colony losses during the winter of the previous year. The COLOSS survey is attached in Annex 3. Here, question no. 20 had special support for the analysis of pesticides residues.

To enable solvent extraction for chromatographic analysis, stored pollen was removed from the comb using a long-handle small scoop and stored at -80 °C in 50ml plastic centrifuge tubes until analysis. Pollen and honey were weighed at room temperature and stored at -20 °C 2 hours before solvent extraction. All pollen samples (20) and 7 honey samples were extracted using the miniaturisation protocol after validation. The extraction method for honey and pollen samples is detailed in Chapter 1.

Target analytes

A set of 33 analytes (table 2.1) were targeted in this study. The target analytes include 13 fungicides, 12 insecticides (including 5 neonicotinoids), 4 herbicides, and 4 acaricides. All compounds analysed here are either known to be widely used in the Irish farmlands (Botías *et al.*, 2015; Ravoet, Reybroeck and de Graaf, 2015; López-Ballesteros *et al.*, 2021), are reportedly associated with Colony Collapse Disorder (vanEngelsdorp *et al.*, 2009; Williams *et al.*, 2010; Rortais *et al.*, 2017; Portus, 2020), or have been described as hazardous for bees and/or wild pollinators (Whitehorn *et al.*, 2012; Botías *et al.*, 2015; Ndakidemi, Mtei and Ndakidemi, 2016; Uhl and Brühl, 2019; López-Ballesteros *et al.*, 2021).

Table 2.1. Tested compounds categorized by type of substance and detection method.

Type	Compound	LCL (mg/kg)	Spike concentration (mg/kg)	MRL (mg/kg)
Fungicide	Boscalid (F)	10.00	99.96	0.15
	Chlorothalonil[2]	10.00	100.04	0.05
	Dimoxystrobin (F)	10.00	100.00	0.05
	Prochloraz (F)	10.00	100.03	0.15
	Tetraconazole (F)	10.00	99.97	0.02
	Cyprodinil (F)	10.02	50.11	0.05
	Epoxiconazole (F)	10.05	50.23	0.05
	Famoxadone (F)	10.01	50.04	0.05
	Fludioxonil (F)	9.86	49.31	0.05
	Imazalil	9.89	49.47	0.05
	Penconazole (F)	9.94	49.72	0.05
	Prothioconazole - desthio (F)	9.93	49.65	0.05
	Pyraclostrobin (F)[1]	9.87	49.33	0.05
Insecticide	Bifenthrin (F)[4]	10.00	99.97	0.05
	Cyhalothrin-lambda (F)[4]	10.00	99.98	0.05
	Cypermethrin (F)[4]	20.00	199.99	0.05
	Deltamethrin (F)[4]	9.99	99.92	0.05
	Dimethoate[3]	10.00	100.03	0.01
	Permethrin (F)	10.00	100.00	0.05*
	Propargite (F)	10.00	100.01	0.05
	Acetamiprid[5]	10.13	50.64	0.05
	Clothianidin[5]	10.05	50.25	0.05
	Imidacloprid[5]	10.01	50.06	0.05
	Thiacloprid[5]	9.91	49.57	0.2
	Thiamethoxam[5]	10.41	52.03	0.05
Acaricide	Fluvalinate-Tau (F)[4]	10.00	100.05	0.05
	Coumaphos[3]	10.00	99.98	0.1
	Chlorfenvinphos (F)[3]	9.85	49.26	0.01
	Methiocarb[1]	9.96	49.80	0.05
Herbicide	**2,4-D**	19.41	97.04	0.05
	MCPA (F)	10.32	51.60	0.05*
	Mecoprop	9.82	49.09	0.05*
	Triclopyr	10.23	51.14	0.05

LCL, Lowest concentration limit. MRLs, maximum residue levels. (*), When the value is unavailable, a default level of 0.05mg/kg is assumed (Regulation (EU) No 283/2013). (F), Fat-soluble. 1, Carbamate. 2, Organochloride. 3, Organophosphate. 4, Pyrethroid. 5, Neonicotinoid. In bold, pesticides detected by LC-MS/MS.

The list of pesticides targeted in this study was also dependant on the capacity of the available instruments for detecting certain chemical substances, and on the availability of pure standards in the Pesticide Residues Laboratory at DAFM. The detection of two other compounds, Cyprodinil and Fludioxonil, was also included in the analysis at a later stage.

Detection of pesticides

Liquid and Gas Chromatography

Chromatography is a technique used to separate components from a mixture (Hage, 2018). This occurs when the components interact with two phases: the mobile and the stationary phase (Hage, 2018). The sample is injected onto the chromatographic system through the column (a tubular structure where the mobile and stationary phases are located) and then partitioned between the mobile and the stationary phase. The components interact with the stationary phase at different rates (Hage, 2018). Components that have less interaction with the stationary phase travel faster than those with strong interaction and spend more time in the mobile phase (Hage, 2018). In contrast, components that interact heavily with the stationary phase go slower and spend more time in the system (Hage, 2018). The time the molecules from a compound spend interacting with both phases cause the molecules to separate (Hage, 2018). Detection of each component occurs therefore at a different time for each molecule also called retention time that is the time the molecule spent interacting with the stationary phase (Hage, 2018). Depending on the mobile phase carrier, chromatography is divided into two types: Gas Chromatography (GC) when the mobile phase is a gas, usually helium, and liquid chromatography (LC) when the carrier is a liquid buffer (Hage, 2018).

Tandem Mass Spectrometry

Stan H. (2005) in his book chapter defines the Mass Spectrum as “a plot of the intensity as a function of the mass-to-charge ratio”. He also describes the base peak as the peak with the highest intensity (Stan, 2005). When a molecule is ionised, the molecule releases an ion (charged atom) that has enough energy to fragment or disassociate the molecule. If the ion has enough energy to break down the molecule, it will produce a series of decompositions in a plot called fragment pathway and several fragment pathways give origin to the patron of fragmentation characteristic of the tested molecule (figure 2.1) (Stan, 2005).

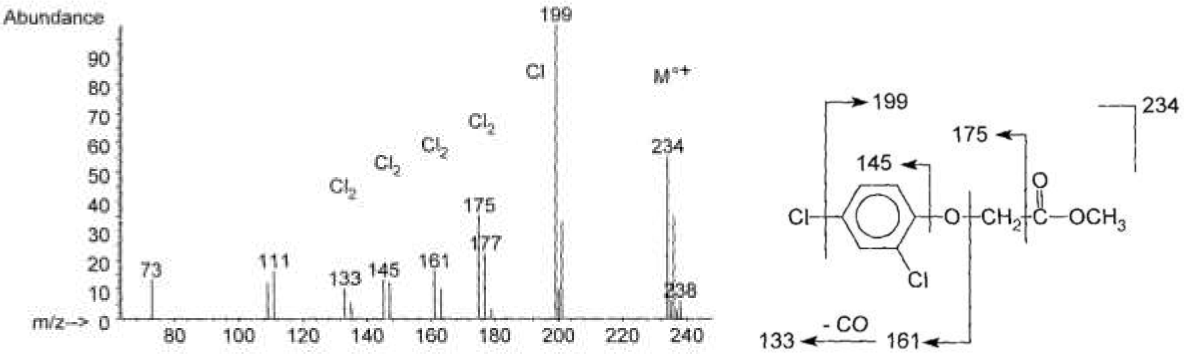


Figure 2.1. Mass spectrum of the methyl ester fraction of the compound 2,4-D.
Left: Mass spectrum of the compound. Right: Fragmentation patron (Stan, 2005).

In tandem mass spectrometry (MS/MS) molecules are ionized to facilitate separation of compounds and detection according to the mass of the molecule (Yates, 2000). In a MS/MS system, two quadrupole mass filters and one collision cell, work together to provide more information about the tested molecule (Stan, 2005). The filters (quadrupoles) can be set to specific mass ratios (m/z) according to the type of compound we are detecting (Doerr, 2013). Initially, the sample is ionized in the ion source (figure 2.2) by the addition of one or more protons through electron impact (Agilent Technologies, 2011). The first quadrupole selects ions that are in the targeted range of mass (Agilent Technologies, 2011). The filtered ions pass to the second quadrupole (collision cell) where they are fragmented by collision with nitrogen atoms that cause energetic excitation of the ions, this process is called collision-induced dissociation (CDI) (Yates, 2000). The fragment ions from the collision cell reach the third quadrupole filter for a second round of filtration where the product ion concentrate. This last filtration phase facilitates the detection of precursor-product ion transitions (figure 2.3) (Agilent Technologies, 2011). At the end, fragments that pass through the third quadrupole filter are identified in a high-energy detector and converted into signals.

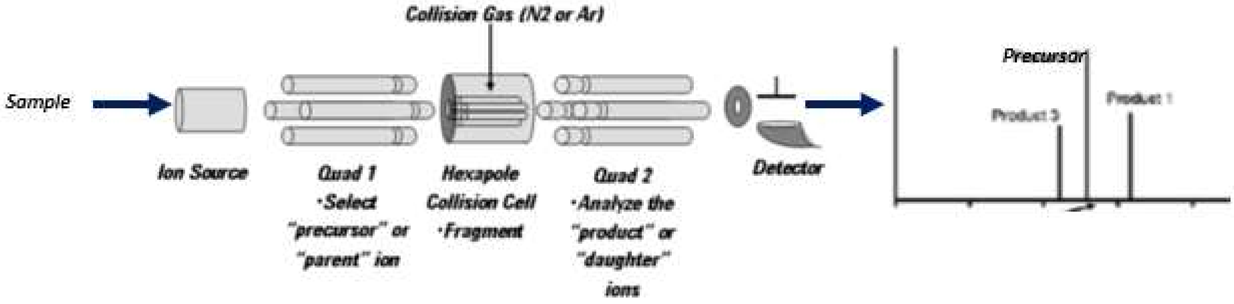


Figure 2.2. Scheme of the tandem Mass Spectrometry Screener
(Agilent Technologies, 2011, modified.)

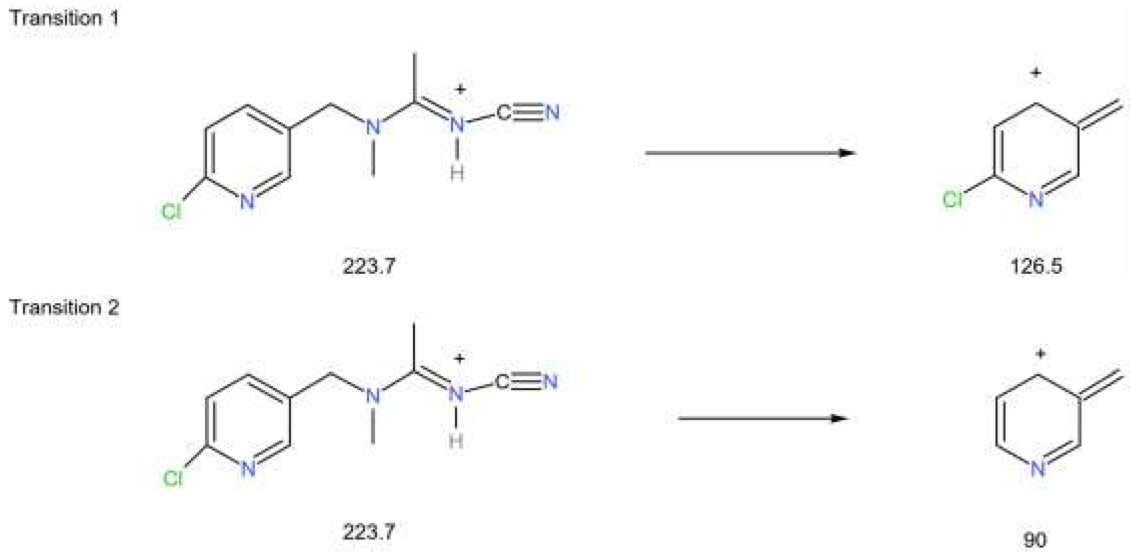


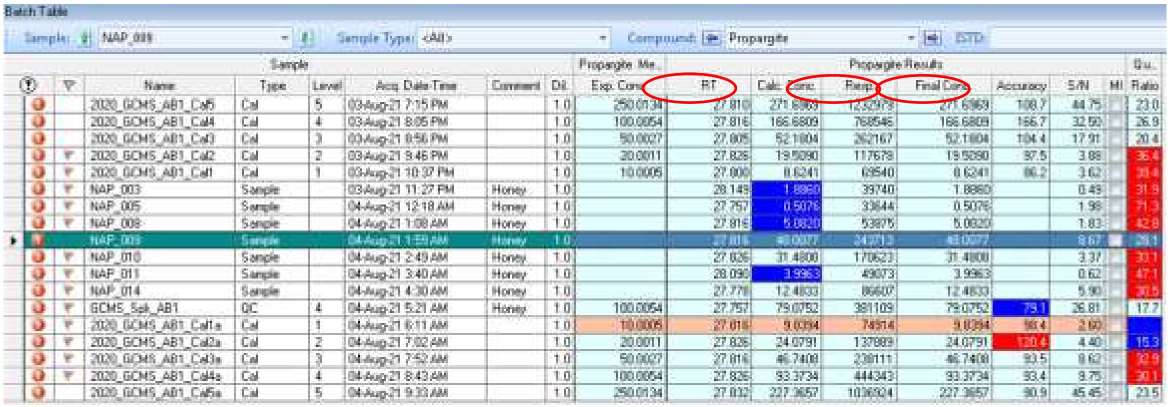
Figure 2.3. Dissociation of the molecule of Acetamiprid (MF = C10H11ClN4, MW = 222.67) into ion transitions. Product ion (transition 1) = 223.7→ 126.5. Product ion (transition 2. T1= 223.7→ 90).

Data analysis and positive samples

All samples were processed with the software Agilent Mass Hunter (Agilent Technologies, no date) and the chromatograms were analysed according to the SANTE document (SANTE/12682/2019) (SANTE/12682/2019, 2019). Briefly, chromatograms from extracted samples should have peaks of similar retention time, shape and response ratio to those generated by the calibration standards and the GC/LC spikes (SANTE/12682/2019, 2019). For gas and liquid chromatography analysis, a sample was considered positive when all the following criteria happened at the same time (Agilent Technologies, 2017):

a) A target transition must be present (green peak in figure 2.5). It is used to determine and quantify the target analyte (Stan, 2005; SANTE/12682/2019, 2019).
b) The acquisition or retention time (RT) of the detected compound corresponds to that of the calibration standards and the GC/LC spike with a tolerance of ± 0.1 min (figure 2.4).
c) A qualifier ion must be present and has to have a ratio between the qualifier tolerance levels (blue dashes in figure 2.5). The qualifier is used for confirmation of structure and the ratio of the two should be within 30% of the quantitation transition (Stan, 2005; SANTE/12682/2019, 2019).
d) The response value has to be similar to that of the calibration solutions at the closest concentration level. When the response is superior to the highest calibrator, the sample requires dilution.
e) Manual integration cannot influence the characteristics of the peak or the assessing criteria.

Additionally, based on the SANTE document, recoveries outside the default range of 60 to 140% were re-analysed (SANTE/12682/2019, 2019). All confirmatory analyses were performed in the GC-MS/MS Orbitrap. Specifications of the instrument are presented by Garvey *et al.*, (2020) (Garvey *et al.*, 2020).



Batch Table

Sample: NAP_009 Sample Type: <All> Compound: Propargite ISTD

Sample						Propargite Me...	Propargite Results							Qu...
Name	Type	Level	Acq. Date-Time	Comment	Dil.	Exp. Conc.	RT	Calc. Conc.	Resp.	Final Conc.	Accuracy	S/N	MI	Ratio
2020_GCMS_AB1_Cal5	Cal	5	03-Aug-21 7:15 PM		1.0	250.0134	27.810	271.6969	1232979	271.6969	108.7	44.75		23.0
2020_GCMS_AB1_Cal4	Cal	4	03-Aug-21 8:05 PM		1.0	100.0054	27.816	166.6809	768546	166.6809	166.7	32.50		26.9
2020_GCMS_AB1_Cal3	Cal	3	03-Aug-21 8:56 PM		1.0	50.0027	27.805	52.1804	262167	52.1804	104.4	17.91		20.4
2020_GCMS_AB1_Cal2	Cal	2	03-Aug-21 9:46 PM		1.0	20.0011	27.826	19.5090	117679	19.5090	97.5	3.88		36.4
2020_GCMS_AB1_Cal1	Cal	1	03-Aug-21 10:37 PM		1.0	10.0005	27.800	8.6241	69540	8.6241	86.2	3.62		33.4
NAP_003	Sample		03-Aug-21 11:27 PM	Honey	1.0		28.149	1.8860	39740	1.8860		0.49		31.9
NAP_005	Sample		04-Aug-21 12:18 AM	Honey	1.0		27.757	0.5076	33644	0.5076		1.98		71.3
NAP_008	Sample		04-Aug-21 1:08 AM	Honey	1.0		27.816	5.0820	53975	5.0820		1.83		42.8
NAP_009	Sample		04-Aug-21 1:59 AM	Honey	1.0		27.816	48.0077	243713	48.0077		8.67		28.1
NAP_010	Sample		04-Aug-21 2:49 AM	Honey	1.0		27.826	31.4800	170623	31.4800		3.37		30.1
NAP_011	Sample		04-Aug-21 3:40 AM	Honey	1.0		28.090	3.9963	49073	3.9963		0.62		47.1
NAP_014	Sample		04-Aug-21 4:30 AM	Honey	1.0		27.778	12.4833	86607	12.4833		5.90		30.5
GCMS_Spk_AB1	QC	4	04-Aug-21 5:21 AM	Honey	1.0	100.0054	27.757	79.0752	381109	79.0752	79.1	26.81		17.7
2020_GCMS_AB1_Cal1a	Cal	1	04-Aug-21 6:11 AM		1.0	10.0005	27.816	9.8394	74514	9.8394	98.4	2.60		
2020_GCMS_AB1_Cal2a	Cal	2	04-Aug-21 7:02 AM		1.0	20.0011	27.826	24.0791	137889	24.0791	120.4	4.40		15.3
2020_GCMS_AB1_Cal3a	Cal	3	04-Aug-21 7:52 AM		1.0	50.0027	27.816	46.7408	238111	46.7408	93.5	8.62		32.9
2020_GCMS_AB1_Cal4a	Cal	4	04-Aug-21 8:43 AM		1.0	100.0054	27.826	93.3734	444343	93.3734	93.4	9.75		30.1
2020_GCMS_AB1_Cal5a	Cal	5	04-Aug-21 9:33 AM		1.0	250.0134	27.832	227.3657	1036924	227.3657	90.9	45.45		23.5

Figure 2.4. Information table of the analysed batch presented by the software Agilent MassHunter.
The response of each sample can be compared to the calibration solutions and spikes (GCMS_Spk_AB1). Samples are bracketed by two sets of calibration solutions. Parameters to be considered for the analysis are retention time (RT), response (Resp.) and Final Concentration. The highlighted sample is positive for Propargite. The final concentration is reported as 0.048 µg/kg.

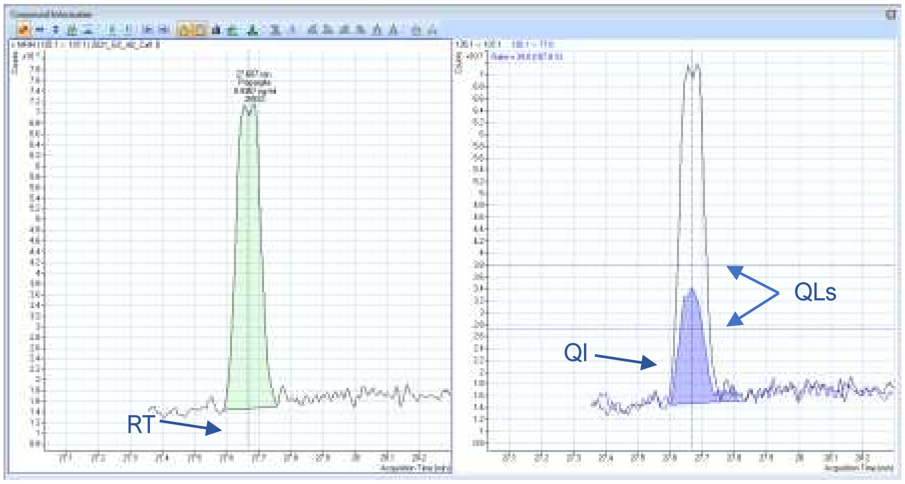


Figure 2.5. Response of Propargite in the calibration standard at the lowest concentration level (10 µg/kg).
The software Agilent MassHunter shows two transition diagrams: Transition 1 (left diagram) that corresponds to the quantitation transition (T1= 135.1→ 107.1), and transition 2 (right diagram) that corresponds to the confirmation transition (T2= 135.1→ 77.0). (RT), Retention time. (QI), qualifier ion. (QLs), qualifier levels.

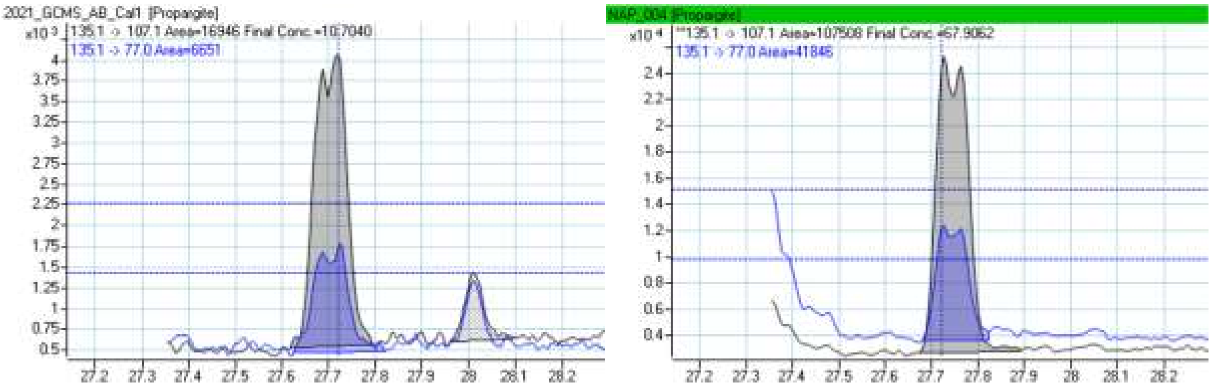


Figure 2.6. Method of analysis to determine a positive sample.
The transition pair of the sample (right) is compared to the transition of the calibration standard at the Lowest Concentration Level (LCL: 10µg/kg) (left). Peak shape, retention time, qualifiers and concentration are compared to define if the sample is positive. Here, the transition pair from the sample meets the criteria to be considered positive for Propargite.

Instrument run conditions

Before running the samples, the instruments were conditioned. Conditioning involves the flow of a carrier (liquid or gas) through the column to remove contaminants from previous runs that could have been accumulated and could influence the outcome of the new run (Hage, 2018). In this study, the GC detector was conditioned with a double injection of a blank sample and one injection of the low calibration level (LCL) standard. The LC system was prepared by a single injection of the screener that is a standard prepared with all the compounds detectable in the LC instrument. In addition, a blank sample, an LC or GC spike, and a double set of calibration standards were injected in every run. Blank samples are also used for quality control of the extraction process and are injected at the beginning of the run leading the first set of calibration standards. The LC and GC spikes are key for quantitation and are injected between the last sample and the second set of calibration standards. The order of injection of the set of calibration standards is vital for constructing the calibration curve and it varies in both systems. In the GC-QQQ, the first set of calibrators is positioned in descending order, and the second one, in ascending order. In the LC-MS/MS, both sets of calibration standards are located in ascending order.

Results

Pesticide detection

We used liquid and gas chromatography coupled with mass spectrometry to determine levels of pesticides in 25 honey samples and 20 pollen samples collected in Autumn 2020 throughout the island of Ireland (figure 2.7). Honey and pollen samples were collected from the same apiaries. However, not all beekeepers participating in the study were able to collect pollen samples, therefore, no pollen samples were available for five of the 25 apiaries assessed in this study.

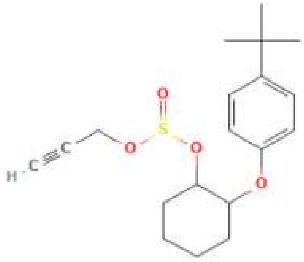
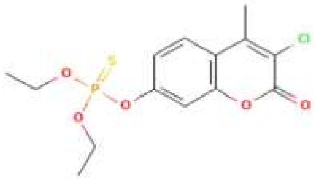
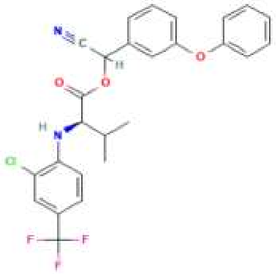
The validated miniaturisation method (see Chapter 1) was used for the extraction of all pollen samples and 7 honey samples. In general, the method presented an acceptable percentage of recovery (60 to 140%) and the RSD% was lower than 20%. Only the batch of 7 honey samples (NAP-003, 005, 008, 009, 010, 011 and 014) presented bad recovery in the LC-MS/MS instrument in positive Electrospray Ionisation mode (ESI+). The recovery for that group of samples was less than 40%.

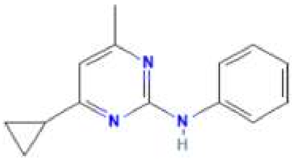
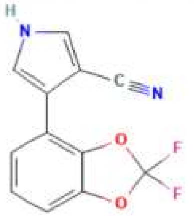
Table 2.2. List of pesticides detected in honey and pollen samples throughout the island of Ireland.

Sample ID	Pesticide	Sample volume (g)	Final Concentration (mg/kg)	R^2	MRL (mg/kg)	LCL (mg/kg)	% Recovery in batch
Honey							
NAP-004 Dublin	*Propargite*	14.99	0.061*	0.995	0.05	0.010	93.60
NAP-009 Kildare	*Propargite*	5.01	0.048	0.989	0.05	0.010	79.10
NAP-010 Kildare	*Propargite*	4.99	0.032	0.989	0.05	0.010	79.10
NAP-013 Down	*Propargite*	15.00	0.185*	0.995	0.05	0.010	93.60
NAP-014 Down	*Propargite*	5.01	0.013	0.989	0.05	0.010	79.10
Pollen							
NAP-006 Louth	**Cyprodinil**	5.01	0.056	0.989	0.05	0.010	103.00
	Fludioxonil		0.047	0.991	0.05	0.009	121.00
NAP-013 Down	*Propargite*	3.41	0.118*	0.988	0.05	0.010	84.30
	Fluvalinate tau		0.028	0.983	0.05	0.010	81.27
	Coumaphos		0.022*	0.980	0.10	0.010	100.90

In blue, samples extracted using the miniaturisation method. In italic, samples analysed by GC-MS/MS. In bold, samples analysed by LC-MS/MS. (*) samples confirmed with high-resolution technology by the GC-MS/MS Orbitrap. MRLs, Maximum Residue Levels. LCL, Lower concentration limit also known as the Limit of Detection (LOD) for this experiment.

Table 2.3. Pesticides detected in honey and pollen samples throughout the island of Ireland.

Propargite (III) ($C_{19}H_{26}O_4S$)	**Coumaphos (Ib)** ($C_{14}H_{16}ClO_5PS$)	**Fluvalinate – Tau (III)** ($C_{26}H_{22}ClF_3N_2O_3$)
		
[2-(4-tert-butylphenoxy)cyclohexyl] prop-2-ynyl sulphite	3-chloro-7-diethoxyphosphinothioyloxy-4-methylchromen-2-one	[cyano-(3-phenoxyphenyl)methyl] (2R)-2-[2-chloro-4-(trifluoromethyl)anilino]-3-methylbutanoate
Sulfite ester	Synthetic organothiophosphate and organochlorine	Synthetic pyrethroid
Group 12 (12C): Inhibitor of mitochondrial ATP synthase	Group 1 (1B): Acetylcholinesterase (AChE) inhibitor	Group 3 (3A): Sodium channel modulator

Cyprodinil (U) ($C_{14}H_{15}N_3$)	**Fludioxonil (U)** ($C_{12}H_6F_2N_2O_2$)
	
4-cyclopropyl-6-methyl-N-phenylpyrimidin-2-amine	4-(2,2-difluoro-1,3-benzodioxol-4-yl)-1H-pyrrole-3-carbonitrile
Aminopyrimidine	Benzodioxol, pyrrole, nitrile and organofluorine
Group D (D1): Inhibition of amino acids and protein synthesis, methionine biosynthesis inhibition	Group E (E2): Signal transduction. Mitogen activate protein (MAP)/Histidine-Kinase in osmotic signal transduction

🟧 Insecticide 🟨 Acaricide 🟩 Fungicide

The table includes chemical structure, IUPAC name, chemical class, and classification by mode of action. In addition, it shows the hazardous classification recommended by WHO: Highly hazardous (Ib), Slightly hazardous (III), and Unlikely to present acute hazard in normal use (U) (Fungicide Resistance Action Committee, no date; Insecticide Resistance Action Committee, no date; World Health Organization, 2020; National Institute of Health, 2021).

We found that 39 samples (86.7 %) were clear, meaning they are not contaminated or they contain no detectable levels of pesticides. Only six samples were found to be positive for pesticides. All positive honey samples contained one compound (table 2.2). In contrast, all positive pollen samples contained more than one compound. Three types of pesticides were detected (table 2.3): one insecticide (Propargite), two acaricides (Coumaphos and Fluvalinate tau), and two fungicides (Fludioxonil and Cyprodinil). Propargite was found in both honey and pollen samples from the same beekeeper, NAP_013, located in Down. The geographical distribution of all apiaries is shown in figure 2.7.

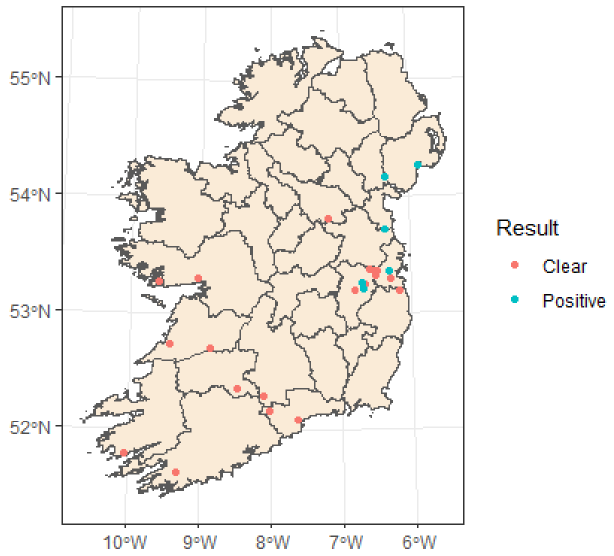


Figure 2.7. Map of the island of Ireland indicating the geographical location of the apiaries sampled.
In red, apiaries where no pesticides were detected. In green, apiaries where pesticides were detected. Internal lines in the map indicate county borders.

Confirmation of pesticide detection by high-resolution analysis

Positive samples with concentrations higher than the MRLs were confirmed in the high-resolution system GC-MS/MS Orbitrap. Here, in order to determine the presence of the compound in the sample, the mass (m/z) of the target compound in the calibration solution was compared to one of the apparently positive samples. A difference in mass of 5 ppm indicates the probability of finding the pesticide in the sample (SANTE/12682/2019, 2019). The lower the difference, the higher the probability that the pesticide is present in the sample. As an illustration, we analysed sample NAP-013 (pollen) for Coumaphos (figure 2.8). We confirmed that the difference in mass was - 1.42 ppm which suggested that the acaricide was present in the sample (figure 2.9).

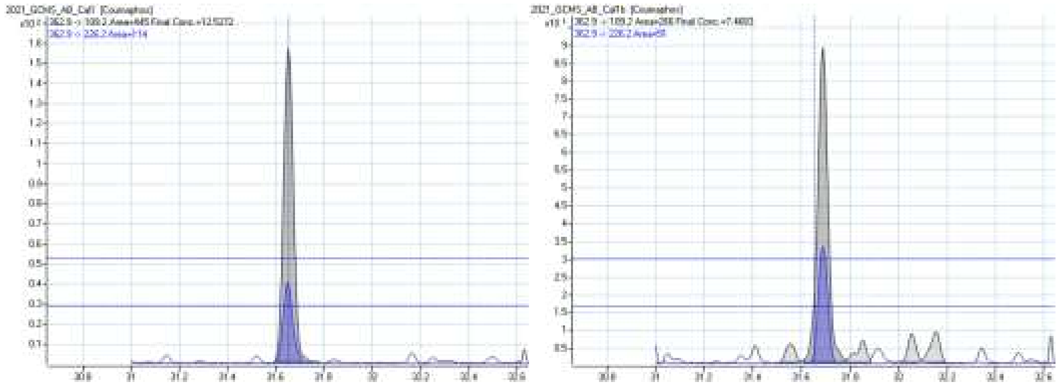


Figure 2.8. Transition diagrams of the pesticide Coumaphos detected in pollen sample NAP-013.
Transition pair of the calibration standard at the Lowest Concentration Level (LCL: 10µg/kg) (left). Transition pair of the sample NAP-013 (right). The peak shape and the quality ion of the sample correspond to that the calibration standard. However, there is a shift in the retention time and an outside of the qualifier and the quantitation ions. Therefore, confirmation with a high-resolution system is required.

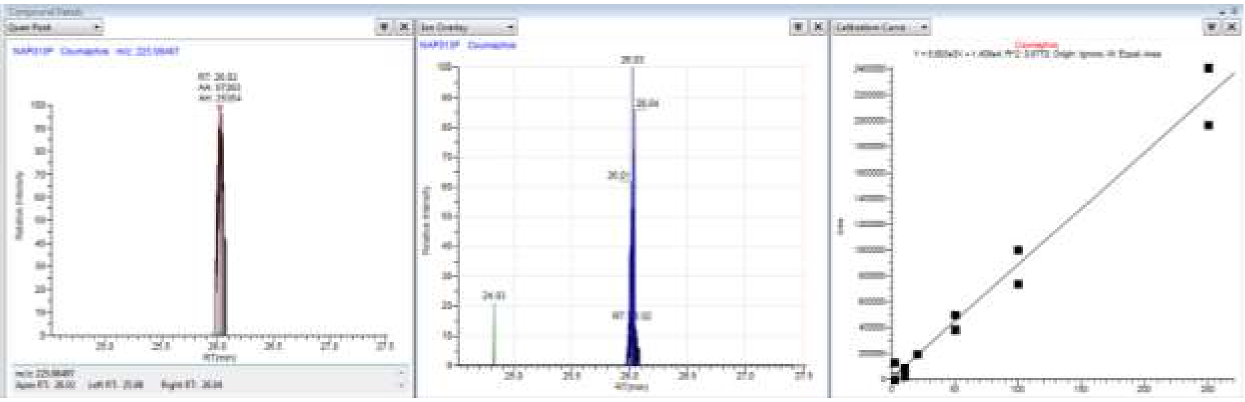


Figure 2.9. Confirmation of the presence of the pesticide Coumaphos using a high-resolution system (GC-MS/MS Orbitrap).

Peak from the sample (left), the mass of the compound is 225.98 m/z. Confirmation peak (middle) represented by an overlay between the qualifier ion and the sample peak. Linearity (right) is represented by the calibration curve (R^2 = 0.977). Screenshot taken from the software Thermo TraceFinder General GC.

The COLOSS form

Analysis of the completed COLOSS questionnaires revealed that Thymol is the preferred method of *Varroa* control by the beekeepers in this study (24 %) (figure 2.10). In addition, analysis of the survey indicates that none of the beekeepers used Coumaphos or Fluvalinate against *Varroa destructor*. However, some information was missing in the surveys completed by beekeepers. Therefore, it was not possible to confirm, by the survey only, that no beekeepers used the mentioned acaricides. The chemical analyses showed that sample NAP_013 was positive for both miticides. The presence of Fluvalinate-Tau is shown in figure 2.11.

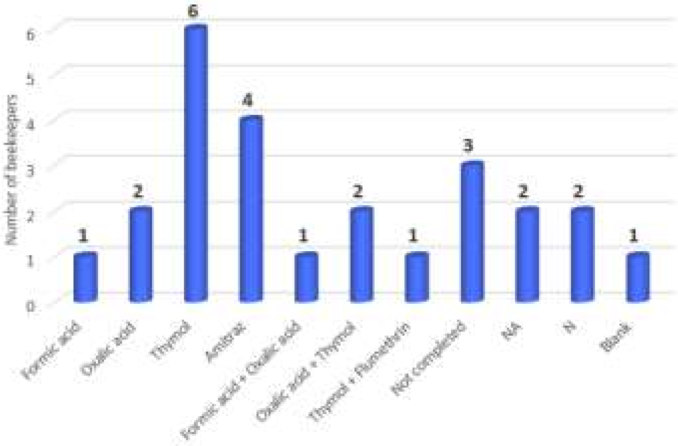


Figure 2.10. Methods of Varroa control applied by the participant beekeepers from April 2019 to March 2020 (COLOSS surveys).

Not completed, used when the beekeeper did not return the form. NA (non-applicable), used for first-year beekeepers. (N), when none method was applied. Blank, when the question was not answered.

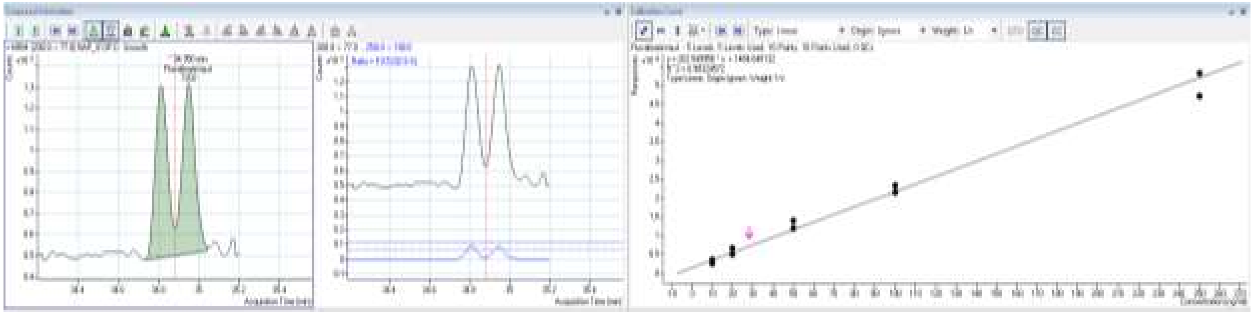


Figure 2.11. Transition diagrams of the pesticide Fluvalinate-Tau detected in pollen sample NAP-013.
Quantitation transition (left). Qualifier transition (right). The peak matches the criteria to be considered positive for Fluvalinate-Tau (peak shape, retention time and qualifier ions) and the curve has good linearity (R^2 = 0.983). Fluvalinate-Tau has an extra layer of confirmation that is represented by two peaks generated by two isomers. In this case, the ratio of the two isomers in the sample is the same as in the standards which confirms the presence of the compound in the sample. Screenshot taken from the software Agilent MassHunter.

Discussion

Monitoring the presence of pesticides in beekeeping resources is essential for determining the pesticide levels that honey bees and other pollinators are exposed to in the environment and within the colony. Moreover, honey and pollen are essential food sources for humans and pollinators, respectively; therefore, pesticides found in these matrices must be monitored to ensure they are safe to consume. In this context, we tested 25 honey samples and 20 pollen samples from different locations throughout the island of Ireland. We aimed to compare pesticide levels in these samples in accordance with the EU regulations that outline the Maximum Residue Levels (MRLs). An adaptation of the mini-Luke method, outlined in chapter 1, and the standard mini-Luke extraction method, were utilised with gas and liquid chromatography-tandem mass spectrometry for pesticides detection. Thirty-three active substances (table 2.1), known to be commonly used across the island of Ireland (López-Ballesteros *et al.*, 2021), were targeted in this experiment. These substances were also targeted because of the potential impact they have on the ecosystem, farmland, and beekeeping (Botías *et al.*, 2015; Ravoet, Reybroeck and de Graaf, 2015; López-Ballesteros *et al.*, 2021).

Matrix analysis

Some special considerations that can influence pesticide detection are the preparation and composition of the sample, and the sample volume. Here, the set of samples from each apiary was pooled and reduced into a single sample, and equal parts were included from each subsample. This procedure was completed individually for the honey and pollen samples. All samples contained particles of bee wax but the honey samples contained higher proportions of wax than the pollen samples. Most honey samples had enough volume for extraction under the standard method, except for 7 samples in which the volume was under 10g. The average sample volume for pollen was 18.56g, meaning most pollen samples could be extracted using the miniaturisation method adapted for 5g. The lower volume of pollen that was processed under the miniaturised method was 3.45g from sample NAP-013. The sample performed well during extraction and pesticide detection. Three pesticides were detected in this sample (Propargite, Fluvalinate-Tau and Coumaphos) meaning that the volume of the sample could be lower than 5g in specific cases.

One group of honey samples (NAP-003, 005, 008, 009, 010, 011 and 014) was particularly challenging for pesticide detection because they contained a high proportion of wax. The lowest recovery level (around 30%) was achieved with these samples when they were analysed by the LC-MS/MS in positive Electrospray Ionisation mode (ESI+) but with acceptable RSD% (< 20%). The presence of wax likely affects the detection of the compound and the transfer of the sample within the column.

Miniaturization method

The extraction method Dutch mini-Luke (acetate extraction) miniaturised and optimised for 5 grams of sample was applied for the detection of pesticides in honey and pollen samples from across the island of Ireland. The validated method (see Chapter 1) was applied to 7 honey samples and 20 pollen samples and, although the screening was done for 346 active substances, the quantitation targeted only 36 pesticides for this experiment. In general, 31 (88.6 %) pesticides fit the linearity with a coefficient of variation (R^2) ranging from 0.962 to 0.998.

Insecticides in honey and pollen samples

The insecticides Propargite, Coumaphos and Fluvalinate-Tau were found in honey and pollen samples. The most frequent compound was Propargite that was present in all the positive honey samples (5), and in one positive pollen sample. The LOQ was 10 µg/kg for the majority (90 %) of the target compounds. In general, insecticide residues were under the MRLs except for Propargite in honey samples NAP-004 and NAP-013, and in pollen sample NAP-013, as shown in table 2.2. Following the guidelines from the SANTE document (SANTE/12682/2019), we reanalysed the samples where the final concentration was higher than the MRLs. Here, the high-resolution system (CG-MS/MS) confirmed that the concentration of Propargite in the samples was above the MRLs (0.05 mg/kg). Our experiment also revealed that in sample NAP-013 the concentration of Propargite in honey (0.185 mg/kg) was higher than in pollen (0.118 mg/kg). It is worth mentioning that the honey sample, NAP-13, was extracted with the standard mini-Luke method and the pollen sample from the same apiary was extracted with the miniaturised version of the method.

Propargite was found to be present in 80% of the positive samples, and was more common in honey samples. To our knowledge, this is the first time Propargite has been detected in honey. The insecticide acaricide has been reported in wax foundation in the US in 2009 and 2011 (Ostiguy *et al.*, 2019), and in bee wax in Belgium in 2015 (Ravoet, Reybroeck and de Graaf, 2015). The presence of Propargite inside honey bee colonies may be caused by resources being brought into the colony by foraging workers (Ravoet, Reybroeck and de Graaf, 2015). This is because it is an acaricide widely used for plant protection in crops such as cotton, tea, fruits (berries, cherries, apples, peaches, etc.), vegetables and ornamentals (Pesticide Action Network, no date; Ostiguy *et al.*, 2019). Previous studies have shown that Propargite has low or no effect on untargeted insects when applied under regulations (Kumar *et al.*, 2005). However, it has been reported that in association with other compounds, Propargite can have a higher toxicity and lethal consequences for bees, especially the Alfalfa leaf-cutting bees, which are thought to be more susceptible to insecticides than honey bees (Johansen *et al.*, 1983). Due to the fact that Propargite is a lipophilic compound, it can be transferred from contaminated wax to other resources such as pollen or honey, as has been demonstrated for other pesticides (Tremolada *et al.*, 2004). Our honey samples had a high content of wax meaning that the insecticide was likely transferred to the samples from the wax.

Coumaphos, another acaricide, was also found in sample NAP-013. In pollen, the content of Coumaphos was found to be below the MRLs. In honey, Coumaphos appeared as a false positive because of a short delay in the retention time and an oversize on the qualifier ion when analysed by the GC-MS/MS. Therefore, the honey sample was re-run in high resolution and the presence of the analyte was rejected. The difference in mass between the calibrator standard and the sample (-28.84 ppm) revealed that the analyte found in the sample does not correspond to Coumaphos. The presence of Coumaphos in honey and pollen has been widely reported. As an example, Lambert et al., 2013, found the acaricide in 78% of the honey and 3.9% of the pollen samples analysed (Lambert *et al.*, 2013). It has been demonstrated that honey and bee wax retain the organophosphate (Coumaphos) for long periods (up to 69 days and 9 months in honey and bee wax, respectively) and that bee wax is the matrix that retains a higher amount of the active ingredient (Korta *et al.*, 2001; Tremolada *et al.*, 2004; Martel *et al.*, 2007). Moreover, it has been reported that Coumaphos is stable in bee wax and that it can be transferred from the comb to honey, honey bees, pollen and brood (Tremolada *et al.*, 2004). Direct contamination occurs when the acaricide is applied in the hive for *Varroa* control and indirect contamination happens when the active ingredient is transferred from the contaminated matrix, generally wax, to other materials (honey or pollen) (Tremolada *et al.*, 2004). Beekeepers tend to recycle the wax used in their honey bee colonies, thus this can increase the persistence of the compound in the hives (Ravoet, Reybroeck and de Graaf, 2015; Gaweł *et al.*, 2019). Additionally, it has been reported that residues in wax might have a high impact on bees because of the volatile nature of the compound, and long-term exposure (Sanchez-Bayo and Goka, 2014).

Coumaphos and Fluvalinate-Tau are frequently associated with the presence of *Varroa* mites in colonies (Johnson, Pollock and Berenbaum, 2009). Both compounds are potent acaricides used to kill the mite and are found in matrices associated with beekeeping (Mullin *et al.*, 2010). As an illustration, Mullin *et al.*, 2010, reported that 47% of tested samples (bee wax and pollen) in North American apiaries contained both acaricides (Mullin *et al.*, 2010). Coumaphos and Fluvalinate-Tau are pesticides with low toxicity for bees (Johnson, Pollock and Berenbaum, 2009). However, it appears that the presence of both compounds in the hive at sublethal levels creates an interaction between compounds (synergism) that can modify the detoxificative metabolism in honey bees (Johnson, Pollock and Berenbaum, 2009). The synergistic interaction leads a competition between active ingredients for accessing the enzyme P450 involved in detoxication (Johnson, Pollock and Berenbaum, 2009). The study presented by Johnson, Pollock and Berenbaum, 2009, suggests that this synergistic interaction can occur in managed bees when Coumaphos and Fluvalinate-Tau are applied at the manufacturer's recommended dosage. Also, Johnson, Pollock and Berenbaum, 2009, suggest that the synergistic impact depends on the cast and age of bees. As an example, larvae are likely more susceptible for they are continuously in contact with bee wax (Johnson, Pollock and Berenbaum, 2009). Rangel and Tarpy, 2015, found that the presence of both compounds in bee wax do not affect the emergence time of queens, but can compromise the queen's fertility by reducing viability and sperm count in the queen's spermatheca. It has been estimated that both acaricides, Fluvalinate-Tau and

Coumaphos, can persist for 5 years in bee wax (Bogdanov, 2004), which suggests a continuous and long-lasting risk for bees. Fluvalinate-Tau and Coumaphos are authorized by the EU and both are considered safe when they are applied under Good Agricultural Practices (GAPs) (European Food Safety Authority (EFSA), 2016; Brancato *et al.*, 2018).

Honey samples from Northern Ireland tested by the Expert Committee on Pesticide Residues in Food (PRiF), report the presence of 10 active ingredients in this matrix (Department for Environment Food and Rural Affairs, 2019). The pesticides detected include veterinary products that are not mentioned in the report but are used in beekeeping against mites and other parasites (Department for Environment Food and Rural Affairs, 2019). As the report mentions all the products were on or under the MRLs. Accordingly, we detected in County Down one positive sample (NAP-013) for Coumaphos and Fluvalinate-Tau, two active ingredients used in beekeeping. Similarly, we reported that both compounds were under the MRLs set at 0.05 mg/kg and 0.10 mg/kg, respectively. As the report mentions, the presence of veterinary products is mostly attributed to in-hive treatments, but it can also be attributed to indirect contamination through recycled wax previously contaminated (Department for Environment Food and Rural Affairs, 2019).

The European Food Safety Authority (EFSA) suggest that bees are exposed to pesticides by two routes, oral and contact (Rortais et al., 2017). Oral exposure is key for bees since bees from all casts, from foragers to larvae, can be exposed to pesticides as the food supplies are continuously distributed throughout the colony (Rortais et al., 2017). In this study, due to the fact that pesticides were detected in honey and pollen, main dietary resources for bees, it is suggested that honey bee colonies in the island of Ireland may be under continuous and long-lasting oral exposure to pesticides.

Fungicides in pollen samples

The fungicides Cyprodinil and Fludioxonil were found to be present in one pollen sample (NAP-006). The concentration of Cyprodinil (0.056 mg/kg) was slightly above the MRLs (0.050 mg/kg). Residues of Cyprodinil have been also reported by Mullin *et al.*, 2010, and Böhme et al., 2018, in pollen samples (Mullin *et al.*, 2010; Böhme *et al.*, 2018). Chlorothalonil and Boscalid are the most common fungicides found in pollen (Mullin *et al.*, 2010). In general, the presence of fungicides in pollen is caused by worker bees collecting pollen from flowers that have been directly sprayed with fungicides in farmlands, home gardens, or roadside meadows (Mullin *et al.*, 2010; Friedle *et al.*, 2021). Indirectly, fungicides can also end up in pollen via spray-generated dust that can be transported by wind gusts contaminating food and water sources (Friedle *et al.*, 2021).

Geographic distribution of clear and positive samples

Honey and pollen samples collected in different geographic regions throughout the island of Ireland were found to be both clear and positive for pesticides. Samples positive for Propargite were found in Dublin (NAP-004), Kildare (NAP-009, 010) and Down (NAP-013, 014), and Cyprodinil and Fludioxonil were found in Louth (NAP-014). As shown in figure 2.7, all positive samples were detected on the East coast of the island. The Northern Ireland threatened bee report, explains that the East and the South-Eastern side, (including County Down), the Northern coast and the County Fermanagh, among others, are essential regions for bees and wild pollinators (Hart, no date). The insects' life depends on climate conditions and the South-East of the island provides the most favourable habitats, sheltered from the wind and mildly cold weather (Hart, no date). Further, County Down on the East of the island is where most of the honey bee colonies are located, but also where the highest number of colonies were lost (27%) during winter 2019-2020, as reported by the Northern Ireland Honeybee Husbandry Survey (2020-21) (Forsythe and Murchie, 2021).

The presence of pesticides in the environment can explain why we detected pesticides in our pollen and honey samples. County Down, for instance, supports 81% of the outdoors vegetable crops grown in Northern Ireland, where the farmland is dominated by carrots and parsnips (Lavery *et al.*, 2019). These crops represent 42% of the crops in Northern Ireland and receive 58% of the pesticide application (measured in weight of active ingredients) in this country (Lavery *et al.*, 2019). It has been reported that honey bees are reliable pollen carriers and probably the most dominant pollinator in the production of hybrid carrot seeds (Gaffney *et al.*, 2018). Honey bees, unlike other wild pollinators, forage on the nectar of the carrot umbels (Gaffney *et al.*, 2018). We did not determine the origin of the pollen of the positive samples, but it may be useful to do so in future work in order to determine exactly which species bees are foraging and where the pesticides are coming from. Honey bees are also important pollinators of several fruits, such as berries, strawberries and Gala apples, produced in Northern Ireland (Hart, no date). In the same region, fungicides and insecticides represent 24% of the plant protection products sprayed (Lavery *et al.*, 2019). Propargite is used as an insecticide for several crops, including fruits, vegetables and ornaments, and Cyprodinil and Fludioxonil are widely used as fungicides on fruits and vegetables (Pesticide Action Network, no date). In Northern Ireland, seed treatments are applied to 54% of carrots and parsnip crops, and the formulation: cymoxanil/ fludioxonil/ metalaxyl-m is the most prevalent (Lavery *et al.*, 2019). Similarly, the Plant Protection Plan reports that Fludioxonil in combination with other fungicides and insecticides is used as a seed dressing in Ireland (Department of Agriculture Food and Marine, 2021). We suggest that the pesticides detected in our samples likely contaminated the pollen particles via two direct routes: 1) by drift droplets that fall over flowers of the treated crops during spraying, and 2) by translocation through treated plants, soil or seeds (Sanchez-Bayo and Goka, 2014). Indirect routs of contamination include water sources or dust where the wind combines the pesticide with the pollen when the pesticide is sprayed when the weather conditions are unappropriated (Rortais *et al.*, 2017).

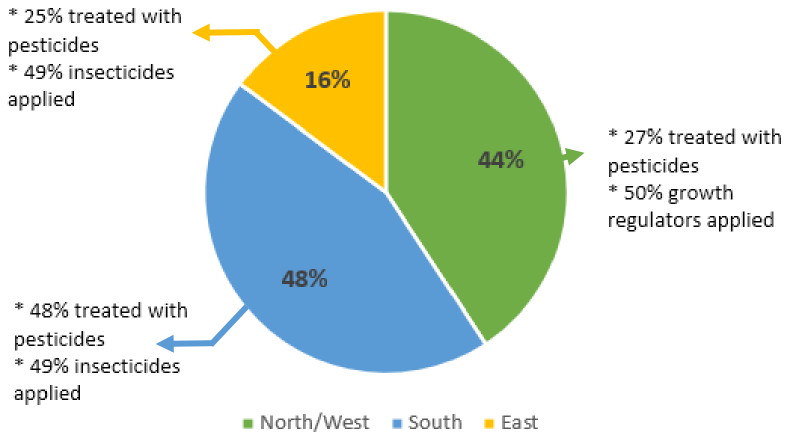


Figure 2.12. Grassland distribution in Ireland.
The chart shows the percentage of grassland growth in each region. In addition, it shows the percentage of area that is treated with pesticides and the type of pesticide mainly applied in each region. North/West: Donegal, Leitrim, Monaghan, Cavan, Westmeath, Longford, Sligo, Mayo, Galway, and Clare South: Wexford, Kilkenny, Waterford, Tipperary, Limerick, Cork, Kerry, and Roscommon. East: Louth, Meath, Dublin, Kildare, Offaly, Laois, Carlow, and Wicklow (DAFM, 2017).

Managed honey bee colonies are not evenly distributed geographically throughout Ireland. County Cork, has the largest number of honey bee colonies in Ireland (15%), followed closely by County Dublin with 11% of all recorded colonies. Kildare, on the other hand, has only 4% of the total national number of colonies (27 040 colonies) (Department of Agriculture Food and Marine, 2019). In Ireland, 90% of the cultivated area is covered by grassland (López-Ballesteros *et al.*, 2021). The southeast of Ireland, and a small region of the northwest, are where there is the highest proportion of arable land, however this is not necessarily where the majority of the pesticides are applied (López-Ballesteros *et al.*, 2021). The East side of the country which includes the following counties: Louth, Meath, Dublin, Kildare, Offaly, Laois, Carlow and Wicklow, supports only 16% of the grassland and fodder crops and supports 25% of pesticides spraying (DAFM, 2017). As it is explained in figure 2.12, in this area, 49% of the pesticides utilised are insecticides (DAFM, 2017).

In comparison to other pollinators, such as bumblebees (*Bombus impatiens*), it has been reported that honey bees benefit more from grassland (Quinlan *et al.*, 2021). Quinlan *et al.*, 2021, suggest that honey bee foragers tend to visit larger crop patches over small patches and visit large grassland areas more frequently (Quinlan *et al.*, 2021). We suggest, therefore, that in the East side of the island of Ireland, bees may be attracted by large patches of crops and grassland where considerable volume of pesticides have been applied.

The COLOSS survey

The COLOSS survey (see annexe 3) was completed by all the beekeepers that supplied samples for this study. The survey is conducted Europe wide annually and is designed to collect data on the number of colony losses experienced by beekeepers in the previous winter, as well as to record factors that may have affected their colonies, such as the application of pesticides for *Varroa* control. In the subset of surveys analysed here, we found that Thymol was the most commonly used substance for *Varroa* control by beekeepers (24%). This organic substance was applied alone or in combination with Flumethrin, but different beekeepers used different application methods and treated their colonies at different times of the year. Other organic acids used by this group of beekeepers included Formic and Oxalic acid. Amitraz was the second most common pesticide, and was used by 16% of beekeepers. We did not detect Flumethrin in any of the samples studied here, and Amitraz was not targeted in our study. Due to the instability of Amitraz in aqueous and acid matrices like honey, the detection of this compound and its metabolites requires an alternative method, such as HPLC (High-Performance Liquid Chromatography) for single residues.

Because not all the beekeepers that provided samples also completed the COLOSS survey, it was not possible to determine if there was a correlation between the information collected from the survey, and the chemical analysis of the positive sample for Coumaphos and Fluvalinate-Tau. However, we suggest that chemical analyses such as those conducted in this study, are a key tool for monitoring pesticide levels in honey bee colony resources, including the effects of the control methods that beekeepers are applying to combat bee pests, particularly *Varroa*.

The present study was the first comprehensive investigation of pesticides in honey bee colony resources throughout the island of Ireland. This work leads the way for future studies and the inclusion of more apiaries from across the island. This work has allowed us to start to better understand one of the key stressors, pesticide contamination, and how it may be contributing to honey bee colony loss, as well as the decline of other pollinators and untargeted insects. It has been reported that continuous exposure to pesticides in the colony through food sources may have a direct impact on bees' physiology at all life stages (Rortais et al., 2017). Pesticides can harm bees' detoxificative metabolism (Johnson, Pollock and Berenbaum, 2009), weaken the immune response (Cuesta-Maté et al., 2021), impair homing behaviour (Woodcock et al., 2017), and reduce learning abilities such as grooming (Decourtye et al., 2004).

General conclusions

In this thesis, it was demonstrated that the Dutch mini-Luke miniaturised method is a robust and useful alternative pesticide extraction method to the standard extraction method, and can be utilised for detecting pesticides in samples that have a limited quantity and availability. The miniaturisation method for the majority of the compounds listed in annexe 1 by meeting the criteria established in the EU SANTE guidance document for accuracy and precision for these compounds. Applying the multi-resides miniaturised extraction method in liquid and gas chromatography-tandem mass spectrometry enabled similar results to those found when the standard method was applied, therefore the new miniaturisation extraction method was confidently applied to the analysis of real samples.

It was found that pollen samples may be more easily contaminated with pesticides than honey samples, as pollen samples were found to contain more than one pesticide. Plant protectors such as insecticides and fungicides, and acaricides may end up in colonies via two routes: 1) Direct route: through the applications of pesticides for *Varroa* control in beekeeping, or via foraging honey bee workers collecting pollen sprayed with pesticides. 2) Indirect route: through the re-use of bee wax by beekeepers, where the wax was previously contaminated with pesticides, via contaminated water sources or topsoil where pesticides have been moved by the wind during spraying.

In addition, it was detected all the positive samples on the East side of the island of Ireland. In the Northern Ireland it is likely due to the fact that the highest number of managed colonies are located in this region. In addition to the fact that this region supports a large area of outdoors vegetable crops where an important number of pesticides is applied. Similarly, in Ireland, the East side of the country is covered by grassland where a big portion of insecticides has been applied.

The extraction method, in combination with the analysis methods, GC-QQQ and LC-MS/MS, can be considered useful monitoring tools for detecting pesticides in beekeeping resources, as well as for detecting the level of plant protectors and veterinary products that honey bees are exposed to in the environment and inside the hive.

Future work

The investigation conducted here will facilitate future studies aimed at detecting pesticides in bee resources, and will thus contribute to determining which stressors are involved in honey bee colony losses, as well as in the decline of wild pollinator populations. We suggest that the miniaturised method is a trustable monitoring tool for determining the presence of pesticides in beekeeping resources, and for evaluating pesticide levels in the environment. Moreover, the methods and the information provided here can be utilised for risk assessment to determine toxicity of the pesticides in honey bees, and to estimate how likely are the founded compounds to cause biological damage in managed bees and wild pollinators.

The results of this study suggest that the new extraction method can be utilised for the routine analysis of beekeeping commodities, as well as for the analysis of other food origin samples such as fruits and vegetables. The method has been validated using the SANTE guidance document, therefore, it can be utilised in official control laboratories where food samples are analysed.

Annexes

Annex 1. List of analytes quantified in this study. The same analytes were utilised for validation of the miniaturisation method.

GC Compound	Spike concentration (mg/kg)	Lowest calibration level (LCL) (mg/kg)	LC Compound	Spike concentration (mg/kg)	Lowest calibration level (LCL) (mg/kg)
1,4-Dimethylnapthalene	100.71		1-Naphthylacetamide	50.01	10.00
4,4-Dichlorobenzophenone	99.98	10.00	Acephate	49.63	9.93
Acephate	99.96	10.00	Acetamiprid	50.64	10.13
Acrinathrin	99.99	10.00	Atrazine	49.93	9.99
Aldrin	99.99	10.00	Atrazine-desethyl	49.78	9.96
Anthraquinone	497.08	50.95	Atrazine-desisopropyl	49.98	10.00
Azinphos-methyl	99.98	10.00	Azoxystrobin	49.63	9.93
Azoxystrobin	100.05	10.01	BAC10	50.34	10.07
Bifenthrin	99.97	10.00	BAC12	49.71	9.94
Binapacryl	100.05	10.00	BAC14	49.75	9.95
Biphenyl	500.36	50.04	BAC16	49.78	9.96
Bitertanol-I	100.04	10.00	Benalaxyl	50.11	10.02
Bitertanol-II	100.04	10.00	Bendiocarb	49.62	9.92
Boscalid	99.96	10.00	Boscalid	49.88	9.98
Bromopropylate	100.00	10.00	Bromacil	49.84	9.97
Captafol	100.05	10.01	Bupirimate	49.71	9.94
Captan	99.99	10.00	Buprofezin	50.44	10.09
Chlordane-cis	99.95	9.99	Carbaryl	50.25	10.05
Chlordane-trans	99.95	10.00	Carbendazim	49.65	9.93
Chlorfenapyr	99.95	10.00	Carbofuran	50.06	10.01
Chlorothalonil	100.04	10.00	Carbofuran 3 Hydroxy	50.28	10.06
Chlorpropham	100.04	10.00	Carbosulfan	50.08	10.02
Chlorpyrifos methyl	99.97	10.00	Chlorantraniliprole	49.53	9.91
Chlorthal-dimethyl	100.05	10.01	Chlorfenvinphos	49.26	9.85
Cyfluthrin	99.99	10.00	Chlorpyriphos	49.48	9.90
Cyhalothrin-lambda	99.98	10.00	Clofentezine	50.04	10.01
Cypermethrin	199.99	20.00	Cyazofamid	48.34	9.67
Cyproconazole	99.95	10.00	Cymoxanil	49.69	9.94
Deltamethrin	99.92	9.99	Cyproconazole I	50.04	10.01
Diazinon	99.95	9.99	Cyproconazole II	50.04	10.01
Dicofol	99.96	10.00	Cyprodinil	50.11	10.02
Dieldrin	100.01	10.00	DDAC	50.32	10.06
Dimethoate	100.03	10.00	Diethofencarb	50.78	10.16
Diphenylamine	498.56	49.86	Difenoconazole	49.85	9.97
Endosulfan-alpha	100.00	10.00	Dimethomorph I	50.64	10.13
Endosulfan-beta	99.99	10.00	Dimethomorph II	50.64	10.13

Endosulfan-ether	100.03	10.00	Dodine	50.07	10.01
Endosulfan-lacton	99.99	10.00	Emamectin benzoate	50.34	10.07
Endosulfan-sulfate	100.03	10.00	Epoxyconazole	50.23	10.05
Endrin	100.04	10.00	Ethiofencarb Sulfoxide	247.20	49.44
Ethoprophos	100.02	10.00	Ethion	50.42	10.08
Etoxazole	99.96	10.00	Ethirimol	50.19	10.04
Fenarimol	100.03	10.00	Etofenprox	49.64	9.93
Fenazaquin	99.96	10.00	Famoxadone	50.04	10.01
Fenbuconazole	99.99	10.00	Fenhexamid	49.97	9.99
Fenitrothion	100.03	10.00	Fenoxycarb	50.10	10.02
Fenpropathrin	100.01	10.00	Fenpropidin	49.99	10.00
Fenvalerate-I	99.98	10.00	Fenpropimorph	49.67	9.93
Fenvalerate-II	99.98	10.00	Fenpyroximate	49.79	9.96
Fludioxonil	99.96	10.00	Fensulfothion	49.74	9.95
Fluvalinate-tau-I	100.05	10.00	Fenthion	50.27	10.05
Fluvalinate-tau-II	100.05	10.00	Fenthion Sulfone	49.74	9.95
Folpet	100.00	10.00	Fenthion Sulfoxide	50.28	10.06
HCH-alpha	99.96	10.00	Flonicamid	49.86	9.97
HCH-beta	100.05	10.00	Fludioxonil	49.31	9.86
HCH-delta	100.03	10.00	Flufenoxuron	50.20	10.04
Hexachlorobenzene	100.05	10.00	Fluopicolide	49.74	9.95
Hexaconazole	99.96	10.00	Fluopyram	100.02	20.00
Iprodione	100.03	10.00	Fluquinconazole	48.96	9.79
Iprovalicarb-I	99.98	10.00	Flutolanil	49.69	9.94
Iprovalicarb-II	99.98	10.00	Flutriafol	50.19	10.04
Lenacil	99.96	10.08	Fluxapyroxad	49.76	9.95
Lindane	100.03	10.00	Fosthiazate	49.95	9.99
MCPA methyl ester	99.95	9.99	Hexythiazox	49.54	9.91
Methamidophos	99.97	10.00	Imazalil	49.47	9.89
Molinate	100.02	10.00	Imidacloprid	50.06	10.01
Omethoate	99.96	10.00	Indoxacarb	50.24	10.05
opDDD	100.01	10.00	Isoprothiolane	50.01	10.00
opDDE	99.97	10.00	Kresoxim-methyl	49.46	9.89
opDDT	99.99	10.00	Linuron	49.92	9.98
o-Phenylphenol	100.04	10.00	Malaoxon	49.87	9.97
Oxychlordane	100.00	10.09	Malathion	49.14	9.83
PCB 101	49.96	5.00	Mandipropamid	49.38	9.88
PCB 118	50.01	5.00	Mepanipyrim	49.60	9.92
PCB 138	49.97	5.00	Metalaxyl	49.05	9.81
PCB 153	49.96	5.00	Metamitron	49.81	9.96
PCB 180	50.03	5.00	Methamidophos	49.93	9.99
PCB 28	50.01	5.00	Methidathion	49.26	9.85
PCB 52	50.03	5.00	Methiocarb	49.80	9.96

Pendimethalin	99.99	10.00	Methiocarb Sulfone	51.45	10.29
Permethrin-I	100.00	10.00	Methiocarb Sulfoxide	47.14	9.43
Permethrin-II	100.00	10.00	Methomyl	50.09	10.02
Phosmet	100.00	10.00	Methoxyfenozide	50.21	10.04
Pirimicarb	100.04	10.00	Metrafenone	50.19	10.04
Pirimicarb desmethyl	99.99	10.00	Molinate	50.17	10.03
ppDDD	100.01	10.00	Monocrotophos	49.58	9.92
ppDDE	99.98	10.00	Myclobutanil	49.86	9.97
ppDDT	100.04	10.00	Oxamyl	49.96	9.99
Prochloraz	100.03	10.00	Paclobutrazol	50.18	10.04
Profenofos	99.99	10.00	Penconazole	49.72	9.94
Propargite	100.01	10.00	Pencycuron	49.57	9.91
Propiconazole-I	99.98	10.00	Phenmedipham	50.00	10.00
Propiconazole-II	99.98	10.00	Piperonyl butoxide	49.13	9.83
Prothiofos	100.03	10.00	Pirimiphos-ethyl	50.08	10.02
Pyridaben	100.03	10.00	Pirimiphos-methyl	49.46	9.89
Resmethrin	1000.08	100.01	Propyzamide	49.11	9.82
Spirodiclofen	100.00	10.00	Proquinazid	49.87	9.97
Tefluthrin	100.00	10.00	Prosulfocarb	249.24	49.85
Tetraconazole	99.97	10.00	Prothioconazole desthio	49.65	9.93
Tetramethrin-I	199.98	20.00	Pymetrozine	49.49	9.90
Tetramethrin-II	199.98	20.00	Pyraclostrobin	49.33	9.87
Tolclofos-methyl	99.94	9.99	Pyrethrins	246.95	49.39
Triadimefon	100.01	10.00	Pyridaben	49.98	10.00
Triadimenol-I	101.16	10.00	Pyridalyl	49.93	9.99
Triadimenol-II	101.16	10.00	Pyrimethanil	50.06	10.01
Trifluralin	100.06	10.01	Pyriproxifen	49.89	9.98
Aclonifen	99.96	10.00	Quinoxyfen	50.34	10.07
Alachlor	99.98	10.00	Spinosyn A	49.87	9.97
Azaconazole	100.01	10.00	Spinosyn D	49.87	9.97
Azamethiophos	99.99	10.00	Spirodiclofen	49.34	9.87
Azinphos-ethyl	99.96	10.00	Spiromesifen	50.15	10.03
Bromophos-ethyl	100.01	10.00	Spirotetramat	49.98	10.00
Bromophos-methyl	100.02	10.00	Spiroxamine	49.57	9.91
Chlorbufam	99.96	10.00	Tebuconazole	50.13	10.03
Chlorobenzilate	100.04	10.00	Tebufenozide	49.59	9.92
Chlozolinate	100.02	10.00	Tebufenpyrad	50.31	10.06
Coumaphos	99.98	10.00	Terbuthylazine	49.81	9.96
Cyanofenphos	100.01	10.00	Thiabendazole	49.43	9.89
Cyanophos	99.95	9.99	Thiacloprid	49.57	9.91
Demeton-S-me-sulfone	100.00	10.00	Thiamethoxam	52.03	10.41
Dichlobenil	99.99	10.00	Thiodicarb	48.28	9.66
Dichlofluanid	99.99	10.00	Thiophanate-Ethyl	48.92	9.78

Dichlorvos	99.95	9.99	Thiophanate-Methyl	48.74	9.75
Dicloran	100.04	10.00	Triazophos	49.61	9.92
Dimoxystrobin	100.00	10.00	Trichlorfon	98.14	19.63
EPN	101.14	10.24	Tricyclazole	49.90	9.98
Etridazole	100.01	10.00	Trifloxystrobin	49.93	9.99
Fenamidone	100.00	10.00	Zoxamide	50.16	10.03
Fenchlorphos	99.97	10.00	2,4,5-T	51.05	10.21
Flucythrinate-I	100.02	10.00	2,4-D	97.04	19.41
Flucythrinate-II	100.02	10.00	2,4-DB	336.96	67.39
Flurtamone	99.99	10.00	Bentazone	46.90	9.38
Flusilazole	99.95	9.99	Bixafen	51.22	10.24
Fonofos	100.04	10.00	Bromoxynil	52.25	10.45
Formothion	99.98	10.00	Chlorfluazuron	52.69	10.54
Furalaxyl	100.02	10.00	Clethodim	51.06	10.21
Heptachlor	100.06	10.01	Clothianidin	50.25	10.05
Heptachlor endo-epoxide,trans	100.02	10.00	Cyclanilide	51.18	10.24
Heptachlor exo-epoxide,cis	99.98	10.00	Cycloxydim	503.62	100.72
Iodofenphos	100.00	10.00	Dichlorprop	52.68	10.54
Isazophos	100.03	10.00	Diflubenzuron	50.34	10.07
Isocarbofos	100.00	10.00	Dinoseb	48.83	9.77
Isodrin	99.97	10.00	Dinoterb	48.86	9.77
Isofenphos	100.04	10.00	DNOC	50.27	10.05
Isofenphos-methyl	99.94	9.99	Endosulfan sulfate	52.52	10.50
Isofenphos-oxon	99.97	10.00	Fenoprop (2,4,5 TP)	52.30	10.46
Mecarbam	100.02	10.00	Fipronil	51.98	10.40
Methacrifos	99.96	10.00	Fipronil desulfynil	52.25	10.45
Methoxychlor	100.00	10.00	Fipronil sulfide	49.97	9.99
Metribuzin	100.01	10.00	Fipronil sulfone	50.03	10.01
Mevinphos	100.03	10.00	Fluazifop	49.48	9.90
Mirex	100.01	10.00	Fluazinam	49.66	9.93
Nitrofen	99.98	10.00	Flubendiamide	48.87	9.77
Nonachlor-trans	100.04	10.00	Haloxyfop	52.49	10.50
Nuarimol	99.98	10.00	Hexaflumuron	51.72	10.34
Oxadixyl	100.01	10.00	Ioxynil	49.58	9.92
Paraoxon methyl	100.01	10.00	MCPA	51.60	10.32
Parathion-ethyl	100.05	10.00	MCPB	51.50	10.30
Parathion-methyl	100.03	10.00	Mecoprop	49.09	9.82
Pentachloroaniline	99.99	10.00	Quizalofop	48.72	9.74
Phenthoate	100.04	10.00	Sulfentrazone	46.84	9.37
Phorate	99.96	9.68	Teflubenzuron	50.42	10.08
Phosalone	99.97	10.00	TFNA	51.72	10.34
Phosphamidon-I	100.01	10.00	TFNG	50.38	10.08
Phosphamidon-II	100.01	10.00	Triclopyr	51.14	10.23

ppDDE	99.98	10.00	Triflumuron	49.86	9.97
Procymidone	99.99	10.00			
Propachlor	99.96	10.00			
Propanil	100.05	10.00			
Propetamphos	100.01	10.00			
Propham	100.01	10.00			
Pyrifenox-I	99.99	10.00			
Pyrifenox-II	99.99	10.00			
Quintozene	99.98	10.00			
Silthiofam	100.00	10.00			
Tecnazene	100.03	10.00			
Tetradifon	100.01	10.00			
Tolyfluanid	99.93	9.99			
Triflumizole	100.04	10.00			
Vinclozolin	100.04	10.00			

Annex 2.

IMPORTANT INSTRUCTIONS FOR SAMPLING FOR THE NATIONAL APICULTURE PROGRAMME

In order to standardise the sampling procedure at the national scale we ask you to carefully follow the instructions below. To ensure your samples remain viable for testing, please only conduct your sampling when you can store your samples in a freezer, or when you have time to take your samples back to your collection point on the same day that you sample, or at most the next day after sampling.

1) Assemble all of your flat packed cardboard boxes (you will need 3 boxes per hive, therefore you will need to assemble 15 boxes if you are sampling from 5 hives, and fewer if you have fewer hives)

2) Using the pencil, fill out the **SURVEY** on colony losses and put it back into the ziplock plastic bag

3) Randomly select 5 HIVES from your apiary/ies (or less if you have fewer hives)

4) Put one coloured sticker on each of your selected hives. Use a different colour for each hive. When you collect samples, match the coloured sticker on the hive with the coloured sticker on the boxes and tube that you put your samples into.

5) For each of your selected hives, sweep a group of adult bees (around 100 bees) into one of the 5 cardboard boxes labelled **BEES**

6) For each of your selected hives, cut out a small portion (approx. 10cm x 10cm if possible, or no less than 5cm x 5cm) of **capped** BROOD COMB and put it into one of the 5 cardboard boxes labelled **BROOD**

7) For each of your selected hives, cut out a small portion (approx. 10cm x 10cm) of comb containing pollen (bee bread) and put it into one of the 5 cardboard boxes labelled **POLLEN**

8) For each of your selected hives, use a clean wooden spoon provided to collect honey (approx. 50 mls, or fill the tube) (without comb) into one of the 5 tubes labelled **HONEY.** If sampling from less than three hives please fill two 50 ml tubes from each hive to ensure sufficient honey volume is available for testing.

9) Place your completed survey, and all of your sample boxes and tubes into the big plastic bag provided

10) Please take the bag containing your samples and survey to your collection point on the same day, or the day after, you do your sampling, OR, please place the bag containing your samples into a freezer on the same day that you do your sampling.

** If you have any questions at any time, please email Julia at julia.jones@ucd.ie

Thank you very much for your help with the National Apiculture Programme sampling

Annex 3. The COLOSS survey.

COLOSS: Honey Bee Colony Loss Survey

2019/2020

Dear Beekeeper,

Your contribution to this annual survey on colony losses is much appreciated. At various points in the questionnaire below, there are points of Information marked in RED. Hopefullythese will explain the logic of the questions that follow. Please try to complete **all** the questions as this will allow not only the number of winter colony losses to be calculated, butwill also contribute to identifying the possible causes for these losses.

Please return your completed questionnaire in its ziplock bag with your sampling kit.

Information: **Completing your personal details (name, address and contact details) is optional but details of the town/county closest to apiary is essential. Please note that any personal information provided will be removed from the file used for processing the data and kept confidential.**

First name ______________________ Last name
______________________ Address
______________________ City
______________________ Eircode
______________________ Email
______________________ **County**
______________ Country **Ireland**

① To describe the location of your main apiary or operation, please state
   a) the name of a city/town/village near to your apiary? __________
   b) and the postal code of the apiary (or a postal code nearby) __________

② How many apiaries do you have? ☐

❸ If you have more than one apiary, are all your apiaries within a distance of about15 km of each other? If you have only **one** apiary, **please answer yes.**

○ Yes ○ No ○ Don't know

Information: Numbers of colonies, losses and status of the colony post-winter.
Please consider winter as the period between the moment that you finished the pre-winter preparations for your colonies and the start of the new foraging season.
In this questionnaire we try to gather information about production colonies. A production colonyis a colony which is queen right and is strong enough to produce honey.

❹ How many **production colonies** did you have **before winter 2019-2020?**

Information: ***In the next questions you are asked for numbers of colonies lost.Please consider a colony as lost if it:***

1. ***died out (or reduced to a few hundred bees) during winter***
2. ***died due to natural disaster***
3. ***was still alive post winter, but had queen problems you couldn't solve (drone laying queensor no queen at all)***

Each colony LOST should be recorded to ONLY one of these three categories.

❺ How many of these (❹) colonies did you lose that were alive but hadunsolvable queen problems. If none, please answer 0.

❻ How many of these (❹) colonies did you lose, because of natural disaster (flooding, storm, fallen tree, cattle/sheep, vandalism, theft, rodents)?

❼ How many of these (❹) colonies died out during the winter period?

❽ How many of the dead colonies (died out group) (❼)

a) … had many dead bees in or in front of the hive?

b) … had no or only a few dead bees in or in front of the empty hive?

c) … had dead workers in cells and no food present in the hive(signs of starvation)?

d) … had dead workers in cells while food was present in the hive?

e) … had none of the above or unknown symptoms?

Note: Each individual dead colony should be categorized into only one of the Groups marked a) to e). The total number of dead colonies in Q7 should be equal to sum of coloniesQ8

❾ How many of the wintered colonies (❹) were weak but queenright after winter 2019-2020?

Information: We would like to calculate increases and decreases in the number of colonies, so ifyou had colonies in spring 2019 and remember how many you had, please answer the followingtwo questions, considering spring as the start of the foraging season:

⑩ How many production colonies did you have in a) spring 2019 (last year)?
b) spring 2020?

Information: Conditions in the colonies, the environment around the apiary, and management:

⑪ How many of the wintered colonies had a new queen in 2019? Don’t know

⑫ To what extent did you observe queen problems in your colonies during the foraging season of 2019 compared to what you usually have?

O More O Normal O Less O Don’t know

⑬ Compared with your colonies with old queens, have your colonies with young queens survivedwinter?

O Better O No differently O Worse O Don’t know / not applicable

⑭ How many of your surviving colonies had a large amount offaeces inside the hive after winter? Don’t know

⑮ Did you migrate any of your colonies at least once for honey production or pollination in 2019?

O Yes O No O Don’t know

⑯ Approximately what proportion of brood combs did you replace with comb foundation per colony in 2019? O 0% O 1-30% O 31-50% O more than 50%

⑰ Did the majority of your bee colonies have a significant flow on one or more of the following plants in 2019?

a) Orchards	O Yes	O No	O Don’t know
b) Oil seed rape	O Yes	O No	O Don’t know
c) Maize	O Yes	O No	O Don’t know
d) Sunflower	O Yes	O No	O Don’t know
e) Heather	O Yes	O No	O Don’t know
f) Autumn forage plant	O Yes	O No	O Don’t know

(**Autumn forage plants = nectar plants growing in land lying fallow: eg mustard)**

⑱ Have you monitored your colonies for Varroa during the period April 2019 - March 2020?

O Yes O No O Don’t know / not applicable

⑲ Have you treated your colonies against Varroa during the period April 2019 – March 2020?

O Yes O No O Don’t know / not applicable

⑳ Could you please indicate the months when you monitored your production coloniesfor Varroa **AND** also indicate when you **STARTED** a *Varroa* treatment or management plan during the period: Apr 2019 – Mar 2020?	**Month in which each measure started**:											
	2019									**2020**		
Method / Product:	April	May	June	July	August	September	October	November	December	January	February	March
Monitoring of Varroa infestation level (e.g. countingmite fall)												
Drone brood removal												
Hyperthermia (heat treatment of brood/bees)												

Other biotechnical method (as e.g. trapping comb, complete brood removal, queen confinement)												
Formic acid - short term												
Formic acid - long term (e.g. MAQS)												
Lactic acid												
Oxalic acid (ApiBioxal) - trickling												
Oxalic acid (ApiBioxal)- sublimation (evaporation)												
Hiveclean/Bienenwohl/Beevital												
Thymol (e.g. Apiguard, ApilifeVar)												
Tau-fluvalinate (e.g. Apistan)												
Flumethrin (e.g. Bayvarol)												
Amitraz (in strips, e.g. Apivar, Apitraz)												
Amitraz (fumigation/aerosol)												
Coumaphos (e.g. Perizin)												
Coumaphos (in strips, e.g. Checkmite+)												
Another chemical product												
Another method												

22 If you gave your colonies a supplemental sugar feed (sugar solution or inverted sugar) last year to prepare for winter, how many kg of sugar (dry matter) did you giveon average per production colony?

23 Do you keep your bees in an area where Varroa has still not been detected?
○ Yes ○ No ○ Don't know

24 Have you noticed bees with crippled/deformed wings in your colonies (during the summer season)? (These are signs of the presence of Deformed Wing Virus, which is spread by Varroa mites).
○ Not at all ○ to a limited extent ○ to a large extent ○ Don't know

25 What particular measures apply for the majority of your beekeeping:

a) Screened (mesh) bottom board in Winter	○ Yes	○ No	○ Don't know
b) Insulated hives in Winter (incl. double walled hives)	○ Yes	○ No	○ Don't know
c) Hives made from synthetic materials	○ Yes	○ No	○ Don't know
d) Certified organic beekeeping	○ Yes	○ No	○ Don't know
e) Queens bred from Varroa tolerant/resistant stock	○ Yes	○ No	○ Don't know
f) Small brood cell size (5.1 mm or less)	○ Yes	○ No	○ Don't know
g) Natural comb (without foundation)	○ Yes	○ No	○ Don't know
h) Purchase wax from outside own operation	○ Yes	○ No	○ Don't know
i) Non-wax foundation in brood chamber	○ Yes	○ No	○ Don't know

26 Did you observe *Vespa velutina* (Asian hornet) foraging for honey bees in your apiary/apiaries? ○ Yes ○ No ○ Don't know

27 How much in Euros (€) do you estimate your costs to be per colony for Varroatreatment in the period April 2019 to March 2020.

On behalf of Julia Jones and the National Apiculture Programme, our sincere thanks for completing thisannual survey and we appreciate the time you have taken to complete it.

Julia Jones
September 2020

References

Agilent Technologies, I. (2011) *7000 Series Triple Quadrupole GC/MS System*. Second Edi. Available at: https://www.agilent.com/cs/library/usermanuals/Public/G7000-90031.pdf.

Agilent Technologies, I. (2017) 'Agilent MassHunter Workstation Software Quantitative Analysis: Quantitation DataSet'. Agilent Technologies, Inc., p. 238.

Agilent Technologies, I. (no date) 'Agilent MassHunter'. Available at: https://www.agilent.com/en/promotions/masshunter-mass-spec.

Anderson, K. E. *et al.* (2014) 'Hive-stored pollen of honey bees: many lines of evidence are consistent with pollen preservation, not nutrient conversion', *Molecular Ecology*, 23(23), pp. 5904–5917. doi: 10.1111/mec.12966.

Aparna, A. R. and Rajalakshmi, D. (1999) 'Honey—its characteristics, sensory aspects, and applications', *Food Reviews International*, 15(4), pp. 455–471. doi: 10.1080/87559129909541199.

Baša Česnik, H., Kmecl, V. and Velikonja Bolta, Š. (2019) 'Pesticide and veterinary drug residues in honey - validation of methods and a survey of organic and conventional honeys from Slovenia', *Food Additives & Contaminants: Part A*, 36(9), pp. 1358–1375. doi: 10.1080/19440049.2019.1631492.

Bernardes, M. F. F. *et al.* (2015) 'Impact of Pesticides on Environmental and Human Health', in *Toxicology Studies - Cells, Drugs and Environment*. InTech. doi: 10.5772/59710.

Bogdanov, S. (2004) 'Beeswax: quality issues today', *Bee World*, 85(3), pp. 46–50. doi: 10.1080/0005772X.2004.11099623.

Bogdanov, S. (2006) 'Contaminants of bee products', *Apidologie*, 37(1), pp. 1–18. doi: 10.1051/apido:2005043.

Böhme, F. *et al.* (2018) 'Pesticide residue survey of pollen loads collected by honeybees (Apis mellifera) in daily intervals at three agricultural sites in South Germany', *PLOS ONE*. Edited by N. Desneux, 13(7), p. e0199995. doi: 10.1371/journal.pone.0199995.

Boncristiani, H. *et al.* (2012) 'Direct effect of acaricides on pathogen loads and gene expression levels in honey bees Apis mellifera', *Journal of Insect Physiology*, 58(5), pp. 613–620. doi: 10.1016/j.jinsphys.2011.12.011.

Botías, C. *et al.* (2015) 'Neonicotinoid Residues in Wildflowers, a Potential Route of Chronic Exposure for Bees', *Environmental Science & Technology*, 49(21), pp. 12731–12740. doi: 10.1021/acs.est.5b03459.

Brancato, A. *et al.* (2018) 'Review of the existing maximum residue levels for tau-fluvalinate according to Article 12 of Regulation (EC) No 396/2005', *EFSA Journal*, 16(11). doi: 10.2903/j.efsa.2018.5475.

Brühl, C. A. and Zaller, J. G. (2019) 'Biodiversity Decline as a Consequence of an Inappropriate Environmental Risk Assessment of Pesticides', *Frontiers in Environmental Science*, 7. doi: 10.3389/fenvs.2019.00177.

Calatayud-Vernich, P. *et al.* (2018) 'Pesticide residues in honey bees, pollen and beeswax: Assessing beehive exposure’, *Environmental Pollution*, 241, pp. 106–114. doi: 10.1016/j.envpol.2018.05.062.

Chamberlain, D. E. *et al.* (2000) ‘Changes in the abundance of farmland birds in relation to the timing of agricultural intensification in England and Wales’, *Journal of Applied Ecology*, 37(5), pp. 771–788. doi: 10.1046/j.1365-2664.2000.00548.x.

Cuesta-Maté, A. *et al.* (2021) ‘Resistance and Vulnerability of Honeybee (Apis mellifera) Gut Bacteria to Commonly Used Pesticides’, *Frontiers in Microbiology*, 12. doi: 10.3389/fmicb.2021.717990.

DAFM, D. of A. F. and M. (2017) *Pesticide Usage in Ireland, Grassland & Fodder Crops Survey Report 2017*. Celbridge, Co. Kildare. Available at: https://www.pcs.agriculture.gov.ie/media/pesticides/content/sud/pesticidestatistics/2GrasslandReport2017FinalVersion290520.pdf.

DAFM, D. of A. F. and M. (2020) *Publication: Beekeeping & Honey*, *Beekeeping & Honey*. Available at: https://www.gov.ie/en/publication/9e1ff-beekeeping-honey/.

Dangles, O. and Casas, J. (2019) ‘Ecosystem services provided by insects for achieving sustainable development goals’, *Ecosystem Services*, 35, pp. 109–115. doi: 10.1016/j.ecoser.2018.12.002.

David, A. *et al.* (2015) ‘Sensitive determination of mixtures of neonicotinoid and fungicide residues in pollen and single bumblebees using a scaled down QuEChERS method for exposure assessment’, *Analytical and Bioanalytical Chemistry*, 407(26), pp. 8151–8162. doi: 10.1007/s00216-015-8986-6.

David, A. *et al.* (2016) ‘Widespread contamination of wildflower and bee-collected pollen with complex mixtures of neonicotinoids and fungicides commonly applied to crops’, *Environment International*, 88, pp. 169–178. doi: 10.1016/j.envint.2015.12.011.

Decourtye, A. *et al.* (2004) ‘Effects of imidacloprid and deltamethrin on associative learning in honeybees under semi-field and laboratory conditions’, *Ecotoxicology and Environmental Safety*, 57(3), pp. 410–419. doi: 10.1016/j.ecoenv.2003.08.001.

Department for Environment Food and Rural Affairs, D. (2019) *The Expert Committee on Pesticide Residues in Food (PRiF). Anual report, 2019.* Available at: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/932348/expert-committee-pesticide-residues-food-annual-report-2019.pdf.

Department of Agriculture Food and Marine, D. (2019) *Beekeepers Census 2019*. Available at: https://www.gov.ie/ga/foilsiuchan/9e1ff-beekeeping-honey/#beekeeping-census.

Department of Agriculture Food and Marine, D. (2021) *Plant Protection Products 2021*. Available at: https://www.pcs.agriculture.gov.ie/media/pesticides/content/register/2021book/Plant Protection Products 2021.pdf.

Doerr, A. (2013) ‘Mass spectrometry–based targeted proteomics’, *Nature Methods*, 10(1), pp. 23–23. doi: 10.1038/nmeth.2286.

European Commission, E. (2021) *Maximum Residue Levels*. Available at: https://ec.europa.eu/info/index_en (Accessed: 11 October 2021).

European Food Safety Authority (EFSA) (2016) ‘Setting of maximum residue levels for amitraz, coumaphos, flumequine, oxytetracycline, permethrin and streptomycin in certain products of animal origin’, *EFSA Journal*, 14(8). doi: 10.2903/j.efsa.2016.4570.

Feltham, H., Park, K. and Goulson, D. (2014) ‘Field realistic doses of pesticide imidacloprid reduce bumblebee pollen foraging efficiency’, *Ecotoxicology*, 23(3), pp. 317–323. doi: 10.1007/s10646-014-1189-7.

Forsythe, I. and Murchie, A. K. (2021) *Northern Ireland Honeybee Husbandry Survey 2020-21*. Available at: https://www.afbini.gov.uk/sites/afbini.gov.uk/files/publications/Honeybee Husbandry Survey Report 2020-21.pdf.

Friedle, C. *et al.* (2021) ‘Pesticide residues in daily bee pollen samples (April–July) from an intensive agricultural region in Southern Germany’, *Environmental Science and Pollution Research*, 28(18), pp. 22789–22803. doi: 10.1007/s11356-020-12318-2.

Fungicide Resistance Action Committee, F. (no date) *Search Fungicides to find FRAC Recommendations*. Available at: https://www.frac.info/ (Accessed: 11 October 2021).

Gaffney, A. *et al.* (2018) ‘Frequent Insect Visitors Are Not Always Pollen Carriers in Hybrid Carrot Pollination’, *Insects*, 9(2), p. 61. doi: 10.3390/insects9020061.

Garrido, P. M. *et al.* (2013) ‘Immune-related gene expression in nurse honey bees (Apis mellifera) exposed to synthetic acaricides’, *Journal of Insect Physiology*, 59(1), pp. 113–119. doi: 10.1016/j.jinsphys.2012.10.019.

Garvey, J. *et al.* (2020) ‘Multi-residue analysis of pesticide residues and polychlorinated biphenyls in fruit and vegetables using orbital ion trap high-resolution accurate mass spectrometry’, *Analytical and Bioanalytical Chemistry*, 412(26), pp. 7113–7121. doi: 10.1007/s00216-020-02844-w.

Gaweł, M. *et al.* (2019) ‘Determination of neonicotinoids and 199 other pesticide residues in honey by liquid and gas chromatography coupled with tandem mass spectrometry’, *Food Chemistry*, 282, pp. 36–47. doi: 10.1016/j.foodchem.2019.01.003.

Hadian, Z., Samira, S. and Yazdanpanah, H. (2019) ‘Pesticide Residues Analysis in Iranian Fruits and Vegetables by Gas Chromatography-Mass Spectrometry.’, *Iranian journal of pharmaceutical research : IJPR*, 18(1), pp. 275–285. Available at: http://www.ncbi.nlm.nih.gov/pubmed/31089362.

Hage, D. S. (2018) ‘Chromatography’, in *Principles and Applications of Clinical Mass Spectrometry*. Elsevier, pp. 1–32. doi: 10.1016/B978-0-12-816063-3.00001-3.

Hart, A. (no date) *Northern Ireland Threatened Bee Report*. Available at: https://cdn.buglife.org.uk/downloads/Northern-Ireland-Threatened-Bee-Report.pdf.

Henry, M. *et al.* (2012) ‘A Common Pesticide Decreases Foraging Success and Survival in Honey Bees’, *Science*, 336(6079), pp. 348–350. doi: 10.1126/science.1215039.

Herbert Jr., E. W. and Shimanuki, H. (1978) ‘Chemical composition and nutritive value of Bee-Collected and Bee-Stored pollen’, *Apidologie, Springer Verlag*, 9(1), pp. 33–40. Available at: https://hal.archives-ouvertes.fr/hal-00890453.

Hernández, F. *et al.* (2013) ‘The role of GC-MS/MS with triple quadrupole in pesticide residue analysis in food and the environment', *Analytical Methods*, 5(21), p. 5875. doi: 10.1039/c3ay41104d.

Insecticide Resistance Action Committee, I. (no date) *The IRAC Mode of Action Classification Online*. Available at: https://irac-online.org/ (Accessed: 11 October 2021).

IUPAC (2019) 'Compendium of Chemical Terminology', in McNaught, A. D. and Wilkinson, A. (eds) *The IUPAC Compendium of Chemical Terminology*. 2nd ed. (t. Research Triangle Park, NC: International Union of Pure and Applied Chemistry (IUPAC). doi: 10.1351/goldbook.S05752.

Johansen, C. A. *et al.* (1983) 'Pesticides and Bees 1', *Environmental Entomology*, 12(5), pp. 1513–1518. doi: 10.1093/ee/12.5.1513.

Johnson, R. M. *et al.* (2010) 'Pesticides and honey bee toxicity – USA', *Apidologie*, 41(3), pp. 312–331. doi: 10.1051/apido/2010018.

Johnson, R. M. *et al.* (2013) 'Acaricide, Fungicide and Drug Interactions in Honey Bees (Apis mellifera)', *PLoS ONE*. Edited by N. E. Raine, 8(1), p. e54092. doi: 10.1371/journal.pone.0054092.

Johnson, R. M., Pollock, H. S. and Berenbaum, M. R. (2009) 'Synergistic Interactions Between In-Hive Miticides in Apis mellifera', *Journal of Economic Entomology*, 102(2), pp. 474–479. doi: 10.1603/029.102.0202.

Kasiotis, K. M. *et al.* (2014) 'Pesticide residues in honeybees, honey and bee pollen by LC–MS/MS screening: Reported death incidents in honeybees', *Science of The Total Environment*, 485–486, pp. 633–642. doi: 10.1016/j.scitotenv.2014.03.042.

Kiljanek, T. *et al.* (2016) 'Multi-residue method for the determination of pesticides and pesticide metabolites in honeybees by liquid and gas chromatography coupled with tandem mass spectrometry—Honeybee poisoning incidents', *Journal of Chromatography A*, 1435, pp. 100–114. doi: 10.1016/j.chroma.2016.01.045.

Komosinska-Vassev, K. *et al.* (2015) 'Bee Pollen: Chemical Composition and Therapeutic Application', *Evidence-Based Complementary and Alternative Medicine*, 2015, pp. 1–6. doi: 10.1155/2015/297425.

Korta, E. *et al.* (2001) 'Study of Acaricide Stability in Honey. Characterization of Amitraz Degradation Products in Honey and Beeswax', *Journal of Agricultural and Food Chemistry*, 49(12), pp. 5835–5842. doi: 10.1021/jf010787s.

Kujawski, M. W. and Namieśnik, J. (2008) 'Challenges in preparing honey samples for chromatographic determination of contaminants and trace residues', *TrAC Trends in Analytical Chemistry*, 27(9), pp. 785–793. doi: 10.1016/j.trac.2008.07.004.

Kumar, V. *et al.* (2005) 'Dissipation behavior of propargite—an acaricide residues in soil, apple (Malus pumila) and tea (Camellia sinensis)', *Chemosphere*, 58(6), pp. 837–843. doi: 10.1016/j.chemosphere.2004.06.032.

Kumar, V., Kumar, P. and Singh, J. (2019) 'Contaminants in Agriculture and Environment: Health Risks and Remediation', in *Contaminants in Agriculture and Environment: Health Risks and Remediation*. Agro Environ Media - Agriculture and Ennvironmental Science Academy, Haridwar, India, pp. 1–301. doi: 10.26832/AESA-2019-CAE.

Laaniste, A. *et al.* (2016) 'Determination of neonicotinoids in Estonian honey by liquid chromatography–electrospray mass spectrometry', *Journal of Environmental Science and Health, Part B*, 51(7), pp. 455–464. doi: 10.1080/03601234.2016.1159457.

Lambert, O. *et al.* (2013) 'Widespread Occurrence of Chemical Residues in Beehive Matrices from Apiaries Located in Different Landscapes of Western France', *PLoS ONE*. Edited by N. Desneux, 8(6), p. e67007. doi: 10.1371/journal.pone.0067007.

Lavery, M. K. *et al.* (2019) *Pesticide usage in Northern Ireland. Northern Ireland Outdoor Vegetable Crops 2019, Survey Report 293*. Available at: https://www.afbini.gov.uk/sites/afbini.gov.uk/files/publications/VEG19 Final Report.pdf.

Lehotay, S. J. *et al.* (2010) 'Comparison of QuEChERS sample preparation methods for the analysis of pesticide residues in fruits and vegetables', *Journal of Chromatography A*, 1217(16), pp. 2548–2560. doi: 10.1016/j.chroma.2010.01.044.

Lennon, J. J. (2015) 'Potential impacts of climate change on agriculture and food safety within the island of Ireland††This paper is one of a series of reviews on "Climate Change and Food Safety – an Island of Ireland perspective".', *Trends in Food Science & Technology*, 44(1), pp. 1–10. doi: 10.1016/j.tifs.2014.07.003.

Lister, A. S. (2005) 'Validation of HPLC methods in pharmaceutical analysis', in Ahuja, S. and Dong, M. W. (eds) *Handbook of Pharmaceutical Analysis by HPLC*. Elsevier Inc., pp. 191–217. Available at: https://reader.elsevier.com/reader/sd/pii/S0149639505800510?token=B59644C2C3781B3C1616E082DB46C173644070A5A3098420CCC13D9896271CECFAF45D28DD801FF366A2C20DD22A3BF5&originRegion=eu-west-1&originCreation=20210923172530.

López-Ballesteros, A. *et al.* (2021) *European Plant Protection Product use: an assessment of data availability and implications for risk assessment on non-target organisms*, *Science of The Total Environment*.

Lozano, A. *et al.* (2016) 'Miniaturisation and optimisation of the Dutch mini-Luke extraction method for implementation in the routine multi-residue analysis of pesticides in fruits and vegetables', *Food Chemistry*, 192, pp. 668–681. doi: 10.1016/j.foodchem.2015.07.065.

Majors, R. E. (Agilent T. (2013) *Sample preparation fundamentals for chromatography*. Wilmington, DE: © Agilent Technologies, Inc. Available at: https://www.agilent.com/cs/library/primers/Public/5991-3326EN_SPHB.pdf.

Martel, A.-C. *et al.* (2007) 'Acaricide residues in honey and wax after treatment of honey bee colonies with Apivar ® or Asuntol ® 50', *Apidologie*, 38(6), pp. 534–544. doi: 10.1051/apido:2007038.

Moe, S. J. *et al.* (2019) 'Effects of an aquaculture pesticide (diflubenzuron) on non-target shrimp populations: Extrapolation from laboratory experiments to the risk of population decline', *Ecological Modelling*, 413, p. 108833. doi: 10.1016/j.ecolmodel.2019.108833.

Mommaerts, V. *et al.* (2010) 'Risk assessment for side-effects of neonicotinoids against bumblebees with and without impairing foraging behavior', *Ecotoxicology*, 19(1), pp. 207–215. doi: 10.1007/s10646-009-0406-2.

Motta, E. V. S., Raymann, K. and Moran, N. A. (2018) ‘Glyphosate perturbs the gut microbiota of honey bees’, *Proceedings of the National Academy of Sciences*, 115(41), pp. 10305–10310. doi: 10.1073/pnas.1803880115.

Mullin, C. A. *et al.* (2010) ‘High Levels of Miticides and Agrochemicals in North American Apiaries: Implications for Honey Bee Health’, *PLoS ONE*. Edited by F. Marion-Poll, 5(3), p. e9754. doi: 10.1371/journal.pone.0009754.

Narenderan, S. T., Meyyanathan, S. N. and Babu, B. (2020) ‘Review of pesticide residue analysis in fruits and vegetables. Pre-treatment, extraction and detection techniques’, *Food Research International*, 133, p. 109141. doi: 10.1016/j.foodres.2020.109141.

National Institute of Health, N. (2021) *PubChem: Explore Chemistry*. Available at: https://pubchem.ncbi.nlm.nih.gov/ (Accessed: 11 October 2021).

Ndakidemi, B., Mtei, K. and Ndakidemi, P. A. (2016) ‘Impacts of Synthetic and Botanical Pesticides on Beneficial Insects’, *Agricultural Sciences*, 07(06), pp. 364–372. doi: 10.4236/as.2016.76038.

Nicolopoulou-Stamati, P. *et al.* (2016) ‘Chemical Pesticides and Human Health: The Urgent Need for a New Concept in Agriculture’, *Frontiers in Public Health*, 4. doi: 10.3389/fpubh.2016.00148.

OERKE, E.-C. (2006) ‘Crop losses to pests’, *The Journal of Agricultural Science*, 144(1), pp. 31–43. doi: 10.1017/S0021859605005708.

Ostiguy, N. *et al.* (2019) ‘Honey Bee Exposure to Pesticides: A Four-Year Nationwide Study’, *Insects*, 10(1), p. 13. doi: 10.3390/insects10010013.

Özkara, A., Akyil, D. and Konuk, M. (2016) ‘Pesticides, Environmental Pollution, and Health’, in *Environmental Health Risk - Hazardous Factors to Living Species*. InTech. doi: 10.5772/63094.

Pariona, A. (2017) ‘Top Pesticide Using Countries’, *WorldAtlas*, April. Available at: https://www.worldatlas.com/articles/top-pesticide-consuming-countries-of-the-world.html.

Parrilla Vázquez, P. *et al.* (2015) ‘A sensitive and efficient method for routine pesticide multiresidue analysis in bee pollen samples using gas and liquid chromatography coupled to tandem mass spectrometry’, *Journal of Chromatography A*, 1426, pp. 161–173. doi: 10.1016/j.chroma.2015.11.081.

Paszko, T. *et al.* (2016) ‘Adsorption and degradation of phenoxyalkanoic acid herbicides in soils: A review’, *Environmental Toxicology and Chemistry*, 35(2), pp. 271–286. doi: 10.1002/etc.3212.

Patel, V. *et al.* (2021) ‘Why bees are critical for achieving sustainable development’, *Ambio*, 50(1), pp. 49–59. doi: 10.1007/s13280-020-01333-9.

Pesticide Action Network, P. (no date) *Pesticide info searcher*, *Pesticide info*. Available at: https://www.pesticideinfo.org/ (Accessed: 5 November 2021).

Pihlström, T. *et al.* (2007) ‘Analysis of pesticide residues in fruit and vegetables with ethyl acetate extraction using gas and liquid chromatography with tandem mass spectrometric detection’, *Analytical and Bioanalytical Chemistry*, 389(6), pp. 1773–1789. doi: 10.1007/s00216-007-1425-6.

Popp, J., Pető, K. and Nagy, J. (2013) ‘Pesticide productivity and food security. A review’, *Agronomy for Sustainable Development*, 33(1), pp. 243–255. doi: 10.1007/s13593-012-0105-x.

Portus, R. (2020) 'An Ecological Whodunit: The Story of Colony Collapse Disorder', *Society & Animals*, pp. 1–19. doi: 10.1163/15685306-BJA10026.

Quinlan, G. M. *et al.* (2021) 'Honey bee (Apis mellifera) colonies benefit from grassland/ pasture while bumble bee (Bombus impatiens) colonies in the same landscapes benefit from non-corn/soybean cropland', *PLOS ONE*. Edited by A. G. Dolezal, 16(9), p. e0257701. doi: 10.1371/journal.pone.0257701.

Rangel, J. and Tarpy, D. R. (2015) 'The combined effects of miticides on the mating health of honey bee (Apis mellifera L.) queens', *Journal of Apicultural Research*, 54(3), pp. 275–283. doi: 10.1080/00218839.2016.1147218.

Rani, L. *et al.* (2021) 'An extensive review on the consequences of chemical pesticides on human health and environment', *Journal of Cleaner Production*, 283, p. 124657. doi: 10.1016/j.jclepro.2020.124657.

Ravoet, J., Reybroeck, W. and de Graaf, D. C. (2015) 'Pesticides for Apicultural and/or Agricultural Application Found in Belgian Honey Bee Wax Combs', *Bulletin of Environmental Contamination and Toxicology*, 94(5), pp. 543–548. doi: 10.1007/s00128-015-1511-y.

Rejczak, T. and Tuzimski, T. (2015) 'A review of recent developments and trends in the QuEChERS sample preparation approach', *Open Chemistry*, 13(1). doi: 10.1515/chem-2015-0109.

Rortais, A. *et al.* (2017) 'Risk assessment of pesticides and other stressors in bees: Principles, data gaps and perspectives from the European Food Safety Authority', *Science of The Total Environment*, 587–588, pp. 524–537. doi: 10.1016/j.scitotenv.2016.09.127.

Rossi, E. M. *et al.* (2020) *Abejas & Agrotóxicos: Recopilación sobre las evidencias científicas de los impactos de los agrotóxicos en las Abejas – Petición ante la Relatoría DESCA de la Comisión Interamericana de Derechos Humanos. (En Español).* Available at: https://www.apiservices.biz/documents/articulos-es/abejas_y_agrotoxicos.pdf.

Sánchez-Bayo, F. *et al.* (2016) 'Are bee diseases linked to pesticides? — A brief review', *Environment International*, 89–90, pp. 7–11. doi: 10.1016/j.envint.2016.01.009.

Sanchez-Bayo, F. and Goka, K. (2014) 'Pesticide Residues and Bees – A Risk Assessment', *PLoS ONE*. Edited by R. N. C. Guedes, 9(4), p. e94482. doi: 10.1371/journal.pone.0094482.

SANTE/11956/2016 (2016) 'Technical guidelines for determining the magnitude of pesticide residues in honey and setting Maximum Residue Levels in honey'.

SANTE/12682/2019 (2019) 'Analytical quality control and method validation procedures for pesticide residues analysis in food and feed'. European Commision - EURL website. Available at: https://www.eurl-pesticides.eu/docs/public/tmplt_article.asp?CntID=727&LabID=100&Lang=EN.

Schulte, F. *et al.* (2008) 'Chemical Characterization and Classification of Pollen', *Analytical Chemistry*, 80(24), pp. 9551–9556. doi: 10.1021/ac801791a.

Sharma, A. *et al.* (2019) 'Worldwide pesticide usage and its impacts on ecosystem', *SN Applied Sciences*, 1(11), p. 1446. doi: 10.1007/s42452-019-1485-1.

Souza Tette, P. A. *et al.* (2016) 'Pesticides in honey: A review on chromatographic analytical methods', *Talanta*, 149, pp. 124–141. doi: 10.1016/j.talanta.2015.11.045.

Stan, H.-J. (2005) ‘Chapter 6 GC-MS. I: Basic principles and technical aspects of GC-MS for pesticide residue analysis’, in *Comprehensive Analytical Chemistry XLIII*, pp. 269–337. doi: 10.1016/S0166-526X(05)80026-1.

van der Steen, J. J. M. (2016) *Beehold : the colony of the honeybee (Apis mellifera L) as a bio-sampler for pollutants and plant pathogens*. Wageningen University. doi: 10.18174/375348.

van der Steen, J. (2020) *Report year 1 (2019) of the INSIGNIA Pilot Study on Environmental Monitoring of Pesticide Use Through Honeybee*. Available at: https://www.insignia-bee.eu/publications/.

Steiner, D. *et al.* (2020) ‘Evaluation of Matrix Effects and Extraction Efficiencies of LC–MS/MS Methods as the Essential Part for Proper Validation of Multiclass Contaminants in Complex Feed’, *Journal of Agricultural and Food Chemistry*, 68(12), pp. 3868–3880. doi: 10.1021/acs.jafc.9b07706.

Steinhauer, N. *et al.* (2018) ‘Drivers of colony losses’, *Current Opinion in Insect Science*, 26, pp. 142–148. doi: 10.1016/j.cois.2018.02.004.

Sulaiman, N. S., Rovina, K. and Joseph, V. M. (2019) ‘Classification, extraction and current analytical approaches for detection of pesticides in various food products’, *Journal of Consumer Protection and Food Safety*, 14(3), pp. 209–221. doi: 10.1007/s00003-019-01242-4.

Thorp, R. W. (1979) ‘Structural, Behavioral, and Physiological Adaptations of Bees (Apoidea) for Collecting Pollen’, *Annals of the Missouri Botanical Garden*, 66(4), p. 788. doi: 10.2307/2398919.

Thorp, R. W. (2000) ‘The collection of pollen by bees’, in Dafni, A., Hesse, M., and Pacini, E. (eds) *Pollen and Pollination*. Vienna: Springer Vienna, pp. 211–223. doi: 10.1007/978-3-7091-6306-1_11.

Traynor, K. S. *et al.* (2021) ‘Pesticides in honey bee colonies: Establishing a baseline for real world exposure over seven years in the USA’, *Environmental Pollution*, 279, p. 116566. doi: 10.1016/j.envpol.2021.116566.

Tremolada, P. *et al.* (2004) ‘Coumaphos Distribution in the Hive Ecosystem: Case Study for Modeling Applications’, *Ecotoxicology*, 13(6), pp. 589–601. doi: 10.1023/B:ECTX.0000037193.28684.05.

Uhl, P. and Brühl, C. A. (2019) ‘The Impact of Pesticides on Flower-Visiting Insects: A Review with Regard to European Risk Assessment’, *Environmental Toxicology and Chemistry*, 38(11), pp. 2355–2370. doi: 10.1002/etc.4572.

vanEngelsdorp, D. *et al.* (2009) ‘Colony Collapse Disorder: A Descriptive Study’, *PLoS ONE*. Edited by J. Brown, 4(8), p. e6481. doi: 10.1371/journal.pone.0006481.

Whitehorn, P. R. *et al.* (2012) ‘Neonicotinoid Pesticide Reduces Bumble Bee Colony Growth and Queen Production’, *Science*, 336(6079), pp. 351–352. doi: 10.1126/science.1215025.

Whitford, F. and Steeger, T. (2017) ‘The Complex Life of the Honey Bee Environmental, Biological, and Chemical Challenges to Colony Health’. Available at: www.edustore.purdue.edu.

Williams, G. R. *et al.* (2010) ‘Colony Collapse Disorder in context’, *BioEssays*, 32(10), pp. 845–846. doi: 10.1002/bies.201000075.

Woodcock, B. A. *et al.* (2017) ‘Country-specific effects of neonicotinoid pesticides on honey bees and wild bees’, *Science*, 356(6345), pp. 1393–1395. doi: 10.1126/science.aaa1190.

World Health Organization, W. (2020) *The WHO Recommended Classification of Pesticides by Hazard and guidelines to classification, 2019 edition*. Available at: https://www.who.int/publications/i/item/9789240005662.

Yates, J. R. (2000) ‘Mass spectrometry’, *Trends in Genetics*, 16(1), pp. 5–8. doi: 10.1016/S0168-9525(99)01879-X.

Yoder, J. A. *et al.* (2013) ‘Fungicide Contamination Reduces Beneficial Fungi in Bee Bread Based on an Area-Wide Field Study in Honey Bee, Apis mellifera , Colonies’, *Journal of Toxicology and Environmental Health, Part A*, 76(10), pp. 587–600. doi: 10.1080/15287394.2013.798846.

Zhang, P. *et al.* (2014) ‘Mixed-mode solid-phase extraction coupled with liquid chromatography tandem mass spectrometry to determine phenoxy acid, sulfonylurea, triazine and other selected herbicides at nanogram per litre levels in environmental waters’, *Journal of Chromatography A*, 1325, pp. 56–64. doi: 10.1016/j.chroma.2013.12.021.

Zioga, E. *et al.* (2020) ‘Plant protection product residues in plant pollen and nectar: A review of current knowledge’, *Environmental Research*, 189, p. 109873. doi: 10.1016/j.envres.2020.109873.