Honey chemical constituents from apiaries in different landscapes in Ireland

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Thesis submitted for the award of Doctor of Philosophy

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January 2019

Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Doctor of Philosophy is entirely my own work, and that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

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Acknowledgements

Firstly I thank the Irish Research Council without which I would not have been able to pursue my research. I would like to thank everyone in the School of Chemical Sciences in DCU, especially the fantastic technical staff, who were always helpful and ready to lend a hand. I am very grateful to everyone in the Plant-Animal Interactions Research Group in Trinity College Dublin for your continued support and excellent advice.

Thanks to my post-graduate colleagues in chemistry. Thank you to my friends Brian, Nicky, Steve, Leanne, Ruth, Aga, Aoife and Kae for your support and for being there throughout the highs and lows. Special thanks to Larisa for being a great friend. I would also like to thank my family, especially G. Marie, my dad Tom, and Niall for their support and encouragement. Niall, having you around makes me feel like I can do anything!

I thank Eva, my daughter for always cheering me up just by being her wonderful self!

I would like to thank Ciprian for your constant love, endless patience, erratic support, and for constantly believing in me!

Above all, I would like to thank my supervisors Dr Blánaid White and Professor Jane Stout for your consistent support both professionally and personally, for your patience and guidance throughout my project and for the many opportunities you gave me.

Finally, I would like to thank Mary Dunne, my mam for her constant encouragement and for being absolutely wonderful; I dedicate this thesis to you.

Contents

List of Abbreviations and Acronyms

Ach	acetylcholine
AIC	Akaike information criterion
AWMSI	area weighted mean shape index
CE	capillary electrophoresis
CICES	classification of ecosystem goods and services
CID	collision-induced dissociation
CLC	CORINE Land Cover
CORINE	Coordination of Information on the Environment
DPPH	2,2-diphenyl-1-picrylhydrazyl
EC	electrical conductivity
EEC	European Economic Community
EFSA	European Food Safety Authority
EU	European Union
EVIP	economic value of insect pollination
FRAP	ferric reducing antioxidant power
GAE	gallic acid equivalent
GC	gas chromatography
GUA	green urban areas
HBA	2,4 hydroxybenzoic acid
HPLC	high performance liquid chromatography
HMF	hydroxymethylfurfural
incl.	including
LC	liquid chromatography
LOD	limit of detection
LOQ	limit of quantification
LPIS	Land Parcel Information System
MRLs	maximum residue levels
MS	mass spectrometry
NaOH	sodium hydroxide
OSR	oil seed rape
PSM	plant secondary metabolites
QE	quercetin equivalent
RP	reversed-phase

RRF	relative retention factor
RU	rutin equivalent
SNH	semi–natural habitats
SPE	solid phase extraction
TAMB	total aerobic mesophilic bacteria
TEAC	trolex equivalent antioxidant capacity
TFC	total flavonoid content
TLC	thin layer chromatography
TPC	total phenolic content
UHPLC	ultra-high performance liquid chromatography
UMFHA	Unique Manuka Factor Honey Association
UV	Ultraviolet

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1	Saorla Kavanagh	Writing
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2	Saorla Kavanagh	Concept/experimental design and analysis, data collection, sample processing, statistical analysis, writing
	Blánaid White	Concept, experimental design and analysis, comments on draft chapter
	Jane Stout	Concept, experimental design and analysis, comments on draft chapter
	Jessica Gunno	Assistance in experimental analysis
	Thayse Marques Passos	Assistance in experimental analysis
3	Saorla Kavanagh	Concept/experimental design and analysis, data collection, sample processing, statistical analysis, writing
	Blánaid White	Concept, experimental design and analysis, comments on draft chapter
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4	Saorla Kavanagh	Concept/experimental design and analysis, data collection, sample processing, statistical analysis, writing
	Blánaid White	Concept, experimental design and analysis, comments on draft chapter
	Jane Stout	Concept, experimental design and analysis, comments on draft chapter
	Michael Henry	Assistance in LC-MS method development and experimental analysis
5	Saorla Kavanagh	Concept/experimental design and analysis, data collection, sample processing, statistical analysis, writing
	Blánaid White	Structure and comments on draft chapter
	Jane Stout	Structure and comments on draft chapter

Abstract

Saorla Kavanagh

Honey chemical constituents from apiaries in different landscapes in Ireland

The chemical composition of honey influences how beneficial it is to human and bee health, and can vary according to its botanical origin. The aim of this research was to investigate the relationships between botanical origin, landscape context and honey chemistry, specifically the physiochemical properties, phenol composition and pesticide content, of Irish honey.

This research is the first to profile phenolic content of Irish honey, and also investigated the relationship between the phenol composition of honey and a) hive location, b) land cover composition and c) harvest season.

The relationship between land-use and neonicotinoid residues in honey was also assessed. Declines in bee populations have caused great concern due to the valuable ecosystem services they provide and neonicotinoids have been implicated in these declines. Due to the wide application and persistence, neonicotinoids are bioavailable to bees, which can result in their presence in honey.

Results confirmed that physiochemical properties and total phenolic content varied according to the floral origin of the honey and hive location. Irish heather honey had similar physiochemical characteristics to Manuka honey. Seventeen phenols were identified and quantified in Irish honey. The results suggest that anthropogenic land-use and harvest season are the main indirect drivers of the phenol composition of honey. Finally, three neonicotinoids were identified in Irish honey, with 70% of samples containing at least one of the three neonicotinoids. Clothianidin and thiacloprid were more frequently detected in honeys from urban habitats and imidacloprid was more frequently detected in honey from rural habitats.

Honey provides an ideal matrix to evaluate the extent to which the landscape contributes to the chemical compounds to which pollinators are exposed. This research supports the growing evidence that some types of agricultural land provide insufficient floral resources for bees and that agricultural land is not the only source of pesticide exposure.

Chapter 1 Literature Review

1.1 The importance of bees to humans

Bees have long fascinated humans with their societies, beauty and behaviour. Bees are also important for the wide variety of ecosystem services they provide (1). While there is a vast amount of literature on ecosystem service classification, quantification and valuation, there is no international consensus on the definition of ecosystem services. Daily *et al.* (1997) define ecosystem services simply as functions provided by nature that improve and sustain human wellbeing (2). In 2010, Haines-Young and Potschin proposed a common international classification of ecosystem goods and services (CICES), where ecosystem goods and services are defined as "*the contributions that ecosystems make to human well-being",* and arise from the interaction of biotic and abiotic processes (3). Since this report was published, a number of expert meetings on valuing ecosystems and integrating them into economic accounting systems organised by the UNSD, the EEA and the World Bank have taken place. In 2013 Haines-Young and Potschin recommended that the CICES be regarded primarily as a way of describing ecosystem outputs as they directly contribute to human well-being (3). It is widely agreed that ecosystem services are fundamental to humanity but are threatened by human activity (1,2,4). Through their interaction with flowering plants, bees can influence a wide range of ecosystem services including provisioning (e.g. food, materials), regulating (e.g. climate regulation, hydrology), supporting (e.g. primary production, soil formation), and cultural (e.g. aesthetic, education). Two noticeable ecosystem services provided by bees are the support of food production through pollination and the production of honey.

Bees are considered the dominant pollinators in many habitats across the world, (5) although non-bee pollinators (flies, beetles, moths, butterflies, birds and bats, among others) also play an important role (6). In temperate regions, most animal pollination is provided by honey bees (*Apis mellifera*), bumble bees (*Bombus spp.),* solitary bees, wasps and hover flies. Globally, a number, of bee species are actively managed however in temperate regions the managed species are primarily the honey bee and a small number of bumble, cavity nesting mason and leaf-cutter bees. In Ireland, managed bumble bees are most commonly used in enclosed production systems (glasshouses and poly-tunnels), while managed honey bees are mainly used for field and orchard crops (7).

Honey bees provide commercially extractable products (e.g. honey, wax, propolis) and pollination services (a wide variety of crops are pollinated by honey bees, (1) and their

visitation has been shown to increase crop yield (8)). In the last decade, honey bee populations have increased on a global scale, however unprecedented colony losses have been reported in Europe and North America, occurring not only at wintertime, but also throughout the year (9,10). Multiple causes of colony loss are suspected, including infection by pathogens (including viruses, bacteria, fungi and parasites (11–13), poisoning by chemical compounds (including pesticides (14–18)), and habitat loss, which has contributed to a reduction in the abundance and diversity of floral resources (19,20). There is growing evidence that many bumble bee species have declined in recent decades, particularly in Western Europe and North America (21,22). Like honey bee losses, it has been suggested that the combination of habitat fragmentation, pesticide use and infection by pests and diseases and the interactions between these are the main causes of bumble bee decline (20).

1.1.1 Pollination services

Pollination occurs as pollen is transferred from the anther (male part) to the stigma (female part) of a flower. This can be achieved by abiotic means (transport in water or by wind) and biotic means (transport by animals). Ollerton *et al.* (2011) estimate that 87.5% of flowering plants benefit from biotic pollination (23). A wide variety of organisms can act as pollinators, including birds, bats, other mammals and insects (5), with insects being the most common. Numerous studies have shown that of all insects, bees are the dominant flower visitors in both in natural and agricultural systems (24–27). In Ireland Stanley *et al.* (2013) report that the highest visitation rate per flower of oilseed rape was by bumble bees (*Bombus lapidarius* and the *Bombus lucorum* sensu stricto group) closely followed by the honey bee (27). Although flower visitation is not equivalent to pollination, it is considered the most important predictor (28).

A large portion of the human diet (1) and essential nutrients (29) come from crops pollinated by insects. Globally, 87 of the leading food crops (accounting for 35% of the world food production volume) depend on animal pollination (1). Pollination is essential for crop products and is proven to increase their seed yield and economic value. Klein *et al.* (2007) report a 75% increase in fruit or seed set with animal pollination for the world's leading food crops (1).

Using a bioeconomic approach based on dependence ratios, it has been estimated by Gallai *et al.* (2009) that the value of pollination in agriculture to the world economy is €153

billion per year (30). This figure is equivalent to almost a tenth of the total value of world agricultural food production. This is one of multiple attempts to quantify the economic value of pollination services. The economic value of beekeeping in Switzerland i.e. the production of honey and other bee products was determined in 2005 to be approximately 60 million CHF (nearly 60 million euro (1 CHF = 0.983775 euro)), and Swiss bee colonies ensured a yearly agricultural production worth about 256 million CHF (252 million euro) (31). The latter figure only considers pollinating services directly relevant for agricultural production; the pollination of non or partly entomophilous cultures, wild plants and the maintenance of wild plant diversity (and of entire ecosystems), were not considered. The value of pollination services to the Swiss economy is therefore more than five times the value of honey production (31,32). Stanley *et al.* estimated the overall value of pollination services to oil seed rape alone in Ireland as €3.9 million per annum (27). Leonhardt *et al.* (2013) estimated the average annual economic value of insect pollination (EVIP) in Ireland to be €20 million (± 10 million), (33) however, this is significantly lower than the estimation by Bullock *et al.* (2008) (at least €53 million) (34). Leonhardt *et al.* calculated EVIP for crops used for food production using the same equation as Gallai *et al.*, which takes into account regional specialisation, geographical context and socio-economic factors (33). The equation is based on the quantity of crops consumed, the dependence ratio of the crop on insect pollinators and the price of the crop. In comparison, Bullock *et al.* based their value on the weightings of crops used for food production. Other authors have estimated value using a single crop e.g. Ricketts *et al.* (2004) estimated the value of stingless and feral honey bees' pollination for coffee production in Costa Rica as US$361 per hectare per year. Ricketts *et al.* measured seed mass, fruitset, and peaberry frequency to assess the effect of pollination (35). Using a combination of these methods may provide a more accurate value for pollination services.

1.1.2 Threats to bees

Bees and the services they provide are threatened by a range of factors (11,12,14,15,20). It has been widely suggested that the leading cause of bee decline is due to a combination of factors including but not limited to habitat loss, pests and diseases and pesticide exposure (20). Agricultural intensification has led to a loss in floral resources mainly due to changes in land management practices. These changes have been shown to negatively affect bees. For example, Fitzpatrick *et al.* (2007) suggest that the reduction in hay making from flower

rich meadows and the increase in silage monocultures is responsible for bumble bee decline in Ireland (36). More recent publications suggest that the use of pesticides particularly the class known as neonicotinoids (neuro-active insecticides), are a serious threat to pollinators, especially bees. Neonicotinoids have been found to decrease the foraging success of the honey bee (16) and have the potential to induce a variety of behavioural deficits in both honey and bumble bees (16,37). These pesticides have also been shown to impair the ability of bumble bees to provide pollination services (38) which could have severe economic implications. Neonicotinoids are not only used in agriculture but are also widely used in horticulture which suggests that bees could be under threat from these pesticides in both rural and urban environments. Urbanisation, i.e. the increase in artificial surfaces, can also lead to a reduction in floral resources for bees; however some urban landscapes can potentially benefit bees by providing increased, plant species richness (39) and nesting sites (40).

Global declines in bee populations have caused great concern due to the valuable ecosystem services they provide. Bees are important pollinators in many habitats and, therefore, the protection of bees in both urban and rural environments is essential, especially with the increasing demand for food due to the surging growing population and the growth targets for Ireland's agricultural sector under Food harvest 2020 (a strategy to chart the direction of agri-food, forestry and fisheries for the next decade in Ireland) (41).

1.1.3 Influence of floral resource quality on bees foraging preference

Plants produce an array of secondary metabolites that have both positive and negative biological effects on other organisms. Each plant family, genus, and species produces a characteristic mix of these chemicals (42). Plant secondary metabolites (PSM) have been found to exhibit anticarcinogenic, anti-inflammatory, antioxidant, antimicrobial and antimutinogenic effects that may be beneficial in preventing diseases (43–45). It has been estimated that over 40% of medicines have originated from PSM (46). A number of screening programmes for these bioactive compounds exist and have led to the discovery of new drugs, for example taxol, which is used for the treatment of various cancers (46). PSM can be classified on the basis of their chemical structure, composition (containing nitrogen or not), the pathway by which they are synthesised or their solubility in various solvents (42,47). Waksmundzka-Hajnos *et al.* (2008) classify PSM into three groups: phenolics, nitrogen containing compounds (or also containing sulphur) and terpenoids (48).

Hooper lists major forage plants for honey bees (49). The species commonly found in Ireland include: *Acer pseudoplatanus* (sycamore), *Aesculus hippocastanum* (horse-chestnut), *Brassica species*, *Calluna vulgaris* (ling heather), *Chamaenerion angustifolium* (rosebay willowherb), *Crataegus monogyna* (hawthorn), *Erica species*, *Medicago sativa* (lucerne), *Malus species*, *Prunus species*, *Rubus fruticosus agg.* (blackberry), *Sinapis arvensis* (charlock), *Taraxacum officinale* (dandelion), *Tilia species*, *Trifolium pretence* (red clover), and *Vicia faba* (broad bean). In a recent study by Carvell *et al.*, (2004) the flower preference for six bumble bee species was *Centarea cyanus*, *Cirsium arvense* (creeping thistle), *Cirsium vulgare* (spear thistle), *Lathyrus pratensis* (meadow vetchling), *Leucanthemum vulgare* (ox-eye daisy), *Lotus corniculatus* (common bird's foot trefoil), *Prunella vulgaris* (selfheal) and *Rhinanthus minor* (yellow rattle) (50), all of which are native to Ireland. These plants contain an array of PSM and are important sources of food for bees in Ireland.

Many plant traits affect the foraging behaviour of bumble and honey bees, more specifically floral traits (flower colour, odour and shape). Nectar rewards are considered a major driver of foraging preference of bees. Wilmer reports that honey bees will go to almost any flowers in most habitats and are considered to be "supergeneralists" (5); however, they have been found to prefer some pollen over others (51). Schmidt suggests that choice is based on pollen odour or taste (51), however it is likely that nutritional value and / or the PSM composition plays a part. According to Schmidt the open and radial flower is the preferred option for the honey bee.

It is widely accepted that both honey and bumble bees tend to forage in an optimal way and focus on flowers that contain the highest nectar / pollen reward in relation to floral handling efficiency (52,53). However, other factors can also affect floral choice; for example, Kessler *et al.* (2015) found that bees had a preference for foods containing neonicotinoids (54). The honey bee and the buff tailed bumble bee (*Bombus terrestris*) actually had a preference for neonicotinoid-spiked sucrose. This study suggests that anthropogenic compounds can override the bee's natural ability for forage selection or that they have some other pharmacological effect on the bees (54).

1.1.4 Honey production

Honey bees (*Apis spp.*) have been managed by humans for thousands of years. This is mainly for the sweet substance they produce, honey, and also for a number of other bee

products that can be harvested from honey bees. In temperate climates, honey is produced mainly from the nectar of flowers and from honeydew. Both originate from the phloem sap of higher plants (55). According to European Union legislation, "Honey is the natural sweet substance produced by *Apis mellifera* bees from the nectar of plants or from secretions of living parts of plants or excretions of plant-sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in honeycombs to ripen and mature" (56). Other insects such as ants, bumble bees and wasps produce honey and the composition of the honey can vary depending on the insect species. Honey properties will be discussed in Section 1.2.

The global production of honey reached 1,511,000 tonnes in 2014 with China being the leading producer (57). Europe, the second leading producer of honey, produced approximately 1/10 of this in 2014, and imported slightly more than it produced. Honey is becoming a more popular commodity; however in Ireland in recent years trade in honey has decreased in value and tonnage. The export value of honey in 2017 was €2,468,000 compared to €3,943,000 in 2015 and €4,032,000 in 2013 (58,59). Currently, Ireland imports 11 times more honey than it exports (59), with demand consistently outstripping native supply as shown in Fig. 1-1. Despite the increase in the export value of Irish honey the quantity of honey exported has remained relatively constant since 2009 (Fig. 1-2). In 2017, Great Britain (44.19%), China (27.41%) and New Zealand (8.77%) accounted for 80.37% of the value of honey imports into Ireland, though it should be noted that the country of import is not always the county of origin for the honey. For example, the majority of the 44.19% of honey imported from the UK is likely to have originated from China, given their global dominance in production terms. The main feature of the Irish honey market is that most of the Irish production comes from small private producers.

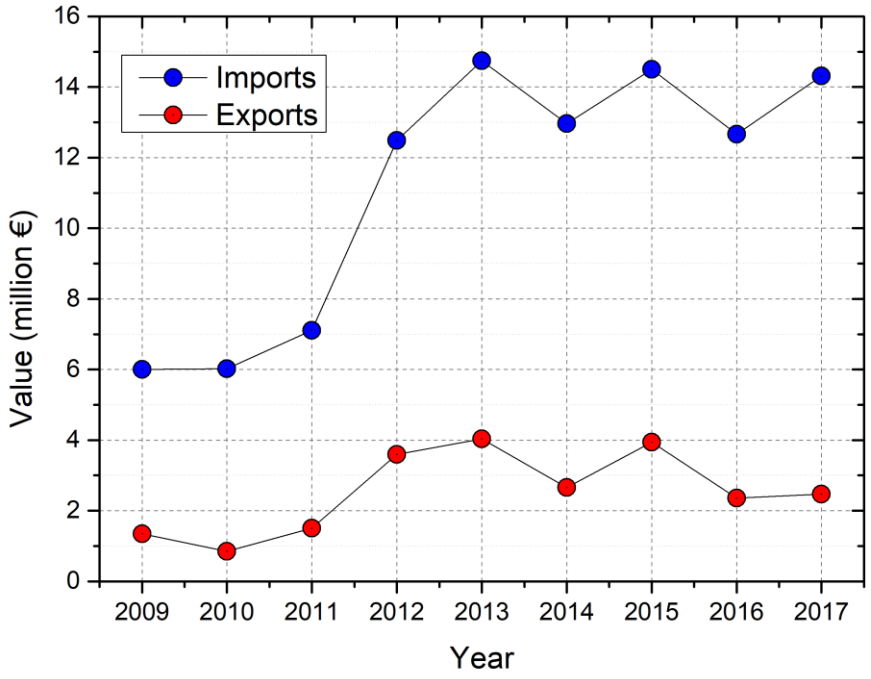


Figure 1-1 Import and export value of honey in Ireland from 2009 to 2017. Data source: Central Statistics Office (58,59).

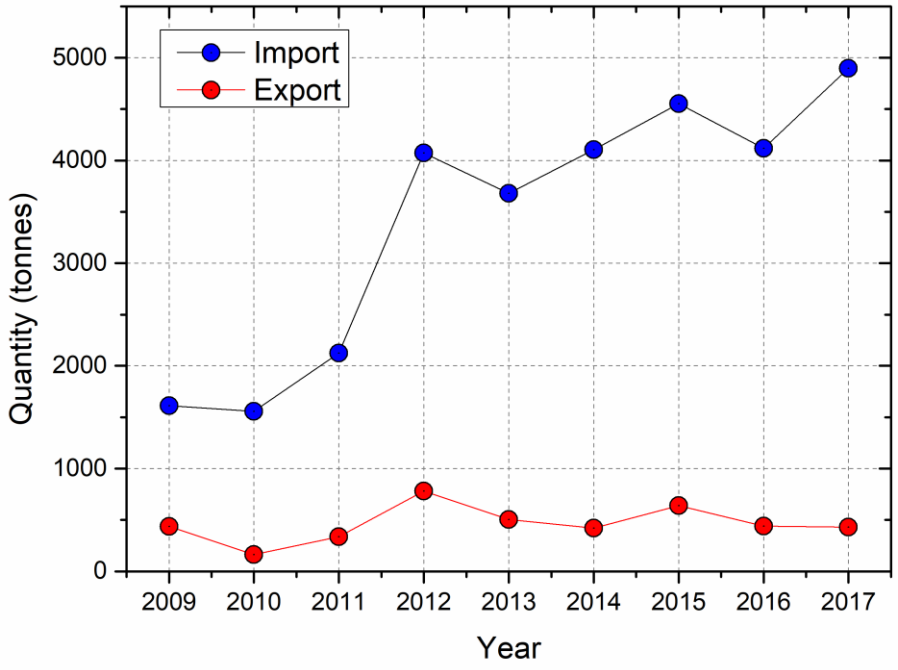


Figure 1-2 Import and export tonnage of honey in Ireland from 2009 to 2017. Data source: Central Statistics Office (58,59).

There are no figures available for the quantity of Irish honey produced in Ireland that is not exported. However, given the Department of Agriculture, Food and the Marine's (DAFM) new registration requirement for Beekeepers and Purchasers and Packers of Honey in Ireland, it is likely that this information will become available in the near future. According to the Federation of Irish Beekeepers, there were 2,800 registered beekeepers in Ireland in 2014 and the average number of hives per beekeeper was three. The number of registered beekeepers in Ireland rose to 3,500 in 2017 and the average number of hives per beekeeper was 6. Given that the average annual crop of one hive is 25kg, an estimated 550 tonnes of honey was harvested in Ireland in 2017. Given that annual colony losses and environmental factors can cause a reduction in harvest, this value is highly speculative. Increasing consumer preferences for products based on honey has led to the expansion of the honey market, varieties of honey and varieties of honey-based foods.

There are fourteen groups of social insects that produce and store honey (60). The insects include all *Apis spp.* (Apinae), almost all Meliponinae, *Bombus spp.* (Bombinae) (excluding the cuckoo bumblebees (subgenus = *Psithyrus*), honey wasps in the Polistinae, and honey ants in the Formicidae and Dolichoderinae (60). The process by which honey bees make honey from nectar is as follows; after foraging, bees return to the hive and pass on the nectar they have collected and stored in their honey stomach to the "house" bees. The forager passes on the contents of her honey stomach to one or several house bees by opening her mandibles with her proboscis retracted and a drop of liquid appears at the base of the glossa. The house bee extends her proboscis fully and sucks up the drop (55). Before the nectar is stored, the water content of the nectar must be reduced. This is achieved by evaporation during the honey ripening process. A bee will pump out the contents of their honey stomach into a flat drop on the underside of the proboscis; she will draw it up again and repeat this process rapidly for 15-20 minutes (55). The nectar is repeatedly exposed to the warm air of the hive and as the water evaporates, the nectar thickens. Bees produce half ripened honey, containing 50 – 60% of dry substance before storing. The bees add a glandular secretion during this process which splits the sucrose of the nectar into fructose and glucose (61). During a strong nectar flow (i.e. when there is a lot of nectar available due to a large number of flowers), the partially ripened honey is stored in the cells of the comb immediately, or after a few transferences from bee to bee (55). The bees then reduce the moisture content by fanning the nectar / honey containing cells. During a moderate or weak flow, the food is passed to many members of the hive and regurgitated repeatedly

before it is stored. Once the honey is ripe, i.e. when it contains 20% water or less, the bees cover the honey cells with a thin layer of wax (61). Honey ripening can take 1-3 days depending on the water content when the honey is first put into the cells, the level to which the cells are filled, air movement within the hive (this depends on the strength on the colony and hive ventilation) and temperature and relative humidity (55,62).

1.2 Honey properties

Honey consists of a concentrated solution of a complex mixture of sugars (63), as well as natural constituents including enzymes, minerals, organic acids, phenols, proteins, vitamins and other phytochemicals (64). The composition and quantity of these constituents vary according to honey floral and geographic origin (64,65), the method used to collect the nectar or honeydew, honey processing and storage (66–68), as well as seasonal and environmental factors (69). Honey can also contain many contaminants mainly due to anthropogenic activities (70), some which may be associated with potential health risks.

The production of honey by the honey bee (*Apis spp.*) involves enzymes activity, primarily secreted invertase. The invertase is secreted by the honey bee from its salivary glands into the honey stomach. This enzyme catalyzes the breakdown of sucrose into glucose and fructose. Enzyme activity varies depending on insect species or genera (60) and thus the properties of the honey will vary depending on the honey producing species.

Honey bees (*Apis spp.*) are the most important source of honey commercially available and research on *Apis* honey is profuse when compared to any other honey producing species. The composition of other honeys (i.e. honey from social bumble bees, stingless bees, wasps and ants) has been studied to a far lesser extent. The next most studied species in terms of honey production are stingless bees. Beekeeping with stingless bees (meliponiculture) is practiced in tropical regions and many studies report on the medicinal uses of the honey produced (71,72). Hunt et al. (1998) report on the nutrients of social wasp honey (71). However, very little research has been published exploring differences and similarities of honey types in terms of honey producing insects. The most recent research in this area, by Graham, compared volatile components in bumble bee and honey bee honey. The honeys analysed shared few similar volatiles, with only 1-phenoxy-2-propanol and butylated hydroxytoluene found in both bumble bee and honey bee honey

(73). Graham concluded that butylated hydroxytoluene may have been present in the sugar additive / water solutions fed to the honey bee and bumble bee colonies as it has been used extensively in the food industry as a food antioxidant (74). The most recent research comparing sugar content in honey types are the studies of Knee and Medler and Maurizio in the 1960's. Knee and Medler observed two types of honey being stored in four different North American bumble bee species and reported different sugar contents between the two honey types (70-87% and 42-52%) (75). The main sugars of the honey bee are fructose and glucose with glucose predominating (F/G ratio 5.31) (55). Maurizio studied the F/G ratio of European bumble bee honeys and found higher fructose contents when compared to honey bee honey (76). Overall however, comparative studies between honey from different insects is limited.

Honeys differ in their chemical composition (volatile constituents, carbohydrates, and phytochemicals), physical properties (colour, viscosity, hygroscopic properties, and pH), taste, and in their different biological activities. Honey bee honey is a functional food (i.e. foods that provide a health benefit beyond basic nutrition) and its properties have been widely researched. The reported biological properties of honey produced by the honey bee (*Apis spp.*) include: antibacterial (77), andtidiabetic (78), anti-inflammatory (79), antimicrobial (80), antioxidant (63), radical scavenging (81), cardio-protective (82) and wound healing (83). It is widely agreed that the botanical origin of honey has the greatest influence on its antioxidant activity, whereas processing, handling and storage (i.e. glass or plastic packaging, pasteurisation, and storage time) can affect the antioxidant activity of honey only to a minor extent (64). For example, Wang *et al.* (2004) compared the impact of heat and filtration on the antioxidant capacity of clover and buckwheat honey. Buckwheat honey was more affected by processing than clover honey in terms of reduction in antioxidant capacity, while the processing of clover honey did not significantly impact antioxidant capacity. The authors conclude that the effect of traditional processing on honey antioxidant capacity varies depending on the honey because of variation in the chemical composition of honeys from different floral sources (68). Because bees forage within certain distances of their colony i.e. are central place foragers, the composition and configuration of the surrounding landscape will also affect honey properties.

1.2.1 Floral Origin

The commercial quality of honey is determined by the floral / botanical origin, as this influences its chemical composition. Some floral honeys are perceived to have a higher quality than others. For example single origin honey (i.e. from a single plant species), has a higher retail price than blended honey. Blended honey is a mixture of two or more honeys differing in floral source, colour, flavour, density or geographic origin (84). The majority of honey sold in Ireland is a blend of EU and non-EU honey. Whether honey is of a single source or consists of a blend, it is desirable to know the complete botanical make up, given that the presence of particular plant species can negatively or positively affect the final product. For example, honey produced from the nectar of *Senecio jacobaea* contains pyrrolizidine alkaloids (85). These alkaloids are capable of binding strongly to nucleophilic centres in tissues or crosslinking DNA which makes them potentially carcinogenic and hepotoxic (86). Therefore, the consumption of honey produced by the nectar of this species may be hazardous to human health and thus is considered to be of poor quality and value. However for a myriad of reasons, both intentional (e.g. food fraud) and accidental, the botanical origin of honey can be mislabelled. It is therefore necessary to develop methods to identify the botanical origin of honey. To determine the botanical origin of honey, pollen identification (melissopalynology and / or metabarcoding) and / or analyses of phenolic compounds is most frequently used. Additionally, it has been suggested that analysis of the volatile compounds (87) and the mineral content (88) in honey may be useful for characterisation of botanical and geographical origin, as both constituents are strongly dependant on these parameters. Additionally, Karabagias *et al.* (2014) report substantial differences (qualitative and semi-quantitative) in volatile compounds among honey samples of different botanical origin (89).

Melissopalynology has been the traditional scientific method in determining the botanical origin of honey. It is based on the identification of pollen by microscopic examination (69). Unfortunately there are many problems associated with this method. For example, different plant species produce different proportions of pollen and the amount of pollen produced can vary from season to season (69). Pollen can be collected from plants that may not be the actual floral source of the honey, as bees will sometimes collect pollen from a flower without collecting the nectar, and vice versa. There are also occasions where pollen may be lost as it can be filtered out of the honey stomach by the bee (76) or filtered by the beekeeper during the processing of the honey. Another limitation associated with this

method is that pollen can be added fraudulently (69). Ireland has few common plant species (90). However, this does not practically limit the variation in flavour and quality of honeys produced, since honey from any given floral source can vary as a result of seasonal climatic variations or geographical location. Downey *et al.* (2005) identified 43 pollen types (present at levels ⩾1%) from 50 Irish honey samples collected in Ireland over two harvest seasons between 2001and 2003 (91). *Trifolium repens* was the dominant pollen type in 19 of the 25 honeys. It was present in a total of 23 samples. *T. repens* (white clover) is a very common plant in Ireland and its presence in Irish honey in large amounts is to be expected. *Rubus fruticosus agg.* (blackberry) was the second most abundant pollen type identified, being the dominant pollen in three of the 25 honeys tested. It was found in 23 samples in total, but in lower amounts than *T. repens*. The authors conclude that the results from this small sample set indicates that in Ireland, *Apis mellifera* feed mainly on a diet of nectar from white clover and blackberry, which is not surprising given the dominance of these two species in Irish pasture systems.

Many authors have suggested that specific phenolic compounds can be used as floral / chemical markers for honey. Ferreres *et al.* (1993) suggest hesperetin and methyl anthranilate as markers of citrus honey because they were found in large amounts in the honey samples (92). Gil *et al.* (1995) suggest that kaempferol is a useful marker for *Rosmarinus* (rosemary) honey and quercetin for sunflower honey (*Helianthus spp.*) (93). *Arbutus unedo* (strawberry tree) honey, a known product of Mediterranean regions, particularly Sardinia, is characterized by its “bitter taste” (94). The antioxidant and anti-radical activities of strawberry tree honey have been attributed to homogentisic acid (95). Homogentisic acid (2,5-dihydroxyphenylacetic acid) has been reported as a chemical marker for the botanical origin of strawberry tree honey (94,96,97) along with (±)-2-cis,4-trans-abscisic acid, (±)-2-trans, 4-trans-abscisic acid and unedone (a derivative of the above acids) (97). Ferreres *et al.* found myricetin, myricetin-3-methyl ether, myricetin-30-methyl ether and tricetin to be the most characteristic compounds in heather honey from the Coimbra region (Portugal) and report that they have not been detected in other honey samples of different plant origin analysed so far (98). These findings were later disregarded by the authors as the compounds themselves or their corresponding glycosides were not detected in heather floral nectar (99). Since this study, myricetin has been reported as a suitable marker for eucalyptus honey (100), as it has been detected in *Leptospermum* honeys from Australia and New Zealand (66) and in chestnut (*Castanea sativa*) and spruce

(*Picea spp.*) honey from Slovenia (101). Instead, Ferreres *et al.* conclude that abscisic acid is a potential marker of heather honeys due to its presence in the corresponding nectar (99). Guyot *et al.* (1999) analysed heather honey from various countries and suggest that *Calluna vulgaris* (heather) honeys can be distinguished from non-heather honeys on the basis of their benzoic acid content (102). The authors found phenylacetic acid exclusively in *Calluna vulgaris* honeys. Guyot *et al.* conclude that the presence of benzoic acid and decanoic acid in a honey reliably indicates a floral origin within the Ericaceae family and that high levels of other compounds such as cinnamic acid, isophorone, and 4-(3-oxobut-1-enylidene)-3,5,5-trimethylcyclohex-2-en-1-one provide further authentication (102).

It is clear from published literature summarised in Table A-1 (Appendix A) that the use of chemical markers in determining honey floral origin can depend on the geographical origin of the honey. However differences in composition have also been attributed to differences in the methods used for detection and quantification (103). It has been suggested that analysing differences in the complete phenolic compound profile may be a more suitable method than the use of any single specific compound.

Mineral and trace element content in honey can also give an indication of the geographical origin as well as environmental pollution (69). Rodriguez-Otero *et al.* (1994) successfully determined the quantity of calcium, chloride, copper, iron, magnesium, manganese, phosphate, potassium, silica, sodium, sulfate as well as environmental contaminant ash contents of 91 samples of honey from Galicia, Spain (104). The authors found that the Galician honeys had higher mineral contents in comparison with other honeys reported in the literature. Fodor and Molnar conclude that the trace element concentration in Hungarian honey is a useful environmental indicator (105). Toxic trace elements have been detected in soil and plants and have even made it into the food chain. Honey can potentially be used to detect environmental contamination (106); however, Bogdanov *et al.* (2007) conclude that variation in trace element content in different honey types from Switzerland is primarily due to botanical origin (107). A honey bee can fly anywhere between 1 metre to 9.5 km from the hive (108) with an average flight path of 5.5 km (108). During flight the bee can pick up trace elements from the air, soil, water as well as floral sources, and so it is possible that the concentrations of trace elements in honey can reflect the amount present in the area accessed by the bee (109). Bee products are the final step of

a bioaccumulation process thus the chemical study of honey may provide useful information about the environmental quality of the area in which the bees feed (110).

1.2.2 Authenticity

The European Union (EU) defines a number of specific rules for honey, supplementing the legislation applicable to foodstuffs under the Council Directive 2001/110/EC relating to honey (56). These rules concern the composition and definition of honey, sales names, labelling, presentation and information on origin. The directive was most recently amended in 2014 (Directive 2014/63/EU) and contains six amendments and 15 new rules on honey.

To comply with legislation, when placed on the market as honey or used in any product intended for human consumption, honey must meet the following composition criteria:

1. Sugar content

1.1 Fructose and glucose content (sum of both)

- blossom honey not less than 60 g/100 g
- honeydew honey, blends of honeydew honey with blossom honey not less than 45 g/100 g

1.2 Sucrose content

- in general not more than 5 g/100 g
- false acacia (*Robinia pseudoacacia*), alfalfa (*Medicago sativa*), Menzies Banksia (*Banksia menziesii*), French honeysuckle (*Hedysarum*), red gum (*Eucalyptus camadulensis*), leatherwood (*Eucryphia lucida, Eucryphia milliganii*), *Citrus spp.* not more than 10 g/100 g
- lavender (*Lavandula spp.*), borage (*Borago officinalis*) not more than 15 g/100 g

2. Moisture content

- in general not more than 20 %
- heather (*Calluna*) and baker's honey in general not more than 23 %
- baker's honey from heather (*Calluna*) not more than 25 %

3. Water-insoluble content

- in general not more than 0.1 g/100 g
- pressed honey not more than 0.5 g/100 g

4. Electrical conductivity

- honey not listed below, and blends of these honeys not more than 0.8 mS/cm
- honeydew and chestnut honey and blends of these except with those listed below not more than 0.8 mS/cm
- exceptions: strawberry tree (*Arbutus unedo*), bell heather (*Erica spp.*), eucalyptus, lime (*Tilia spp.*), ling heather (*Calluna vulgaris*), manuka or jelly bush (*leptospermum*), tea tree (*Melaleuca spp.*)

5. Free acid

- in general not more than 50 milli-equivalents acid per 1 000 grams
- baker's honey not more than 80 milli-equivalents acid per 1 000 grams

Honey authenticity is also defined by the Codex Alimentarius standard (111) and by different national legislations. Turkey for example published the Turkish food codex Notification No. 2012/58 on honey. This notification sets the rules and procedures for hygienic and technically appropriate production, processing, conservation, transport, storage and marketing of honey. The Notification covers honey produced by the honey bee (*Apis mellifera*) and includes legislation on the product features of honey and provisions related to food additives, pesticide residues and veterinary drug residues (112). This Notification consists of 19 articles and an annex on product features of honey. In comparison, the Department of Agriculture, Food and the Marine in Ireland has published documents on bee health and on the labelling of honey (113). The Irish documents are sources of information and not legislation. In Ireland honey legislation is based on the Council Directive 2001/110/EC relating to honey.

Adulteration techniques of honey are based on two different principles: ‘dilution’ of honey by water addition, and extension with sugar and syrups (e.g. beet sugar, corn syrup, high fructose corn syrup) (69). Other adulterations include: bee feeding with sugar syrup and / or artificial honey and mislabelling with regards to the floral or geographical origin. Honey adulteration can result in higher commercial profits for the producer but a reduced nutritional value for the consumer (114). Higher commercial profits are the leading cause of honey adulteration, as evidenced in sales of Manuka honey, a floral honey originating in New Zealand. Due to its commercial value and limited availability, more manuka honey is being sold on the market than is actually produced (UMFHA, the main trade association of New Zealand manuka honey producers estimated that while approx. 1,700 tonnes are produced annually, over 10,000 tonnes of honey marketed as manuka are sold worldwide)

(115). It is assumed that blending and adulteration has led to this counterfeiting. It is estimated that over 85% of worldwide manuka honey is counterfeit, and honey samples showed the highest level of non-compliance (samples that exceed the legal limit) for antibacterial contamination of all animal foodstuffs tested by EFSA (European Food Safety Authority) in 2011 (116). Other non-compliant results were reported under the subgroups for amitraz, copper, lead and tin and diethyltoluamide. Given the extent to which honey adulteration occurs, comprehensive analytical methods to fully characterise the origin and composition are required.

1.2.3 Bioactive compound profile

"Bioactive compounds" are extra-nutritional constituents that typically occur in small quantities in foods (117). They are being intensively studied in honey to evaluate their positive effects on human health. The most commonly studied group are phenolic compounds. Phenols are secondary metabolites of plants and are generally involved in defence against insects, ultraviolet radiation, pathogens (118) and allelopathy (inhibition of the growth of other plant competitors) (119). They are considered one of the most important groups of compounds occurring in plants (120) and can be broadly subdivided into phenolic acids, flavonoids, stilbenes and lignans, based on their chemical structure. The most commonly studied phenols in honey are flavonoids and phenolic acids and their core structures are illustrated in Fig. 1-3. Flavonoids are the largest and most studied group of phenols and are subdivided into multiple sub classes. Phenolic acids arise from the phenylpropanoid acids metabolism in plants and can be broadly classed as either hydroxybenzoic or hydroxycinnamic acids. Many plants contain a vast number of phenols and each plant has a distinctive profile (69).

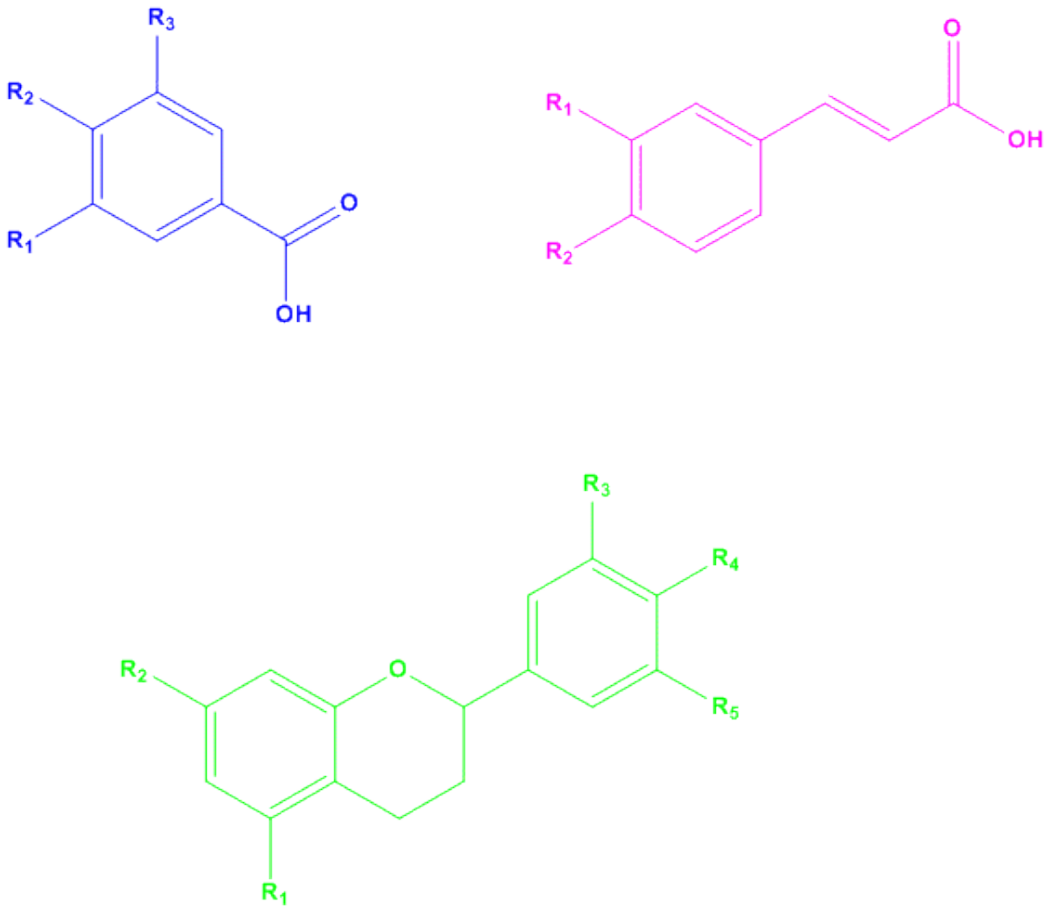


Figure 1-3 Core structure of the different phenol groups most commonly studied in honey - phenolic acids: hydroxybenzoic acids in blue and hydroxycinnamic acids in pink, and flavonoids in green.

More than 150 phenolic compounds have been reported in honey, including phenolic acids and flavonoids, with common examples shown in Fig. 1-4 (121). Gallic acid (an example of a hydroxybenzoic acid), caffeic acid (hydroxycinnamic acid) and luteolin (an example of a flavonoid) have been detected in honeys from many countries around the world (122–124). These compounds have at least one aromatic ring in which at least one hydrogen is substituted by a hydroxyl group (119). Various studies have shown the correlation of antioxidant potential of honey and concentration of total phenolics present (64,101).

Honeys with dark colour typically have a higher total phenolic content and thus a higher antioxidant capacity.

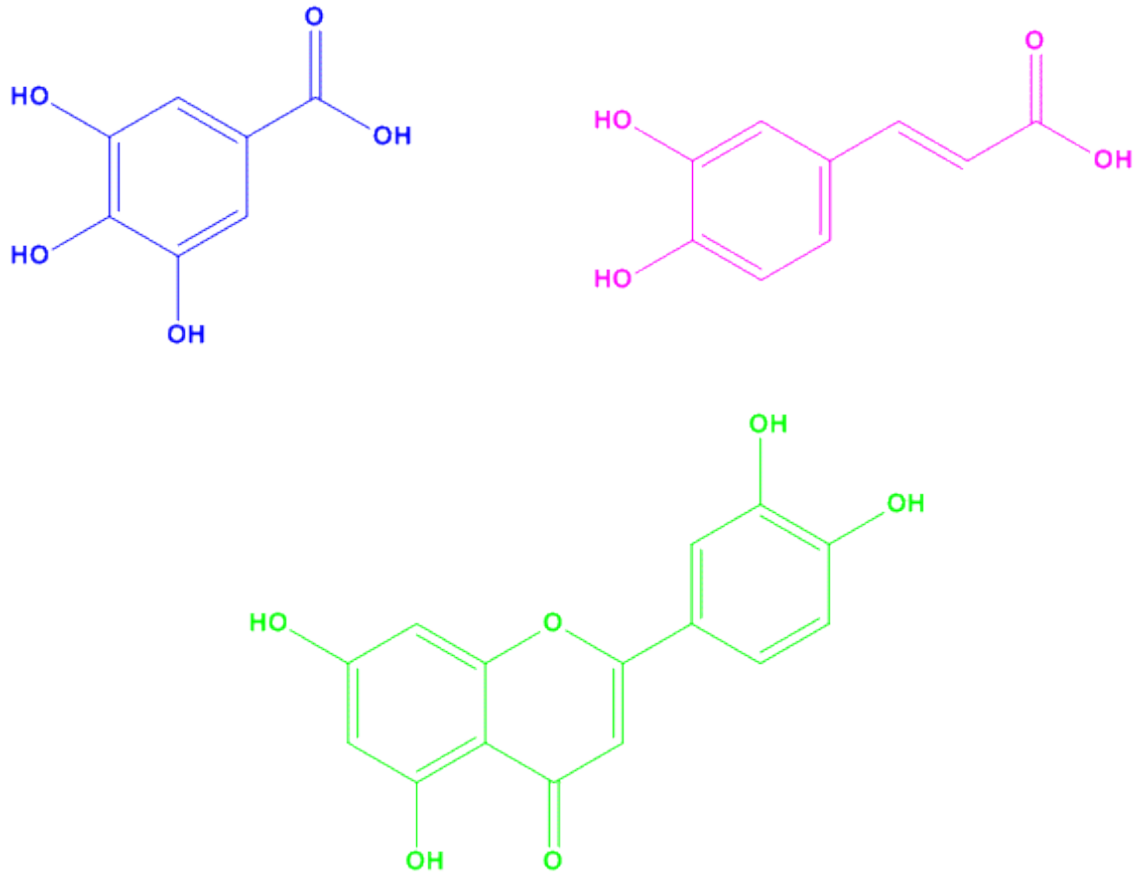


Figure 1-4 Chemical structures of different phenols found in honey – gallic acid in blue, caffeic acid in pink, and luteolin in green (phenolic acids: hydroxybenzoic acids in blue and hydroxycinnamic acids in pink, and flavonoids in green).

Honey phenols originate from the plants from which nectar is collected and by the contact with propolis in the hive (125). Phenolic acids and flavonoids have been comprehensively researched in honey (103,120–122,124,126,127) and as discussed in section 2.1 are considered potential markers of the botanical origin. Both compound classes are used to assess honey quality. The flavonoids in honey have been identified as predominantly flavanones and flavanones / flavanols (128). The total amount of flavonoids or total flavonoid content (TFC) is commonly measured in milligrams of quercetin equivalent / 100g of honey (mg QE/100g) or milligrams of rutin equivalent / 100g of honey (mg

RU/100g). The chosen flavonoid is just a representative and as such, any flavonoid can be used. The total amount of flavonoids found in honey from various countries has been quantified (Appendix A, Table A-2), with TFC results ranging from 3.52 mg QE/100g honey for Argentinian multi-floral honey to 22.45 mg QE/100g for Tunisian mint honey. Interestingly, Eucalyptus honey from Tunisia was reported to have a TFC over twice that of Eucalyptus honey from Spain. As all Tunisian TFC values reported were considerably higher than all others reported, the results may indicate artificially high values.

In addition to TFC, the total phenolic content (TPC) is also regularly used to define honey quality and is commonly measured in milligrams of gallic acid equivalent / 100 g of honey (mg GAE/100g). For example, Beretta *et al.* (2005) found that strawberry tree honey contained the highest TPC (78.96 ± 1.38 mg GAE/100g) and clover honey contained the lowest (6.71 ± 0.56 mg GAE/100g) (64). Table A1-3 lists the TPC for honeys of different floral and geographical origins. The lowest and highest TPC values reported in the literature come from Turkish Rhododendron honey and range from 0.24 to 141.83 mg GAE/100g (129) (Appendix A, Table A-3). As both these values were reported in the same study, artificial overestimation of TPC is unlikely, and so the vast difference in TPC values are most likely attributable to differences in geographical location or variations in honey harvesting time.

The main phenols reported in honey are flavonoids (apigenin, chrysin, galangin, hesperetin, kaempferol, luteolin, myricetin, pinobanksin, pinocembrin, quercetin, and tricetin), phenolic acids (caffeic, chlorogenic, coumaric, ellagic, ferulic, gallic, homogentisic, phenyllactic, protocatecuic, syringic and vanillic acids) and their derivatives (125).

The constituents in honey that are responsible for the observed antioxidant effects are flavonoids, phenolic acids, ascorbic acid, catalase, peroxidase, carotenoids, and products of Maillard reactions (130). The extent of these constituents varies depending on the floral and geographical origin of the honey. In addition, processing, handling, and storage of the honey can influence the composition (68). Several studies on the identification and quantification of the antioxidant components of honey bee products have been reported all over the world (131). Irish honey has not been profiled for constituents with potential health benefits. There are three main types of Irish honey marketed; heather, multi-floral and rape. Among the different types of honey, the antioxidant potentials of heather honey

have been previously reported for many European countries (102). Heather honey produced from *Erica* or *Calluna* species has been reported to have high levels of total phenolic content when compared to other honeys (132). The potential of heather honey as a natural resource to look for new medicines for the treatment of mycotic infections has been reported (133). While multi-floral and rape honeys from Ireland have not been analysed it can be estimated based on international studies that multi floral honeys (expected to be of blackberry, chestnut, clover and dandelion origin) should have a TFC in the region of 5.5 – 7.7 mg QE / 100g (Appendix A, Table A-2) and a TPC in the region of 6.71 – 108.21mg / 100 GAE (Appendix A, Table A-3).

1.2.4 Potential health benefits of phenols

Although phenols play no known role in nutrition (non-nutrients), many of them have the potential to be beneficial in preventing disease and protecting the stability of the human genome (43,134). Therefore, the consumption of foods high in phenols like honey has the potential to be beneficial to human health. Studies have shown that the anticancer effects of phenols are facilitated by the ability of phenols to counteract, reduce, and repair damage resulting from oxidative stress and inflammation (135,136). For example, pre-treatment and co-administration of p-coumaric acid is a promising therapeutic intervention in managing doxorubicin (an anticancer chemotherapy drug) induced cardiotoxicity (137). This phenolic acid has also been shown to activate immune and detoxification signalling pathways in honey bees. Luteolin, quercetin and kaempferol have been shown to inhibit ovarian cancer cell growth *in vitro* (138). Caffeic and rosmarinic acids have been shown to have anti-inflammatory and analgesic properties (139). Ellagic acid and its metabolites have preventative and therapeutic potential against human cancers. The potential health benefits of phenols are vast, with the efficacy of a number of phenolic compounds currently being investigated in clinical trials, including, caffeic acid (phase 4) ellagic acid (phase 1 & 2), kaempferol (phase 2), luteolin (phase 1), quercetin (phase 1, 2 & 3) and rutin (phase 1) (140). The potential health benefits of phenols can depend on the concentration used and at high concentrations some phenols can negatively affect human health. For example, benzoic acid is commonly used as a food preservative and acceptable daily intakes were established by the World Health Organization at 5 mg/kg (141). Therefore EU regulations control the maximum levels of benzoic acid and its salts for use

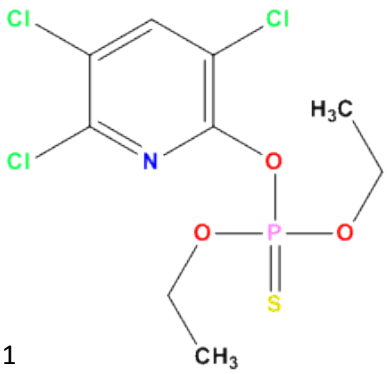
in food stuffs (142). The potential health benefits of phenols are discussed further in Chapter 3.

1.2.5 Anthropogenic compounds

Beehive matrices (honey, honey bee, pollen, propolis and wax) are recognised as appropriate sentinels for monitoring anthropogenic contamination in the environment (143–145). Studies on the use of honey bees and bee products for environmental monitoring date back to 1935 (143,146). Environmental pollutants such as pesticides (147), radioactive elements (148) and heavy metals (106) have been detected in honey and bee products. Cadmium, copper, lead and zinc have been detected in honey from Poland and Finland (149). The authors of the Finnish study conclude that under Finnish conditions, honey and pollen are not good bio-indicators of heavy metal pollution and bees are more suitable for indicating industrial and urban heavy metal pollution. In contrast the results from a Polish study suggest that honey may be useful for assessing the presence of environmental contaminants, namely heavy metals (106). Trace element (incl. heavy metals) concentrations in honey have been shown to correlate with contamination in the environment (150,151). Honey may not be the most suitable matrix to use as a bio-indicator of heavy metal pollution in the environment but it can offer an indication.

Bees are exposed to anthropogenic compounds from the air, water and floral resources. The source of these compounds is mainly from industry and transport (environmental pollutants) and agriculture practices (pesticides). Plant protection products, namely fungicides, herbicides and insecticides are collectively known as pesticides. There are a large number of pesticide classes and families, each with a particular behaviour against pests and diseases, specific properties concerning their environmental effects (persistence, biodegradability and accumulation) and specific toxicity levels.

Pesticides can be applied directly to crops as a foliar spray or granular feed or can be used as a seed dressing. Fungicides account for 60% of the total agrochemical market in Ireland, followed by herbicides (26%) and insecticides (3%) (152). Seed treatments are not included in these figures and account for 3% of the market (152). An estimated 1,144,043 kg of pesticides were used on arable crops in Ireland in 2012 (153). There are five groups of insecticides used for the protection of arable crops in Ireland: carbamates, neonicotinoids, organophosphates, pyrethroids and pyridine azomethines (153). The chemical structure of representative examples from each group can be seen in Fig. 1-5.



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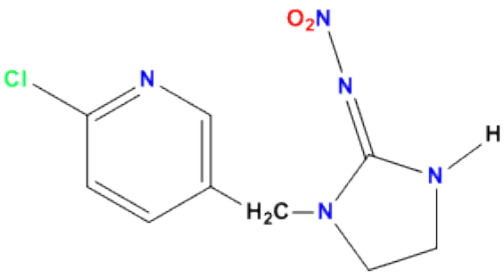

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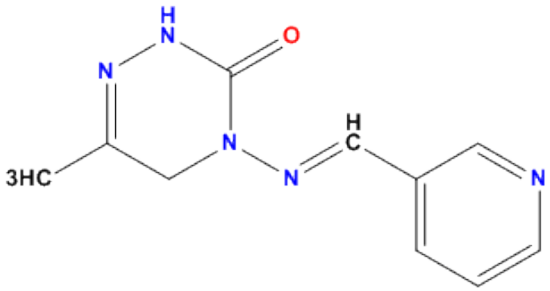

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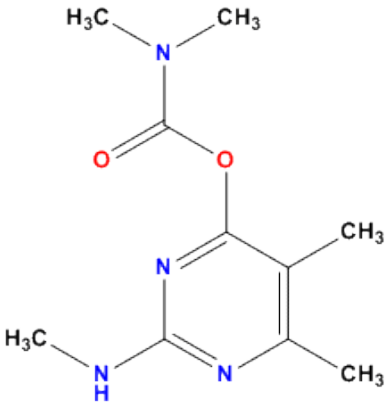

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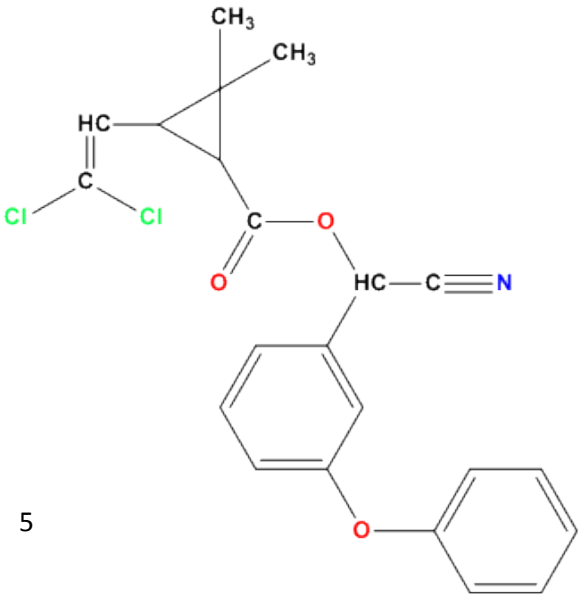

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Figure 1-5 Examples of the five groups of insecticides used for the protection of arable crops in Ireland: 1) Chlorpyrifos (organophosphate), 2) Imidacloprid (neonicotinoid), 3) Pymethrozine (pyridine azomethines), 4) Primocarb (carbamate) and 5) Cypermethrin (pyrethroid).

Neonicotinoids are the most widely used group of insecticides in the world and can be classified into one of three chemical groups based on their pharmacophore moieties (154), N-nitroguanidines (imidacloprid, thiamethoxam, clothianidin, and dinotefuran), nitromethylenes (nitenpyram), and N-cyanoamidines (acetamiprid and thiacloprid) (155,156). They act systematically, travelling through the plant tissue, protecting all parts. They are nicotinic acetylcholine receptor antagonists, i.e. they inhibit the action of acetylcholine (ACh), a neurotransmitter. Neonicotinoids provide effective pest control in crop management as they bind strongly to the nicotinic acetylcholine receptors in the central nervous system of insects, causing nervous stimulation at low concentrations and receptor blockage, paralysis and death at higher concentrations (157). Due to their wide application, persistence in soil and water and potential for uptake by succeeding crops and wild plants, neonicotinoids are bioavailable to pollinators in sub-lethal concentrations for most of the year (158). This can result in the presence of neonicotinoids in honey bee hives. Figure 1-6 depicts potential routes of exposure of neonicotinoids to bees in an agriculture environment. Bees can be exposed to pesticides directly, if their flight path is through a field undergoing spraying or through the dust created during seed sowing, or indirectly during foraging for pollen, nectar and water.

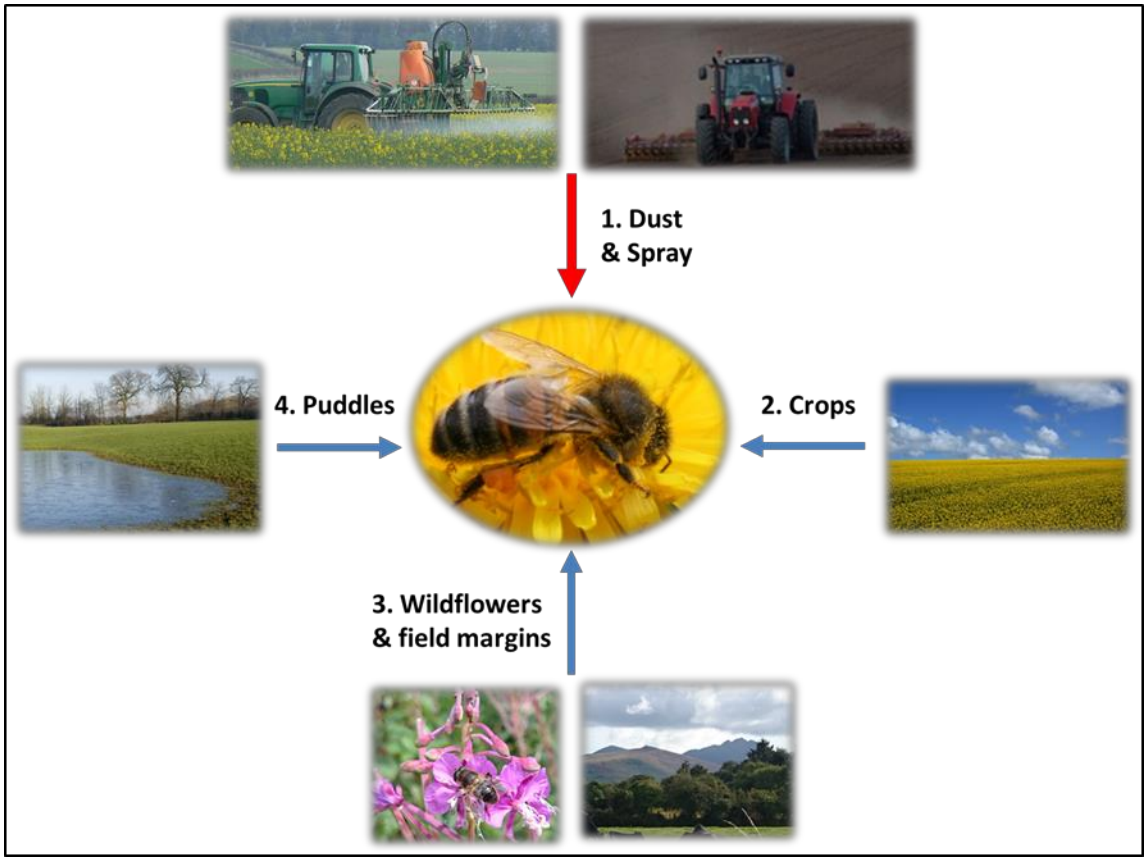

Figure 1-6 Routes of pesticide exposure to bees in an agricultural environment; 1) Direct contact via dust or foliar spray, 2) Uptake of pollen or nectar from treated crops, 3) Uptake of pollen or nectar from wildflowers and flowers in field margins and gardens and 4) Puddles of water in farmyards.

There has been a significant increase in the use of neonicotinoids in crop protection in recent years and their occurrence within honey bee matrices may become more frequent. Systemic insecticides, including neonicotinoids, have been demonstrated to present high acute and chronic toxicity in bees (14,16,159). Neonicotinoids have been found to decrease the foraging success of the honey bee (15) with the potential to induce a variety of behavioural difficulties (160) and other negative effects (17,18,144,161,162). Worldwide, fewer studies have been conducted on bumble bees, and those that have been published have conflicting results (37,163–166). Whitehorn *et al.* (2012) suggest that neonicotinoids may be having a considerable negative impact on wild bumble bee populations across the developed world (37). In contrast, Tasei *et al.* (2000) concluded that the survival rate and reproductive capacity of *Bombus terrestris* was not likely to be affected by prolonged

ingestion of nectar produced by sunflower after seed-dressing treatment with imidacloprid (166). The contrasting results are likely due to differences in imidacloprid dosage as well as the different methods used to assess the effects of neonicotinoids on bees. Whitehorn *et al.* recorded a difference in the number of queens produced between treated and untreated hives, whereas Tasei *et al.* measured worker survival, brood size and larval development. Tasei *et al.* did not measure queen production because the queen was not inseminated and therefore any brood resulted in a haploid male progeny. Since nearly all synthetic insecticides are broad spectrum, and have the potential to affect a range of insect taxa, Neonicotinoids are not the only class that have demonstrated negative effects on bees. Pyrethroids can induce adverse acute sub-lethal effects in bees (14,15). Residues of neonicotinoids (70,145,147,167,168) and pyrethroids (145,167,168) have been found in honey samples from many countries around the world (Austria (70), France (145,167), Italy (169), Poland (147) USA (168)).

Different pesticide residues have been detected in honey from many countries. For example, 30 pesticide residues, including neonicotinoids have been detected in Polish honey (147). The pesticide levels found in honey samples varies considerably and is commonly below 0.5mg kg^{-1} (170). Organochlorine, organophosphorus, acaricides, fluvalinate, coumaphos and bromopropylate are the most common pesticides detected in honey samples (170). Lambert *et al.* (2013) detected 28 chemical residues in honey from different landscapes in Western France (145). The most frequently detected residues (in more than 10% of samples) were coumaphos (78.0%), amitraz II (68.8%), carbendazim (64.5%), phosmet (12.8%) and cyproconazole (11.3%). This is to be expected as coumaphos, amitraz II and carbendazim are commonly used by beekeepers to control the parasitic mite *Varroa destructor*. Lambert *et al.* also detected thiamethoxam which has recently been identified by the EFSA as a risk to bees (116) and imidacloprid. Blasco *et al.* (2003) assessed pesticide residues in honey from Spain and Portugal. The authors report that most of the pesticides found in honey were organochlorines (171), however, this is in contrast to a French study where organophosphorus compounds were detected (145). A potential reason for this difference may be due to the ban of these pesticides in Europe in the 1970s. Portugal and Spain did not join the EU until 1986 and so the use of organochlorines within these countries is likely to be more recent compared to France. Secondly there is a ten year gap in the research, Portugal / Spain (2003) and France (2013) and it is less likely that these residues would occur in honey in a more recent study because

of changes in popularity of different products and due to changes in the legislation regarding these products. Different national regulations have established maximum concentrations of pesticide residues (maximum residue levels - MRLs) permitted in honey (172). There is however a lack of homogeneity causing problems in international marketing and trade (172). For example Germany, Italy, and Switzerland have set MRLs for amitraz, bromopropylate, coumaphos, cyamizole, flumetrine, and fluvalinate, which oscillate between 0.01 and 0.1 mg kg^{-1} in Germany, between 5 and 500 mg kg^{-1} in Switzerland, and 10 mg kg^{-1} in Italy (173). EU legislation has regulated the MRLs for veterinary drugs in honey, three acaricides: amitraz, coumaphos, and cyamizole, which are 0.2, 0.1, and 1 mg kg^{-1}, respectively (174) but pesticide MRLs in honey are not included in the Codex Alimentarius (111). The EC pesticide database however, contains current MRL values for a number of pesticides in honey. The European Economic Community (EEC) has acknowledged that residues in honey might also be derived from pesticide treatment of honey providing plants or environmental contamination and in 1996 the EU Committee agreed that this issue was of low priority (175). Given recent EFSA reports linking pesticides to human developmental neurotoxicity as well as the enormous literature available on the negative effects on bees, this priority designation should be re-examined (176). European Member States recently agreed to ban the use of imidacloprid, thiamethoxam and clothianidin in all crops grown outdoors, which is likely to reduce the concentration of these compounds in honey; however these compounds are likely to be replaced by other insecticides which may also have negative effects on bees. It has been suggested that sulfoximine based insecticides are the most likely successor for neonicotinoids. A recent study has shown that bumble bee colonies exposed to sulfoxaflor (a sulfoximine based insecticide) produced fewer workers and fewer reproductive offspring compared to untreated colonies (177). It has therefore been recommend that a broad evidence base needs to be assessed prior to the development of policy and regulation of pesticides new to the market (178).

Many anthropogenic compounds have been detected in honey, some of which pose a threat to bee health. Irish studies have examined concentrations of veterinary antibiotics for bee protection (179,180). Radonikovic *et al.* (2013) detected semicarbazide in 9 out of 271 samples of honey, some of which were Irish honeys, but the origin of the positive samples is not stated. Crop protection insecticide contamination in Irish honey has not been examined. It is not known therefore if agricultural pesticides are present in Irish honey.

Pesticides are beneficial, in that they provide crop protection, food preservation and disease control; however, pesticides are toxic by design and some groups have been determined to pose serious risks to human health and the environment (181).

1.3 Honey analysis methods

Several methods for analysing and detecting phenols (69) and pesticides (182) in honey have been developed . For the determination of any compound in honey three basic steps must be carried out: isolation from the sample matrix; analytical separation; and detection (identification and quantification)(183). The recovery/isolation step usually involves solid-phase extraction (SPE) and / or solvent extraction. Separation is achieved by high performance liquid chromatography (HPLC), capillary electrophoresis (CE), or gas chromatography (GC). The most common mode of separation uses reversed-phase (RP) systems typically with a C18 column and various mobile phases (120). Detection can be achieved by UV (ultraviolet) absorption and various mass-spectrometric methods. Factors determining optimum separation methods include detection of large number of analytes with high separation efficiency, a short analysis time and low solvent consumption.

A number of standardised methods exist for the analysis of honey authenticity. The current literature related to analytical procedures that allow the determination of phenolic acids, flavonoids and pesticides in honey as well as methods of honey authentication will be briefly discussed.

1.3.1 Authenticity methods

Routine physiochemical analysis can be used to assess the authenticity of honey. The Codex Alimentarius lists the parameters used to assess authenticity variables, which include electrical conductivity, sugar, moisture, free acid and water-insoluble content, as discussed in Section 1.2.2. Methods to analyse these parameters have been validated and harmonised by the International Honey Commission (184) and can be used within the scope of the Codex Alimentarius Honey Standard (111) and the European Union Honey Directive (56). The Bureau of Indian Standards (IS) has a higher number of variables for describing honey authenticity and these incude: specific gravity, ash content, acidity, HMF (hydroxymethylfurfural) content, moisture, total reducing sugars, sucrose and fructose-glucose ratio, optical density and total count of pollens and plant elements, as well as honey sampling protocols (185).

In terms of the purity of honey, a refractometric method is regularly used for the analysis of moisture, and conductivity is widely accepted for analysing honey's electrical conductivity. Melissopalynology can be used to assess honeys floral origin by counting pollen grains, however due to increasing advances in DNA analysis, metabarcoding is also a suitable method for identifying the plant composition of honey (186). Sugar content is a reliable indication of the age of honey and the Fehling method is commonly used to measure the reducing sugar content of honey (mainly fructose and glucose) as well as the apparent sucrose. The specific rotation of honey can be determined by means of a polarimeter and is frequently used to differentiate between honeydew honeys and blossom honeys (187). A number of techniques can be applied to assess whether honey has been adulterated with syrup including high performance liquid chromatography (HPLC) with UV detection (188) and low field nuclear magnetic resonance (114). The colour of honey is assessed by optical comparison using the Pfund scale (189).

Honey authenticity can depend on the honey's country of origin and / or country of sale. It can also be based on purity (ash content, electrical conductivity and insoluble solids in water), maturity (sugar content and moisture) and deterioration (HMF content, acidity and diastase activity), and in Europe it is defined by the Codex Alimentarius (111). These variables can be used to assess if honey has been adulterated by syrups, meets legislation with regard to human consumption and whether the honey is of the indicated floral origin.

1.3.2 Sample preparation

For chromatographic analysis of phenols or pesticides in honey, pre-concentration of analytes together with separation of other polar compounds is required. The preparation of a uniform sample extract enriched in all components of interest and free from interfering matrix components (e.g. the sugar content of honey) is the aim of any extraction procedure (120). Suitable sample preparation for the isolation of pesticides or phenols in honey is a vital step in any analytical procedure. It allows for the pre-concentration of analytes as well as the removal of unwanted matrix components. For the analysis of phenols or pesticides in honey, sugars must be removed. Sample components of interest are separated from the sample matrix by applying the sample mixture to a suitable solid sorbent and eluting the desired components (190). The high sugar content of honey has been reported as the main problem associated with isolating specific compounds from honey (191). A number of methods have been developed for sample clean-up and pre-concentration for honey

analyses in terms of isolating phenolic compounds and pesticides. Solid Phase Extraction (SPE) is the most commonly used method for phenolic acid and flavonoid isolation and the 'QuEChERS method' (Quick Easy Cheep Effective Rugged and Safe) is the favoured method for the isolation of pesticides (182). Other extraction methods used in the analyses of honey include; liquid-liquid extraction (LLE) (96), dispersive liquid-liquid micro-extraction (DLLME) (192,193), microwave-assisted extraction (MAE) (183), ultrasonic extraction (UE) (183) and supercritical fluid extraction (SFE) (194).

SPE is considered one of the simplest, most effective and versatile methods of sample preparation for the analysis of phenols in honeys (190). It utilises low cost, pre-packed disposable cartridges, a low amount of solvent and a shorter sample preparation time (195). In SPE, sorbants are initially used to trap compounds of interest, followed by selective elution of these compounds for further analysis. Judicious choice of sorbent maximises the extent of matrix removal initially and subsequent elution of the compound of interest. For the analysis of phenols in honey the most popular sorbants for SPE are the hydrophobic Amberlite XAD-2 resin and the RP C-18 resins and cartridges with a variety of methods published for a wide range of phenols. Several solvents are used in a SPE procedure for conditioning, washing and elution. Optimisation of each of these solvents leads to improvements in method efficiency (171). Michalkiewicz *et al.* (2008) tested different solid sorbants used in SPE to determine the most appropriate in terms of the recovery of target analytes (specifically phenolic acids and flavonoids) in honey (190). The highest recovery was achieved using an Oasis HLB micocolumn followed by washing the sorbent with 50 mL of acidified water however, only 8 phenols where analysed in this study (190). Many studies report on the isolation of phenolic extracts from honey by adsorption onto hydrophobic Amberlite XAD-2 and subsequent elution with methanol (66,100,131,196). Other studies have utilised SPE-C18 cartridges and contradictory to Michalkiewicz *et al.* this method is considered to provide higher recoveries of analytes and give a reduction in solvent consumption (197). Kaškonienė *et al.* (2009) detected 11 phenols in honey using C-18 cartridges. SPE-C18 cartridges are inexpensive disposable extraction columns and this technique is widely regarded as a simpler technique when compared to extraction with Amberlite XAD-2.

In terms of pesticide analysis in food, generally, dispersive-SPE is preferred and is more commonly referred to as the QuEChERS method (145,159). QuEChERS consists of

extraction and purification with mixtures of salts adapted to the matrix and the substances to be extracted. This method is very diverse and can be used to target one family of pesticides in honey (198) or in a multi-residue analysis (145,159). See Chapter 4 for a more in-depth discussion.

1.3.3 Analysis and detection of phenols

Several methods for analysing phenols in honey have been developed. These include: colorimetric reactions (81,199), gas chromatography (GC) (200), capillary electrophoresis (CE) (201), and more commonly high performance liquid chromatography (HPLC) (132,202). With HPLC, the most common mode of separation for the analysis of phenols in honey uses reversed-phase (RP) systems typically with a C18 stationary phase (120,132,202). Detection is commonly achieved by UV absorption and / or mass spectrometry (MS). HPLC-UV is considered the most widely used analytical technique for characterising phenolic compounds in honey (197) and limits of detection (LOD's) and limits of quantification (LOQ's) typically range from 0.024 - 0.104 mg L^{-1} and 0.072 - 0.312 mg L^{-1}, respectively (203,204). UHPLC-UV is a relatively new development in LC and reported detection and quantification limits are typically an order of magnitude lower than in HPLC, 0.001 - 0.008 mg L^{-1} and 0.003 - 0.028 mg L^{-1} respectively (192). UHPLC is also advantageous over HPLC in terms of system efficiency and analysis time. This is achieved as the ultra-high pressure system allows for the use of small particle packed columns with small diameter (205), however instrumentation costs are higher. The HPLC mode most widely used is reversed-phase HPLC. The stationary phase usually consists of a non-polar octadecylsilane (C_{18}) bonded phase while the mobile phase is typically a polar solvent. An elution mobile gradient is frequently used for the determination of honey phenolic profiles. This is mainly due to the complexity of the phenolic profile found in honey. Identification of isolated compounds can be achieved with UV detection and / or MS. Detection is achieved by comparing retention times and / or mass spectra with those of authentic standards. Although several studies have been conducted on the identification and quantification of phenols in honey from around the world (102,125), there is currently no published research on the identification of phenols in Irish honey.

1.3.3 Analysis and detection of neonicotinoids

Gas chromatography has been extensively used for the analysis of pesticides in honey (145,159,167,194). Its high separation power and wide availability of selective detectors such as electron capture (EC), nitrogen phosphorus (NP), and MS make it a desirable technique. However, there have been some difficulties reported with the pesticide analysis of honey using GC. For example, the LOQ are not always sufficiently low enough to quantify pesticides in the concentrations that are toxic to bees (167). To suitably evaluate the toxicity of pesticides, LOQ have to reach at least 1 ng g^{-1} (167). In recent years, LC has emerged as an alternative technique, especially for polar and thermolabile pesticides due to its improved LOD and LOQ (171).

Using LC-MS different classes of pesticides have been detected in honeys from many countries including France (145), Poland (147) and Spain (204). Paradis *et al.* (2014) used coupling chromatography (LC and GC) with tandem MS to analyse 22 insecticides (specifically, neonicotinoids, pyrazoles and pyrethroids) in honey with LOQ below 1 ng/g. More recently, Mitchell *et al.* (2017) conducted a worldwide study of neonicotinoids and identified at least one neonicotinoid in 75% of samples analysed using UHPLC-MS/MS. Although this study analysed 198 honey samples, there was no Irish honey included in the study.

1.4 The Irish Landscape

Because bees are central place foragers, and forage within a finite range of their nest, the floral resources within this range are expected to affect the bioactive and anthropogenic constituents in honey. The Irish landscape is dominated by agriculture, which comprises approximately 65% of the total land area (206). Of this, 81% is pasture (intensive grassland) although there is regular conversion between grass and arable land (207). Field boundaries provide important habitats for wildlife in agricultural landscapes and in Ireland and consist mainly of hedgerows, which provide essential resources for bees (208) (however dry stone walls dominate in some areas, particularly in County Roscommon). Land management practices such as fertilisation, grazing and cutting can have negative effects on biodiversity in farmland (209). Intensive grasslands support fewer plant (210) and bee (211) species compared to plant and bee communities found in semi-natural

grassland (which only comprise less than 1% of of the total land area in Ireland (212)). From 1990 to 2000, arable land and permanent crops increased in area by 35%, followed closely by artificial surfaces which increased by 31% (212). These changes were largely at the expense of pasture and mixed farmland (213). Since 2000 there has been a further expansion of artificial surfaces, however the percentage of cropland has remained the same (214). Due to the increase in arable land and artificial surfaces, the proportion of semi–natural habitats (SNH) has decreased. SNH in Ireland can be broadly categorised as: scrub and / or herbaceous vegetation associations and open spaces with little or no vegetation. Scrub and / or herbaceous vegetation associations can be broken down into natural grasslands, moors and heathland, sclerophyllous vegetation and transitional woodland scrub. It is within these habitats that high proportions (in terms of abundance and diversity) of native flora can be found (215). There are a dozen species of plants in the west and southwest of Ireland that are not found elsewhere in northern Europe. These species comprise the "Lusitanian flora" which is found in southern and western Ireland and in northern Spain and Portugal (and occasionally Brittany) (216). One example of a particularly species rich region in terms of flora in Ireland is the Burren, a unique habitat in Ireland which represents an example of a glaciated karst landscape, located in north Clare and south Galway and is noted for its unusual and species rich flora. Over 70% of the Irish Flora is known from this relatively small area (217).

1.4.1 Impacts of land use on floral resources for bees

Agricultural intensification has led to the loss and fragmentation of semi-natural habitats (SNH) as well as changes in land management practices such as increased fertilisation and pesticide use. This in turn has led to a loss of floral resources for bees. It is well documented that habitat degradation and destruction, intensive agriculture, overgrazing and selective harvesting have negative effects on floral resources for bees (5). In the UK about 40% of hedgerows have been lost since the 1930's and thousands of hectares of wildflower meadows (5). The quantification of the loss of Irish hedgerows is absent, however there is extensive data available on Irish semi-natural grasslands (212). Between 1990 and 2000, the EPA reported that the area of natural grassland decreased while arable and permanent pastures increased by over 30% (213). In the Irish Semi-Natural Grassland survey of 2007-2012, 27 out of 361 semi-natural grassland Annex 1 sites decreased in area (212). Plant species diversity is higher in natural grassland compared to arable land or

pastures (215) and in a more recent report the EPA states that species rich grasslands are under threat and are listed in the habitats of most pressing concern (218). Carré *et al.* (2009) showed that agricultural intensification may not lead to the extinction of all bees in agroecosystems, but instead may change its community composition, with an increase in the most resilient (less vulnerable to landscape change - based on a 'vulnerability index') bee sub-genera and loss of the more vulnerable species (219), a trend which had also previously been observed (220,221). Kleijn *et al.* (2015) report that it is these common bee species that are responsible for the majority of crop pollination and crop-visiting bee communities rarely contain threatened species (222). Carré *et al.* conclude that for certain crop systems, some wild bee taxa may not be affected by SNH in the surrounding landscape and some might even be favoured by land use intensification. A significant limitation of this study is that it did not take into account other processes of agricultural intensification such as pesticide use, which can negatively affect bee populations. It is unlikely that any bee species would benefit from land use intensification given the negative effects of pesticides on bees, however, it is likely that many species will be able to persist under agricultural expansion and many can be easily enhanced by simple conservation measures (222).

Many flowering entomophilous crops benefit from pollination by bees and supply floral resources to bees, for example fruit trees such as apple orchards, as well as oil seed rape and beans. Other crops, for instance carrot, onion and potato, carry flowers which attract foraging insects but the production of the edible parts of the plant is situated in its root zone and hence, these crops are not dependent on pollination. Cereal crops are not dependent on pollination by insects and provide little floral resources for pollinators, although honeybees often harvest pollen from maize (*Zea mays)*. Cereal crops can however be a source of negative effects for bees. Stewart *et al.* (2014) found neonicotinoids present in the nectar of wildflowers growing in crop field margins (223). The average sample distance from the field edge was 20 m. Depending on the types of crops grown, the pollination service requirement can vary as well as the effect on the bee (positive – providing food or negative – poisoning via pesticides). David *et al.* (2016) report that pesticide exposure to bumble bee colonies was lower in urban areas compared to rural areas (224). Agrochemicals have negative effects on bees, both directly and indirectly. Many studies have reported on the link between the systematic use of persistent neurotoxic insecticides in agriculture and horticulture and the decline in bee populations. Fipronil (a

phenylpyrazole), imidacloprid, thiamethoxam (neonicotinoids) and deltamethrin (a pyrethroid) are toxic to bees and are suspected to be involved in honey bee colony losses (158,167). Henry *et al.* (2012) report that nonlethal exposure of honey bees to thiamethoxam causes high mortality due to homing failure at levels that could put a colony at risk of collapse. Neonicotinoids are a group of active substances first sold on the market in 1991 and Imidacloprid is currently the second most widely used agrochemical in the world (155). Whether bees forage directly on crops containing these neurotoxins or on plants that have taken them up 'accidentally', the bees are carrying these toxins into their colonies.

An increase in artificial surfaces (urbanisation) can lead to the destruction of potential floral resources for bees, however, some urban landscapes have been shown to benefit bees. Baldock *et al.* (2015) compared pollinator communities using quantified flower-visitation networks in three landscapes: urban, farmland and nature reserves. Bee abundance did not differ between landscapes and a higher bee species richness was observed in urban compared with farmland sites (39). The higher bee species richness may be due to the lower amounts of pesticides being used in urban areas, or due to the diversity and year-round availability of floral resources and overwintering, nesting and mating habitats, as well as potential benefits from urban heat island effects. There are currently no data available on how much pesticide is used in urban areas but it is presumed to be less than that used in agricultural areas. However, due to the domestic use in gardens and by urban authorities and traces in plants grown in garden centres (225), there may be more than previously anticipated. Botías *et al.* (2017) reported a higher level of agrochemicals in bees foraging in agricultural landscapes compared with bees foraging at urban sites. However, the highest levels and frequencies of detection for neonicotinoids and the most frequently detected fungicide boscalid were recorded in urban bumblebees (226).

It is clear from the literature that land use has significant impacts on floral resources for bees. The intensification of agriculture has led to the current prophylactic use of neonicotinoids which has in turn maximised exposure of these neurotoxic pesticides to the environment where they are taken up by bees. The loss of floral resources from land use change coupled with the increased use of pesticides has negative effects on bee heath. It is therefore paramount that land management practices change in order to protect these important pollinators.

1.4.2 Land uses and spatial patterns for the provision of flowers and bees

With the current concerns of pollinator decline comes a crucial need for improving pollinator habitats. Bees provide a vital service to both natural ecosystems and farming and therefore should be offered a high level of protection given the potentially far-reaching effects of their decline. Many methods and land management techniques have been implemented, and shown to have positive impacts on bees and their floral resources (227).

Forest edges, grasslands rich in flowers and riparian areas offer suitable forage sites for bumble and honey bees. Forest edges adjacent to more open land have been shown to have a positive impact on bees and small patches of these habitats are particularly important. Woodland and grassland provide nesting habitats for bumble bees and feral honey bees (228). Svensson *et al.* (2000) conclude that nest-seeking queens were most frequently observed along forest and field boundaries, in open uncultivated areas (229). Hopwood *et al.* (2008) conclude that management of roadside vegetation via the planting of native species significantly increased the abundance and richness of wild bees (227). Marginal habitats such as hedgerows or roadsides are important forage sites for bees especially in highly human-impacted environments (227). Railway lines and hedgerows have also been shown to positively impact bee species (230).

Carré *et al.* assessed the effect of habitat type on bee communities in annual entomophilous crops in Europe. Some bee taxa were positively affected by urban habitats only, others by semi-natural habitats only, and others by a combination of semi-natural, urban and crop habitats (219). At the European scale neither abundance nor diversity were shown to be affected by the proportion of semi-natural habitats (219). However, the authors found differences in bee abundance in relation to landscape context at the country scale. Bee abundance increased significantly with the proportion of semi-natural habitats in the UK and bee diversity increased significantly with the proportion of semi-natural habitat in Poland. Some crop and urban habitats (e.g., pasture and sport and leisure facilities) were found to have positive effects on bee diversity in Germany, Poland and Sweden. In the UK the abundance and diversity of wild bees increased with transitional woodland-shrub habitats (219). Steffan-Dewenter *et al.* (2002) found that honey bee abundance increased in Germany when the proportion of semi-natural habitats decreased and the abundance of honey bees was positively associated with urban habitats (231). Tscharntke *et al.* (2005) report that the presence of semi natural habitats in agricultural landscapes increases nesting

sites and floral resources for bees (232). Honey bee abundance will always be affected by beekeeping practices and as such are not ideal taxa for assessing the effects of land use on bee abundance. Plant and animal interactions are highly complex and diversified and for this reason it is advisable to include an assessment of a wide number of taxa when studying the effects of landscape on bee abundance.

Mass-flowering crops can supplement foraging habitats of bees. Higher colony densities of bumble bee species have been found in agricultural landscapes with large proportions of oilseed rape (233). However, flowering continuity is very important for the life cycle of bumble and honey bees and such agricultural flowering crops do not offer this resource. In a later study, the same authors report that oilseed rape has a beneficial effect on colony growth but does not increase sexual reproduction and conclude that this may be due to food plant scarcity later in the colony cycle (234).

To maximise agricultural returns the cost / benefits of controlling pests must be weighed against the cost of promoting bees. While the levels of pesticides bees are exposed to in the natural environment are usually too low to induce death, the sub lethal effects can be extreme (38).

There are numerous strategies which can improve bee forage in the urban and rural environment. For example: planting flowers with accessible and high quantity and quality rewards, cutting during non-flowing periods, reducing or stopping the unnecessary use of pesticides. Extending field margins and other uncultivated areas on farmland could encourage bees by increasing floral resources and nesting habitats (235). Kells and Goulson concluded that to ensure the continued survival of bumble bee species a variety of field and forest boundary types need to be conserved (228). An alternative management of roadside verges may enhance the availability of habitats for bees by increasing floral resources (236). By supplying or maintaining the substrates that provide nest sites and by providing specific forage plants, native pollinator populations will be given a chance to increase in number (5). Incorporating organic crop fields into conventionally managed agricultural landscapes can also provide food resources to encourage greater pollinator species richness (237). Currently around £400 million per annum is spent on Agri-environment Schemes in the UK (no figures available for Ireland) and there is still evidence of pollinator decline. In Ireland, the past five years have shown significant advances in relation to the sustainable management of land use for the provision of flowers

and bees, including initiatives such as the All Ireland Pollinator Plan and the Irish National Action Plan for the Sustainable Use of Pesticides (Plant Protection Products). Given the exponential rate at which research is being conducted both nationally and internationally, the next five years are likely to be equally, if not more, active. The recent decision by the European Member States to ban the use of imidacloprid, thiamethoxam and clothianidin in all crops grown outdoors is one of many steps taken to protect bees.

To summarise, in order to provide for bees and support the ecosystem services they provide, an improvement in the quality and quantity of plant and animal niche habitats is required in conjunction with a reduction in the use of toxic pesticides.

1.4.3 Established methods for landscape analysis

Landscape structure is defined by two components: composition and configuration. Landscape composition is the number and the amount of the different habitat types that can be measured inter alia by the proportion of specific habitat, or by Shannon's diversity index. Landscape configuration is the spatial arrangement of these landscape elements. Landscape configuration can be measured by mean patch size, edge density and / or mean patch shape variability and landscape configurational heterogeneity is calculated using the area weighted mean shape index (AWMSI).

There are many techniques used in research to analyse the landscape. Aerial photographs or satellite remote sensing can be used in conjunction with national maps and / or field work (238,239). The CORINE (Coordination of Information on the Environment) Land Cover (CLC) is commonly used in ecological studies to characterise the landscape (219,239,240). CLC is a common classification system of geo-spatial information that is available for all European Member States. It is based on satellite images and categorises land cover into 44 classes within three hierarchal levels. The minimum map unit is 25 ha (241). Therefore the resolution is not sufficient to define fine-scale landscape complexity for ecological investigations. As such, this method is more suited to larger scale studies. For smaller scale studies, ground-truthing and classifying the landscape using a vegetation classification system is more suitable. The most widely used classification system in Ireland is Fossitt's Guide to Habitats in Ireland (215). The method used for characterising the landscape will depend on the research question and the available resources. Lambert *et al.* compared apicultural matrices between four different landscapes in western France

(145). The landscape was classified into one of four contexts based on susceptibility to contamination by pesticides and veterinary drugs; rural-grassland landscapes (characterised by high length of hedges and numerous grassland plots), rural-cultivated landscapes (characterised by large plots of crops and a low hedgerow network), urban landscapes (characterized by large urban areas and some rural areas), and islands free from high levels of anthropogenic activities. The authors did not disclose what method was used to characterise these landscapes. Panseri *et al.* (2014) had a similar approach and characterised the landscape in relation to its potential pesticides sources (169).

For this research, CLC 2012 will be used to characterise the landscape around hive sites. Because this research is based on an ecological process at a specific point in time, CLC may not be 100% accurate as it is only renewed every six years. Therefore further validation of this characterisation may be required.

1.5 Conclusion

Bees are important for the delivery of multiple ecosystem services and require protection to ensure pollination of a wide range of plants of importance to humans, including crops. Bee abundance and diversity has been shown to depend on the surrounding landscape and the floral resources within. The honey produced by bees is directly influenced by the nectar and pollen the bees collect from floral resources. Therefore analysis of this honey may provide a detailed insight into the foraging behaviour of bees. This can be applied to both natural and anthropogenic constituents of the honey. The extent to which honey composition correlates with the surrounding landscape has not yet been investigated; however, because bees forage within fixed distances of their colony the composition and of the surrounding landscape is likely to affect honey properties. If such a correlation were established it would enable honey constituent profiles to be characterised and used as an indicator of bee exposure to anthropogenic compounds and bee foraging patterns and preferences. There is also a potential for these constituent profiles to be used as indicators of bee health and therefore signal both the effectiveness of pollination services and resultant overall crop yields. Additionally, honey constituent characterisation could be used to identify potential health benefits of Irish honey by elucidating their bioactive profiles. This could increase the economic value of Irish honeys.

1.6 Aim of this work

The aims of this project are to identify bioactive (phenols) and anthropogenic (neonicotinoids) constituents in honey bee honey, and to determine the extent to which honey chemistry is related to the environment foraged by the bees. Honey constituent profiles were characterised according to the landscape composition, physiochemical properties, phenolic content and pesticide composition.

The following hypotheses were tested:

- A different physiochemical make up is found in honeys depending on floral composition and / or land use;
- Different compositions of phenols are found in honey depending on landscape composition and hive location;
- Different concentrations of pesticide residues are found in honey from hives in rural areas compared to urban areas.

Objectives

To achieve these aims, the objectives of the research are to:

1. Collect and catalogue a variety of honey bee honey, both commercially available and that from colonies across Ireland;
2. Characterise the landscape using CORINE 2012 data series to a 5 km radius of each of the 76 honey sampling sites;
3. Develop and utilise methods to investigate the chemical composition (in terms of total phenolic content) and physiochemical parameters (honey electrical conductivity, moisture, pH, total sugar and colour) of different types of Irish honey and compare these with international honeys;
4. Develop and utilise analytical separation techniques to identify the phenolic composition of honey;
5. Determine the presence and concentration of neonicotinoids in Irish honey.
6. Explore the relationships between composition of surrounding landscape and honey phenolic and pesticide content.

The chemical composition and physiochemical parameters of different types of Irish honey were analysed and compared with international honeys (Chapter two). In addition, microbial contamination and microbial stability of Irish multi-floral honey was assessed. Finally, the relationship between the physiochemical properties and chemical composition of honey and honeys floral origin was assessed.

In Chapter three the phenolic composition of honey was analysed using HPLC-UV and the relationships between honey phenolic composition (presence/absence of phenolic compounds and their concentrations) and a) hive location (degree of urbanisation), b) landscape composition surrounding the hive and c) harvest season (when samples are taken) were assessed.

Neonicotinoids have been found to decrease the foraging success of the honey bee [16] with the potential to induce a variety of behavioural deficits [125] and other negative effects [18,19,111,126,127]. In Chapter four I analysed the composition of three neonicotinoids in Irish honey (clothianidin, imidacloprid and thiacloprid) using LC-MS. The relationship between the presence of neonicotinoids and the surrounding landscape in terms of amount of semi-natural habitat, agricultural land and artificial surface was assessed.

In the final chapter, I discussed the main findings of this work in relation to the relevant literature and made suggestions for future research. Lastly, how this research informs the wider context of bee and human health and the potential outcomes of this research are elucidated.

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Chapter 2 Physicochemical properties and phenolic content of honey from different floral origins and from rural versus urban landscapes

Manuscript published: Kavanagh S, Gunnoo J, Passos Marques T, Stout JC, White B. Physicochemical properties and phenolic content of honey from different floral origins and from rural versus urban landscapes. Food Chem. 2019;272:66–75.

Abstract

The composition of honey influences how beneficial it is to human health. This study evaluated the physiochemical properties and total phenolic content (TPC) of single vs. multi-floral Irish and selected international honeys, and whether properties varied according to hive location. Oilseed rape honey had the lowest TPC of Irish unifloral honeys. Heather honey had the highest TPC, similar to Manuka honey (Mean ± SD = 68.16±2.73 and 62.43±10.03 respectively), and the TPC of ivy honey was approximately half that of heather. Urban multi-floral honeys contained higher TPC (28.26±13.63) than rural honeys (20.32±11.54). Physiochemical properties varied according to floral origin, and whether hives were in urban or rural sites. Irish heather honey had similar physiochemical characteristics to Manuka honey. This first examination of Irish honey confirms that TPC and physiochemical properties vary with honey type and hive location, and suggests that Irish heather honey should be examined for potential health benefits.

2.1 Introduction

Honey produced by honey bees (*Apis spp.*) can promote many beneficial biological processes in humans, including: antioxidant (1), antibacterial (2), antidiabetic (3), anti-inflammatory (4) and antimicrobial activity (2), as well as free radical scavenging (5), and wound healing (6). Of these, food antioxidants have been shown to reduce oxidative stress and thus prevent oxidative damage (7), and are thus of great interest for promoting human health and wellbeing. The constituents of honey that are responsible for antioxidant effects are flavonoids, phenolic acids, ascorbic acid, catalase, peroxidase, carotenoids, and products of Maillard reactions (8). Phenolic acids and flavonoids have been comprehensively researched in honey (9–13) and can be used to assess honey quality. Several studies have shown that antioxidant activities are strongly correlated with the concentration of total phenolics. This was confirmed for seven single origin Italian honeys (14), four types of Romanian honeys (15) and seven types of Slovenian honeys (16). A study of Portuguese honeys demonstrated that the phenolic content of honey is not only responsible for its antioxidant properties, but also for its anti-microbial effects (17).

It is widely agreed that the botanical origin of honey has the greatest influence on its phenolic content and thus antioxidant activity, and some floral honeys are perceived to have a higher quality than others. For example, some-single origin honeys (i.e. from a single plant taxa, e.g. Manuka honey (*Leptospermum scoparium*) from New Zealand and heather honey (*Calluna* / *Erica spp.)* from northern Europe) have a higher retail price than blended honey (a mixture of two or more honeys) (18). The purity of the honey also plays a role in its quality and Manuka honey has varying UMFs (Unique Manuka Factors) whereby the designated factor, price and potential health benefits correlate positively (19).

In this study, we focus on honey produced in Ireland. The main feature of the Irish honey market is that most of the production comes from small private producers: according to the Federation of Irish Beekeepers, there were 2,800 registered beekeepers in Ireland in 2014 and the average number of hives per beekeeper was three (Federation of Irish Beekeepers, 2015, personal communication). Given the maritime (relatively cool and damp) climatic conditions of Ireland, the production of honey is limited, and the majority of Irish beekeepers only harvest honey once a year. Despite this, trade in honey increased in value and tonnage in Ireland between 2009 and 2013. Within these years Ireland imported seven times more honey than it exported (20). Increasing consumer preferences for products

based on honey has led to the expansion of the honey market, resulting in a greater number of varieties of honey and varieties of honey-based foods. This has also led to increased demand for single-origin honeys (such as heather and ivy (*Hedera spp.*)) particularly those with perceived health benefits. Being able to identify particular characteristics in honey which are associated with honey's health benefits, and rapidly assessing these characteristics in Irish honey could help beekeepers to market their honey appropriately and increase its commercial value.

The main aim of this study was to investigate the chemical composition (in terms of total phenolic content) and physiochemical parameters (honey electrical conductivity, moisture, Ph, total sugar and colour) of different types of Irish honey and compare these with international honeys. In addition, microbial contamination and microbial stability of Irish multi-floral honey was assessed. The comparison of Irish single-origin honeys (heather, ivy and oilseed rape (*Brassica rapa*) and multi-floral honeys has to our knowledge never previously been made, nor has a comparison of honey from urban vs. rural hive sites. In addition, the relationship between total phenolic content and physiochemical parameters was assessed to enable rapid assessment of Irish honey quality. Results confirmed that total phenolic content (TPC) could be used to differentiate between honey types. Urban multi-floral honeys had a higher TPC than rural multi-floral honeys. Irish heather honey had the highest TPC of all Irish single origin honeys, and had a higher TPC than Manuka honey. It was illustrated that honey colour correlates with electrical conductivity and with TPC, although ivy honey was an exception to this. While ivy was the darkest Irish honey analysed, its TPC was less than that of heather and Manuka honeys. To our knowledge this is the first time that ivy honey and Irish honey have been comprehensively researched.

2.2 Materials and Methods

2.2.1 Sample collection

One hundred and thirty-one Irish honey samples from *Apis mellifera* were collected directly from beekeepers between 2013 and 2015, from 78 locations across Ireland (Fig. 2-1). The majority (124) of samples were multi-floral honeys (55 from urban and 69 from rural locations), three were heather (*Erica* and/or *Calluna spp.)* honeys, two were ivy (*Hedera spp.*) honeys and two were oilseed rape (OSR) honeys. Honeys were classified

according to landscape context (urban / rural) (21) or honey floral source (information provided by beekeepers). Eight international honeys were compared with Irish honeys. These consisted of two blended honeys (EU & non EU) purchased from local supermarkets in Dublin, Ireland, one Romanian acacia (*Robinia pseudoacacia*) purchased from a beekeeper in Vânju Maru, Romania, two Kenyan acacia honeys (one light and one dark) purchased from local beekeepers in Lake Baringo, Kenya and three Manuka honeys (UMF 5+, MGO 250+, MGO 550+) purchased from a local health food store in Dublin. The Kenyan and Romanian honeys were included in this study to provide international context and were chosen because the countries were visited during the time of analysis and samples were collected by the authors. Once collected, all honey samples were stored in the fridge between 0° and 4° C in amber containers prior to analysis (5,22,23).

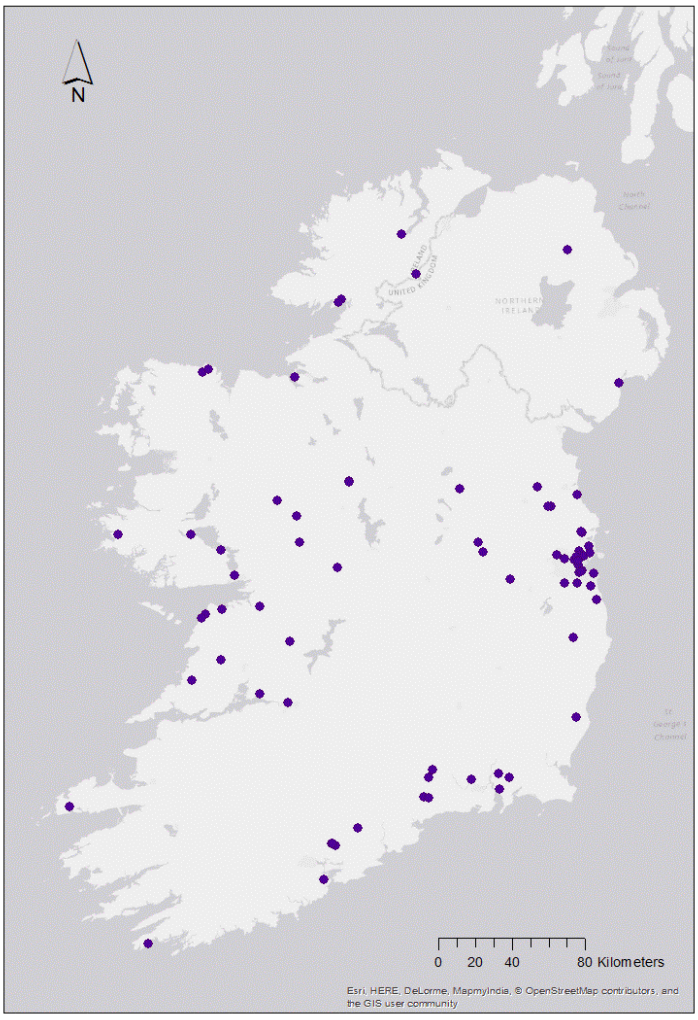


Figure 2-1 Location of honey sampling sites across Ireland.

2.2.2 Estimation of total phenolic content

All 124 multi-floral and seven single-origin honeys from Ireland, plus the eight international honeys, were analysed using a modified Folin-Ciocalteu method (24,5) to determine total phenolic content (TPC). Each honey sample was dissolved in deionised water (10% w/v) and filtered through Whatman No. 1 filter paper (pore size 11µm) (Whatman International Ltd. Maidstone, England). This solution (0.5 mL) was mixed with 2.5 mL of 0.2 N Folin-Ciocalteu reagent for 5 min, and 2 mL of 75 g/L sodium carbonate was then added. After incubation for one hour at room temperature the absorbance of the reaction mixture was measured at 760 nm against a methanol blank (Varian Cary® 50 UV-Vis Spectrophotometer, Agilent, the Netherlands). The mean of three replications was recorded and results were expressed as mg of gallic acid equivalents (GAE) per 100 g of honey. Quantification was based on a six-point calibration curve of gallic acid (5).

2.2.3 Physiochemical properties

The physicochemical parameters (electrical conductivity, moisture, pH, total sugar and colour) and TPC of 35 honey samples were determined. The 35 samples included all single origin honeys, ten rural and ten urban multi-floral honeys, chosen at random.

2.2.3.1 Electrical conductivity

Honey samples were diluted to a 20 % (w/v) solution and filtered using Whatman No. 1 filter paper (pore size 11µm) (Whatman International Ltd. Maidstone, England). The electrical conductivity of each honey solution was measured in triplicate using a YSI Professional Plus Quatro probe (YSI Inc. Kent, UK).

2.2.3.2 Moisture content

Moisture content of all honey samples was measured in triplicate using a VWR® handheld refractometer (VWR International Ltd., Ireland), and the mean refractive value for three readings was recorded.

2.2.3.3 pH

A pH meter (HI2210, Hanna Instrument, Hanna Instruments Ltd., UK) was used to measure the pH of a 10% (w/v) solution of honey prepared in deionised water. Each sample was measured in triplicate.

2.2.3.4 Total sugar content

Honey was diluted in deionised water to a 25% (w/v) solution and filtered through Whatman No. 1 filter paper (pore size 11µm) (Whatman International Ltd. Maidstone, England). The total sugar content was measured in triplicate using a VWR® handheld refractometer (VWR International Ltd., Ireland) (25).

2.2.3.5 Colour

The method reported by Naab *et al.* 2008 was used to measure the colour of honey (26). Honey samples were dissolved in deionised water (50% w/v) and filtered through Whatman No. 1 filter paper (pore size 11µm) (Whatman International Ltd. Maidstone, England). The absorbance of the honey solution was recorded at 635 nm against a methanol blank. Each sample was measured in triplicate using a Varian Cary® 50 UV-Vis Spectrophotometer (Agilent, the Netherlands). Results were expressed using the Pfund scale.

2.2.4 Microbial contamination & stability

A microbial contamination assessment was carried out on 9 multi-floral Irish honeys using previously reported methods (2,27). Honey samples were selected based on the results obtained from the analysis of pH, moisture content and TPC as these are the variables most likely to affect microbial growth. Specifically three honey samples of each variable representing low, medium and high values were selected for analysis. Honey samples (10 g) were diluted in 90 ml of peptone water. Trypticase Soy Agar media (TSA) was used for the primary screening of bacterial contamination (27). Honey samples were incubated at 37 °C for 48 hours. Aerobic mesophilic bacteria were counted using standard plate count agar (PCA) and incubation at 30 ℃ for 48 hours (2). Moulds and yeasts followed the ISO 21527-2:2008 protocol, however, Saboured Dextrose Agar containing 10 mg/L of tetracycline was used as the growing media instead of Dichloran 18% (28). Microbial counts were expressed as colony-forming units per gram of honey (cfu/g) and results were expressed per ISO 7218 (29). All microbial tests were performed in triplicate. The stability of each of the honeys was assessed by comparing the results obtained from honeys analysed immediately after removal from storage (at 4 °C) with honeys analysed after treatment (removed from cold storage and kept at 27 °C for 7 days).

2.2.5 Reagents and standards

All chemicals and reagents were analytical or HPLC grade. Water was deionised using a Mili-Q water purification system before use to a resistance of 18 MΩ cm @ 298 K. Methanol, phosphoric acid, gallic acid, Folin-Ciocalteu Phenol Reagent, amber vials and screw caps and tetracycline were purchased from Sigma Aldrich (Ireland). VWR Syringe filters with nylon membrane, pore size 0.45 μm, 25mm were purchased from VWR International Ltd, Ireland. All media were purchased from Thermo Fisher Scientific (Ireland) and sterile plates were purchased from Cruinn Diagnostics Ltd. (Ireland).

2.2.6 Statistical analysis

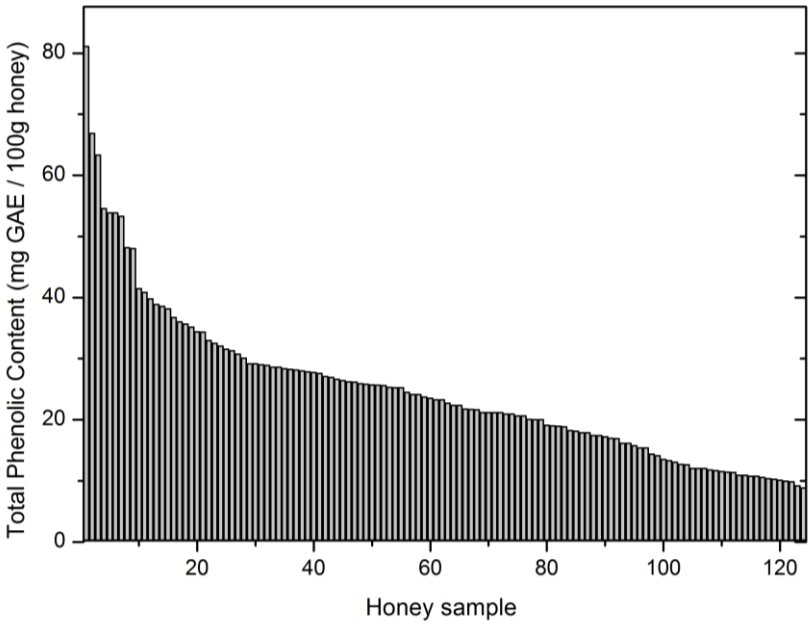
All analyses were carried out in R-3.2.5. Correlations between colour, electrical conductivity and TPC were investigated using Spearman's Rank Correlation because data were not normally distributed. Multinomial logistic regression was performed to assess the best parameter to differentiate between honey types. Strongly correlated variables were excluded from the analysis. Post-hoc Wilcoxon signed rank tests were used to determine where significant differences lay for the microbial stability assessment. Finally, correlations between moisture, pH and TPC and microbial growth were investigated using Spearman's Rank Correlation.

2.3 Results and Discussion

2.3.1 Total phenolic content

The total phenolic content (TPC) of Irish multi-floral honeys (including urban and rural honeys) ranged from 2.59 to 81.10 mg of gallic acid equivalent (GAE) / 100 g of honey, (n= 124, Mean ± SD = 23.84 ±13.07) (Fig. 2-2 (a)). This TPC range is similar to the ranges typically found for European honeys (15,22). A large proportion of the samples (over 85%) were within the range of 10 to 50 mg GAE / 100 g of honey, (n= 106, Mean ± SD = 23.66 ±8.62). The variation in TPC was most likely due to differences in geographical location and / or variation in honey harvesting time (May 2014 to October 2015). Silici *et al.* (2010) reported a range of TPC from 0.24 to 141.83 mg GAE/100 g for rhododendron honeys from the Black Sea region of Turkey, and found a difference in TPC depending on the geographical origin of the honey (30). The difference in TPC in our study could be linked to landscape context, specifically the principal land use type surrounding sampled hives. There was significantly less TPC in rural (Mean ± SD = 20.32 ± 11.54, n = 69) vs. urban honeys (Mean ± SD = 28.26 ±13.63, n=55; t= 3.25 df= 106 $p<0.01$). Given that there is higher floral resource diversity in some urban landscapes (31) it is possible that this could explain the higher TPC. Additionally, there might be plant species in the urban landscape, that contribute to a higher phenolic content in honey, which are less abundant or not present in most rural landscapes.

a)



b)

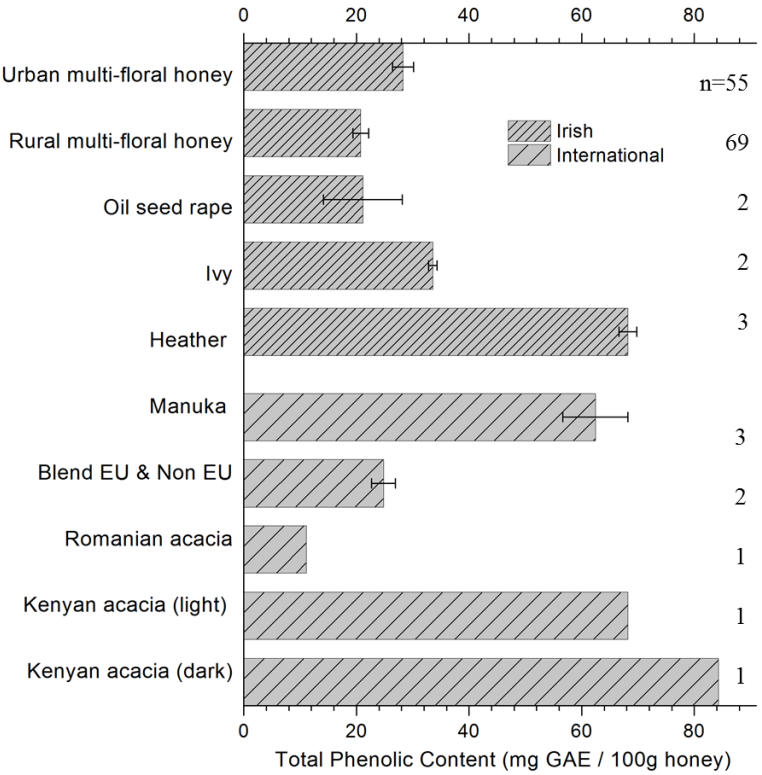


Figure 2-2 (a) Mean total phenolic content measured in mg of gallic acid equivalent per 100 g of honey for all Irish multi-floral honeys (n=124), (b) Mean total phenolic content and standard error of different Irish honey types (multi-floral urban and rural, oilseed rape (*Brassica rapa*), ivy (*Hedera spp.*) and heather *Calluna / Erica spp.*) compared with international honeys (Manuka (*Leptospermum scoparium*) blended, Romanian acacia (*Robinia pseudoacacia*), and Kenyan dark (*Vachellia drepanolobium*) and light).

A difference was observed in the TPC of honey types depending on their floral origin (Fig. 2-2 (b)). For Irish single origin honey, heather honey had the highest mean TPC (68.16 ± 2.73 mg/100 g) and OSR honey had the lowest (21.15 ± 7.02 mg/100 g). Kaškonienė *et al.* (2009) also found heather honey to have a higher mean TPC (20.12 ± 0.55 mg/100 g), compared to rape honey (32). Kús *et al.* (2014) and others, have also reported that OSR honeys have a lower TPC than heather and other honeys. The difference in values between those studies and the values determined here may be attributed to multiple factors including geographical origin, 'purity' of honey and storage conditions. The mean TPC of Irish OSR honey determined here is similar to that reported by Kús *et al.* (18.3 ± 3.61 mg/100 g). Irish ivy honey had a similar mean TPC to Irish urban multi-floral honey. This value cannot be compared with international honeys because, to our knowledge, this is the first record of TPC for ivy honey.

Of the international honeys, Kenyan dark acacia honey had the highest TPC (84.30 mg/100 g), while light acacia was determined to have a lower TPC of 68.22 mg/100 g. Manuka honey is currently the most valued honey on the market and is considered to be one of the most medicinally effective honeys due to its antibacterial activity (19). Many studies report that the antibacterial activity of honey is correlated to the total phenolic content (14–17). In our study, the TPC for Manuka honey ranged from 50.88 to 68.98 mg/100 g. This is lower than that previously reported by Alzahrani *et al.* (89.91 mg/100 g) (33) and Stephens *et al.* (90.30–270.60 mg/100 g) (34). The different values are most likely due to the different Manuka honey brands used in each of the studies. In this study the mean TPC of heather honey (68.16 ± 2.73 mg/100 g)) is higher than the mean TPC of Manuka honey (62.43 ± 10.03 mg/100 g)). The relationship between the bioactivity of honey in terms of its anti-microbial and antioxidant properties with its TPC has been comprehensively reported. Honeys antioxidant activity has been determined using a variety of recognised antioxidant assays (35–37). The ferric reducing antioxidant power (FRAP) assay is a measure of overall antioxidant capacity, while 2,2-diphenyl-1-picrylhydrazyl (DPPH) and high trolex (a Vitamin E analogue) equivalent antioxidant capacity (TEAC) assays measure capacity to scavenge radicals. All are recognised valid methods used to determine the antioxidant activity of food and beverages. Honeys with high TPC have been shown to exhibit significant antioxidant capacity using the FRAP, DPPH and TEAC assays (11,36). The anti-microbial properties of honey are attributed to honeys ability to inhibit microbial growth and honeys with high TPC have been shown to inhibit the growth of a number of

bacteria including *Staphylococus aureus*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa* and *Escherichia coli* (27) and a number of fungi including *Candida albicans*, *Candida krusei*, and *Cryptococcus neoformans* (38). Given the previously reported correlations between honeys TPC and its antioxidant activity (27,36), and between honeys TPC and its anti-microbial effects (17), this result highlights the potentially beneficial antioxidant and anti-microbial capacity of Irish heather honey.

2.3.2 Physicochemical parameters

EC was relatively low for ivy and OSR honey compared with other honeys and sugar content was variable but similar for the two honey types. Very little variation in physiochemical properties was observed within the ivy and within the OSR honey samples. The results reported by Devillers *et al.* (2004) indicate that OSR honey has low variation in moisture, pH and sugar and a larger variation with regards to EC (n=96) (39). While our OSR honey sample size (n=2) is low compared to that of Devillers *et al.*, our results concur with theirs. Ivy honey has not previously been characterised, but the lack of variation in the ivy honey is likely to be due to the lack of other floral resources for bees while ivy is in flower (usually late August to October). It is unlikely that this is the case for OSR as there are many species of plants available for bees in the Irish landscape while OSR is in flower (40). The low variation in physiochemical properties for OSR honey is likely due to the abundance of OSR in the landscape and the bees' tendency to exclusively forage on this crop while it is in flower (40). Sugar and pH values did not vary markedly within the international honeys analysed; however pH varied noticeably between Irish honeys. Honey colour varied within and between all honey types (Table 2-1). The variations in colour, EC, pH and sugar for the Manuka honey samples are likely attributed to the varying "Manuka factor" and therefore the purity. Similarly, the variation in the colour, EC, pH and sugar values for the heather honeys may also be due to the purity of the honey.

Table 2-1 Physicochemical parameters of 35 single-origin and multi-floral honey samples. EC = Electrical Conductivity.

Type	Colour (Pfund mm)	EC (µS/cm)	Moisture (%)	pH	Sugar (% Brix)
		International			
Blend	61.39	233.33	21.13	4.10	72.08
Blend	69.19	150.20	18.53	4.03	79.58
Kenya	251.60	374.90	18.30	3.92	70.42
Kenya	116.66	230.77	18.73	3.73	65.83
Manuka 5+	171.74	791.00	19.87	4.52	85.42
Manuka 250+	186.68	610.00	19.40	4.43	80.42
Manuka 500+	165.94	455.47	20.03	4.29	85.42
		Irish			
Heather	60.96	460.07	24.92	4.83	69.58
Heather	121.58	637.00	24.40	4.67	66.25
Heather	138.52	670.00	21.63	4.55	80.00
Heather	154.35	645.33	22.37	3.72	70.00
Ivy	162.82	236.63	23.70	3.91	76.25
Ivy	154.02	260.70	24.80	3.79	65.42
OSR	70.76	194.47	17.75	3.77	77.08
OSR	108.88	184.33	21.53	4.22	74.58
Rural	85.47	296.60	19.20	3.24	75.00
Rural	142.43	441.50	18.10	3.86	80.42
Rural	107.98	495.93	19.30	4.64	75.83
Rural	65.52	227.93	18.50	4.84	76.67
Rural	105.98	635.67	23.30	4.49	78.33
Rural	62.06	256.20	18.13	3.66	75.83
Rural	93.05	506.00	19.20	4.01	80.00
Rural	81.90	363.20	21.40	3.59	70.83
Rural	87.25	346.63	19.67	3.53	75.83
Rural	85.13	345.40	24.20	3.60	70.42
Urban	114.23	125.60	20.37	4.51	74.17
Urban	147.61	312.07	20.40	4.14	85.00
Urban	71.42	220.57	18.27	4.27	77.92
Urban	83.57	192.43	17.00	3.58	75.42
Urban	132.73	617.67	19.33	3.90	69.17
Urban	143.43	605.00	20.90	3.83	68.75
Urban	154.35	585.00	20.33	3.98	65.42
Urban	158.14	587.00	20.60	3.53	75.00
Urban	111.33	343.93	16.10	3.79	82.08
Urban	98.06	444.93	12.90	3.89	76.25

2.3.2.1 Electrical conductivity

A wide range in electrical conductivity (EC) was found between honey types and a high variation within some honeys was detected (Fig. 2-4). Manuka and heather honeys had the highest mean EC values (618.82 ± 21.34 and 605.27 ± 91.88 µS/cm) respectively (Fig. 2-3). OSR honey had the lowest mean EC values (189.4± 7.17 µs/cm). EC is considered a good criterion of the botanical origin of honey (41) and it is determined in routine honey control instead of the ash content (mineral content) (42). The EC measurement depends on the ash and acid content of honey; the higher ash and acid content, the higher the resulting conductivity (43). Acquarone *et al.* suggest that the high ash content of honey (and thus high EC) is related to geographical origin and is likely due to the high mineral content of the soil (41). All honey samples analysed were within the standard limit (not more than 800 µS/cm) and are therefore in compliance with the EC criteria for honey placed on the market for human consumption (44,45). Values recorded here are similar to those previously reported for multi-floral and single origin honeys (25,41).

2.3.2.2 Moisture content

Moisture content of honey samples analysed were not all within the standard limit required by the European Union's Council Directive 2001/110/EC relating to honey whereby the moisture content in general should not be more than 20%. Eight of the 20 Irish multi-floral honey samples were not in accordance with the directive (Fig. 2-3). Honey can readily absorb moisture from the air and it is possible that the high humidity levels in Ireland contributed to the high moisture levels. It has been previously reported that honeys harvested early in the season can have higher moisture values than honeys harvested later in the year (26) however, this cannot be the case here as all eight samples which exceeded the threshold were harvested late in the season. The higher moisture content is likely due to the honey being unripe (not capped by the bees) when it was extracted. Honey with high moisture is more likely to ferment which can reduce the honey's shelf life and alter its taste. There are exceptions to the EU directive for some single origin / unifloral honeys, e.g. heather honeys should not have more than 23% moisture. One of the four heather samples was above this threshold. The moisture values for Manuka honey reported here are similar to those recorded by Alzahrani *et al.* (33) (19.2%) but very different to those reported by Moniruzzaman *et al.* (11.59% ± 0.12). Manuka honeys sampled in our study have similar moisture contents to Irish heather honeys (Fig. 2-3).

2.3.2.3 pH

All honeys analysed were found to be acidic. The pH range determined was 3-4.5, similar to that of previous studies (25,42). Manuka and heather honeys had a similar pH and were the least acidic of the honeys analysed. Ivy honey had a lower pH compared to heather and Manuka honeys (Fig. 2-3). The acidic nature of honey influences its anti-microbial activity (2) and also has an important role in honey's shelf life.

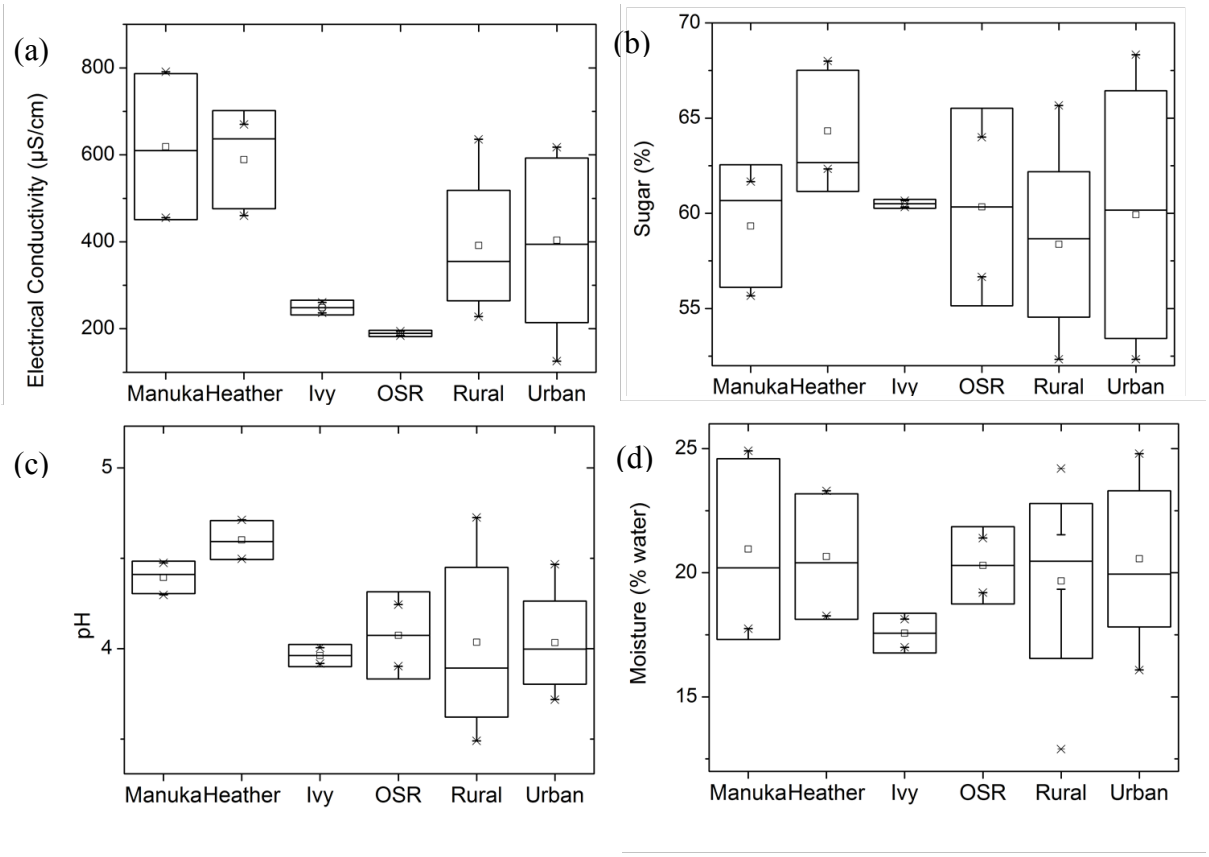


Figure 2-3 Electrical Conductivity (a), sugar, (b), pH (c) and moisture (d) for Irish honey types (heather (n=3), ivy (n=2), OSR (oil seed rape) (n=2), multi-floral rural (n=10) and multi-floral urban (n=10)) compared to Manuka honey (n=3). Box plots represent the median (horizontal line in the box), standard deviation (bottom and top box lines) and the mean (small square in the box) and the range (x).

2.3.2.4 Total sugar content

All Irish honeys were in accordance with the European Union's Council Directive 2001/110/EC relating to the sugar content of honey. Sugar levels detected in the study are also similar to those reported in previous studies (2). Overall, heather honey had a higher

amount of sugar compared to Manuka honeys (Fig. 2-3). Moniruzzaman *et al.* (2013) report a lower % sugar (60.93%) for Manuka honey compared to the result in this study (83.75 ± 2.89). Again, this difference is likely attributed to the Manuka honey factor and / or the Manuka honey manufacturing brand.

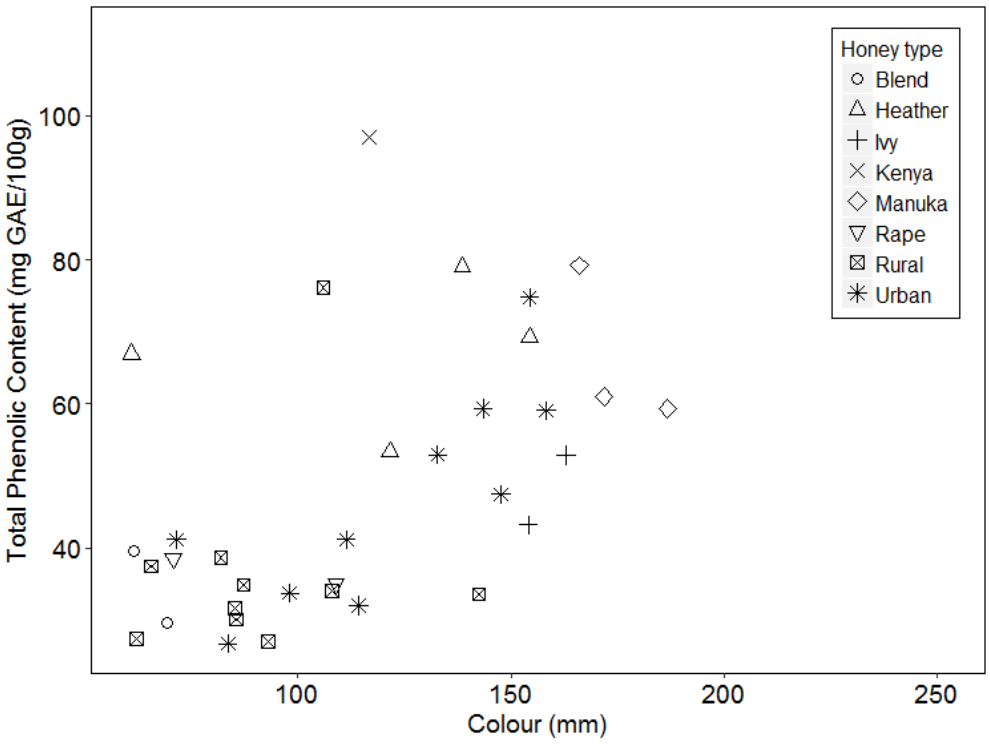
2.3.2.5 Colour

Honey colour ranged from white to dark amber according to the Pfund scale, with darker honeys scoring higher on the scale. Of the Irish honeys, ivy honey had the highest Pfund value (162.82 mm) (Table 2-1). Overall blended honeys had the lowest Pfund value and Manuka honeys had the highest. Honeys with dark colour typically have a higher TPC and thus a higher antioxidant capacity (16,46). Honey colour is related to botanical origin and composition and it is used in the classification of unifloral honeys. The colour of honey can however be affected by the management practices of the beekeeper, specifically by how frequently the wax is changed. Honey colour can also be affected by contact with metals and exposure to high temperatures and light. Therefore it was of interest to determine whether honey colour was a suitable indicator of TPC.

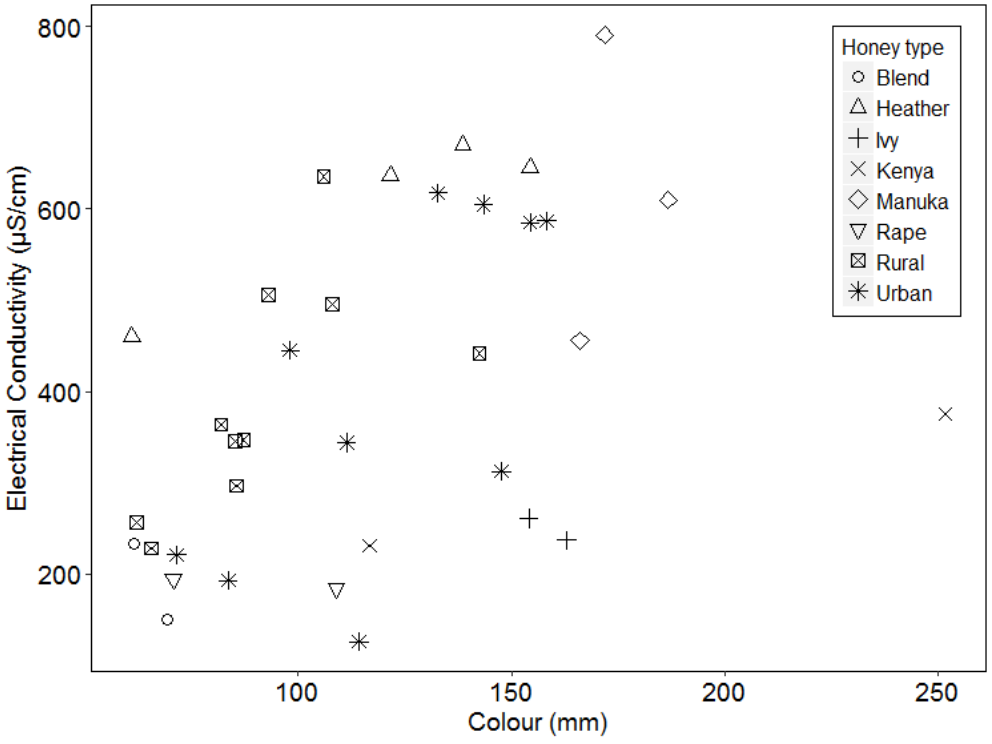
2.3.3 Relationship between TPC and physiochemical properties

For the honey samples analysed in this study a strong statistically significant positive correlation was observed between honey colour and TPC ($r = 0.6$, $n = 36$, $p<0.001$). This correlates with previous findings, e.g. Bertoncelj *et al.* (2007), Beretta *et al.* (2005), (16,46). Manuka and heather honeys were darker in colour and also had a higher TPC (Fig. 2-4 (a)). Electrical conductivity is related to the colour and flavour of honey, with a higher EC associated with a darker colour and stronger flavour (47,48). Results from this study are in agreement with other studies in the literature, and show a moderate statistically significant positive correlation between honey colour and EC ($r = 0.53$, $n = 36$, $p<0.001$) (Fig. 2-4 (b)). This study also found a moderate statistically significant positive correlation between honey EC and TPC ($r = 0.55$, $n = 36$, $p<0.001$) (Fig. 2-4 (c)). From these correlations we can conclude that for Irish honeys, the darker the honey the higher both the EC and the TPC. Given that darker coloured honeys typically have a higher TPC and thus a higher antioxidant capacity, beekeepers could use honey colour as an indication of TPC and therefore structure pricing of the honey accordingly.

a)


Honey type
Blend
Heather
Ivy
Kenya
Manuka
Rape
Rural
Urban
Total Phenolic Content (mg GAE/100g)
100
80
60
40
100
150
200
250
Colour (mm)

b)


Honey type
Blend
Heather
Ivy
Kenya
Manuka
Rape
Rural
Urban
Electrical Conductivity (µS/cm)
800
600
400
200
100
150
200
250
Colour (mm)

c)

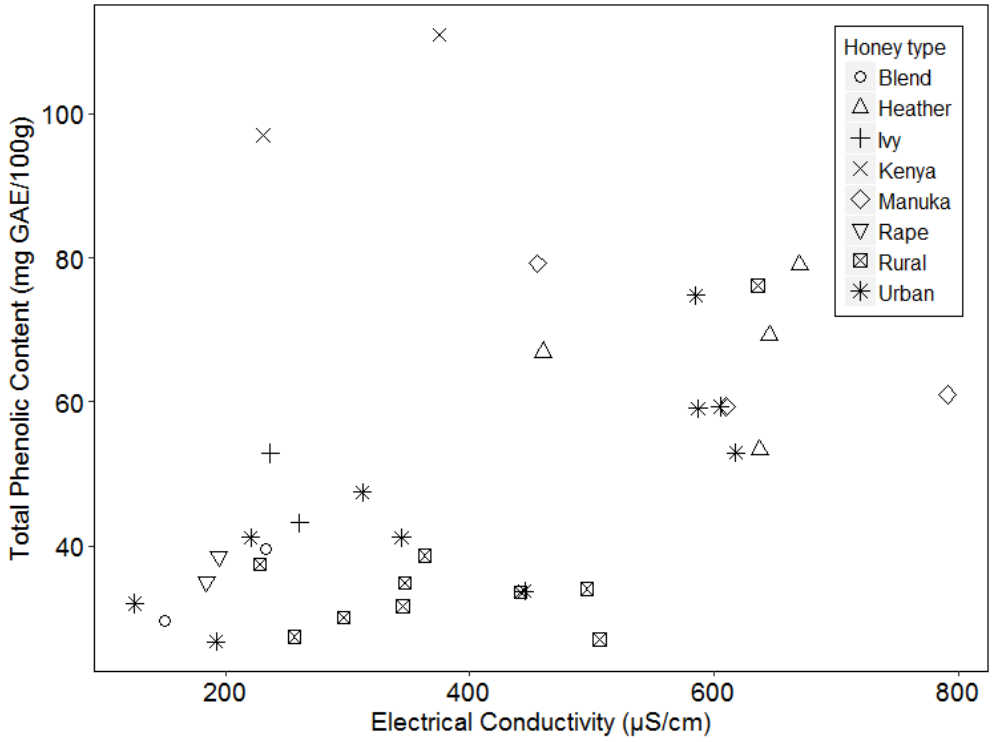


Figure 2-4 (a) Relationship between honey colour and total phenolic content (TPC); (b) Relationship between colour and electrical conductivity (EC); (c) Relationship between electrical conductivity (EC) and total phenolic content (TPC).

Interestingly, rural multi-floral honeys were shown to exhibit noticeably different physiochemical characteristics to all other samples analysed: with the exception of one rural honey sample (which turned out to be heather honey), all other rural samples had both relatively low TPC and colour (Fig. 2-4 (a)). Therefore, multinomial logistic regression was used to determine the best parameter to differentiate between rural honey and other honey types. Because colour strongly correlated with TPC, the latter was excluded from the analysis. pH was the parameter that best differentiated Irish multi-floral rural honeys from all other honey types analysed (rape, heather, ivy, Kenyan and Manuka ($p<0.001$)) except multi-floral urban honeys (Table 2-2). From this model and given that the EC is related to the acidity of the honey (25), pH measurement can be considered the most cost effective and efficient method to use to predict honey type. No parameter tested was significant with regards to differentiating multi-floral rural honeys from multi-floral urban honeys. This is likely due to the high variability of the urban honey samples.

Table 2-2 Results from multinomial logistic regression testing relationships between multi-floral rural honeys and all other honey types using the physiochemical variables, EC, moisture, pH and colour. The magnitude of the coefficient value indicates the importance of the variable at discriminating between rural honeys and all other honey types. Significant relationships are denoted with an asterisk (*p <0.001), (residual deviance: 18.69; AIC: 88.69).

	Coefficients				
Honey Type	Intercept	Colour	EC	Moisture	pH
Urban	-2.26	0.11	0.00	-0.57	0.55
Blend	240.65	-8.58*	-1.04*	45.84*	-79.79*
Heather	-821.87	1.34*	-0.08*	22.34*	46.45*
Ivy	-167.18	8.66*	-2.36*	83.98*	-521.15*
Kenya	524.29	9.12*	-2.04	39.55*	-494.95*
Manuka	-516.65	8.23*	-0.04*	-80.07*	188.75*
Rape	199.78	-0.37*	-0.53*	11.24*	-71.64*

2.3.4 Microbial contamination & stability

In all analysed honey samples, the level of aerobic mesophiles and moulds and yeasts was low (Tables 2-3 & 2-4). In samples where growth had occurred, the number of cfu/g of honey was lower than 4 CFU/g of honey. The results shown here are similar to those previously published (2,27). Honey can be subject to microbial contamination from a number of sources, including the honey bee's digestive tract, nectar, pollen, dust and the beekeeper during honey processing and storage (2,27). In relation to the number of colonies recorded, higher levels of contamination were observed for both moulds and yeasts and TAMB (total aerobic mesophilic bacteria) when honeys where subject to treatment (removed from cold storage to 27 °C for 7 days prior to analysis as opposed to remaining refrigerated at 4 °C until analysis). No statistically significant difference in the means was found for moulds and yeasts, (p=0.48); however, a statistically significant difference was observed for the TAMB (p=0.006). This suggests that storage temperature can have an effect on the microbial stability of honey. Finally no statistically significant correlation was observed between honeys, pH, moisture or TPC with the number of colonies observed for both moulds and yeasts and the TAMB either before or after treatment.

Table 2-3 Microbial analysis of Irish multi-floral honeys (moulds and yeasts).

Honey Sample	No.[a]	Count (cfu/g)[a]	No.[b]	Count (cfu/g)[b]	Moisture	pH	TPC
16	0.33	present <4 /0.1x10^{-1}	0.67	present <4 /0.1x10^{-1}	19.20	3.24	30.00
17	0.33	present <4 /0.1x10^{-1}	0.33	present <4 /0.1x10^{-1}	18.10	3.86	33.56
18	0.33	present <4 /0.1x10^{-1}	0.67	present <4 /0.1x10^{-1}	19.30	4.64	33.99
19	0.33	present <4 /0.1x10^{-1}	0.00	<1 /0.1x10^{-1}	18.50	4.84	37.44
20	0.33	present <4 /0.1x10^{-1}	0.33	present <4 /0.1x10^{-1}	23.30	4.49	76.13
29	0.00	<1 /0.1x10^{-1}	0.00	<1 /0.1x10^{-1}	17.00	3.58	26.71
23	0.00	<1 /0.1x10^{-1}	0.67	present <4 /0.1x10^{-1}	21.40	3.59	38.65
25	0.33	present <4 /0.1x10^{-1}	0.00	<1 /0.1x10^{-1}	24.20	3.60	31.58
35	0.67	present <4 /0.1x10^{-1}	0.67	present <4 /0.1x10^{-1}	12.90	3.89	33.72

Abbreviations: No. = Number of colonies in the plate; [a] = Before treatment: analysed once removed from cold storage; [b] = After treatment: analysed after 7 days of storage at 27℃.

Table 2-4 Microbial analysis of Irish multi-floral honeys (TAMB).

Honey Sample	No.[a]	Count (cfu/g)[a]	No.[b]	Count (cfu/g)[b]	Moisture	pH	TPC
16	0.33	present <4 /0.1x10^{-1}	0.67	present <4 /0.1x10^{-1}	19.20	3.24	30.00
17	0.67	present <4 /0.1x10^{-1}	1.00	present <4 /0.1x10^{-1}	18.10	3.86	33.56
18	0.67	present <4 /0.1x10^{-1}	1.67	present <4 /0.1x10^{-1}	19.30	4.64	33.99
19	1.00	present <4 /0.1x10^{-1}	1.33	present <4 /0.1x10^{-1}	18.50	4.84	37.44
20	0.00	present <4 /0.1x10^{-1}	1.33	present <4 /0.1x10^{-1}	23.30	4.49	76.13
29	2.00	present <4 /0.1x10^{-1}	2.33	present <4 /0.1x10^{-1}	17.00	3.58	26.71
23	0.00	present <4 /0.1x10^{-1}	1.00	present <4 /0.1x10^{-1}	21.40	3.59	38.65
25	1.00	present <4 /0.1x10^{-1}	2.00	present <4 /0.1x10^{-1}	24.20	3.60	31.58
35	0.00	present <4 /0.1x10^{-1}	0.33	present <4 /0.1x10^{-1}	12.90	3.89	33.72

Abbreviations: No. = Number of colonies in the plate; [a] = Before treatment: analysed once removed from cold storage; [b] = After treatment: analysed after 7 days of storage at 27℃.

2.4 Conclusion

Our findings confirm that the differences in phenolic profiles of honey can be attributed to the floral origin of the honey. Multi-floral honeys from different landscapes (i.e. containing different land uses and therefore a different composition of floral resources) and single origin honeys were demonstrated to contain different phenolic contents. Irish heather honey had the highest TPC of the three Irish single origin honeys whilst OSR honey had the lowest. This study has also confirmed that multi-floral honeys have different TPC depending on the landscape in which the hives are located, with urban multi-floral honeys containing a higher TPC than rural multi-floral honeys. It is likely that it is not only the abundance of one particular species but the diversity of the floral resources that affects the TPC of honey. In addition to identifying the types of honey with high TPC and thus antioxidant activity, our findings could also promote beekeeping in Ireland by influencing the commercial value of these honeys.

This study is the first to show that Irish heather honey has similar physiochemical characteristics to Manuka honey and has a higher mean TPC. Given the high demand for, and retail value of, Manuka honey due to its attributed health benefits, the potential health benefits of Irish heather honey should be investigated further.

Multinomial logistic regression confirmed that the physiochemical properties used here (EC, moisture, pH and colour) can be used to distinguish between floral honey types but not between multi-floral honeys from different landscapes (i.e. rural and urban honeys) and pH is the factor that is most beneficial in differentiating between honey types. Therefore pH measurement could be a useful tool for beekeepers to use to characterise their honey and thus set appropriate market prices.

To our knowledge this is the first time that data for ivy honey has been reported. The properties of ivy honey were distinctive in that it was the darkest Irish honey measured but had a relatively low EC. Although it was illustrated that honey colour correlates with EC and with TPC, ivy honey was an exception to this. While ivy was the darkest Irish honey analysed, its TPC was less than that of heather and Manuka honey. The pH and sugar content were similar to that of other honeys analysed. The average moisture content for multi-floral honeys was above the value required by EU legislation. If the ivy honey market continues to expand, we recommend that EU legislation should change to include ivy honey as a single origin honey.

Characterising honey based on floral origin is important for beekeepers and for the commercial honey market. The results of this study have shown that EC, pH, colour and TPC can be used to characterise Irish honeys of different floral origin and differentiate between honey types. Finally this research has shown that heather honey has the highest TPC of all Irish unifloral honeys and this is similar to high TPC honeys internationally, indicating that the antioxidant capacity of heather honey may be similar to that of Manuka honey.

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Chapter 3 Agricultural intensification and urbanisation affect phenol composition of *Apis mellifera* honey

To be submitted for publication "Agricultural intensification and urbanisation affect phenol composition of *Apis mellifera* honey". Target Journal: Proceedings of the Royal Society B.

Abstract

The phenol composition of honey influences how beneficial it may be to human and bee health, but varies according to its botanical origin. Variation in land-use and thus vegetation composition is likely to influence honey properties, but this has not previously been examined. We sampled multi-floral honey from 76 *Apis mellifera* hives from urban, rural and intermediate locations across Ireland, identified and quantified phenolic content using HPLC-UV, and classified surrounding land-use, up to 5km around hive sites. We evaluated the relationship between the phenol composition of honey and a) hive location (urban, rural or intermediate), b) land cover composition surrounding the hive and c) harvest season (when samples were taken). More phenols were present, and their concentration was greater, in honeys from urban centres, and the amount of green urban areas had an overall positive relationship with phenol presence and concentration. In addition, fewer phenols were present and their concentrations were lower in honeys from rural areas, where the amount of pastures had an overall negative relationship with phenol presence and concentration. Some phenols were completely absent in honey from hives located in landscapes with high proportions of pasture. It is tempting to conclude that urban honeys are better than rural honeys in terms of the number and concentration of phenols detected and quantified. But urban environments are known to contain more pollutants than some rural areas. Temporal changes in phenolic composition of honey were also observed: some phenols were absent from honeys harvested early or late in the season but the TPC was higher in honey harvested late in the season. Our results suggest that anthropogenic land-use and harvest season are the main indirect drivers of the phenol composition of honey.

3.1 Introduction

The quantity and chemical composition of the honey produced by bees is directly influenced by the nectar and pollen collected from flowers. Since the availability and composition of flowers in the landscape is influenced by land-use (1), and because bees forage within fixed distances of their colony (2), it is likely that anthropogenic changes in land-use will influence honey production and the quality of the honey for bee, and human, consumption. Agricultural land has previously been linked with negative impacts on the nutritional value of bee bread and protein availability to *Apis mellifera* (honeybees) (3). Donkersley *et al.* (2014) also found that semi-natural habitats (SNH), such as broad-leaved forests and natural grasslands, positively correlate with bee bread protein. However, the extent to which honey composition and quality varies with landscape context has not yet been investigated.

Agricultural intensification has led to a loss and fragmentation of SNH worldwide, increased monoculture cropping and changes in management practices, such as increased fertilizer and pesticide use (4). This has led to a loss of floral resources for bees (5), as well as a decrease in diversity and the homogenisation of those resources that are available (6). Different agricultural habitats provide varying floral resources for bees and previous studies have reported differences in plant species richness depending on crop type (7) and management practices (8). For example, mass-flowering crops such as oilseed rape provide a high abundance of floral resource, if only for the short period during flowering (9,10). In pasture systems, organic dairy fields have been shown to have a higher diversity of insect pollinated plants, and a greater abundance of floral resources, compared to more intensively managed conventional fields (8). In general, less intensively managed farmland is more florally diverse than more intensively managed areas (11,12). Although an increase in artificial surfaces (through urbanisation) would be expected to lead to a reduction of floral resource availability for bees, many urban habitat types have actually been shown to benefit bees (13). Baldock *et al.* (2015) found higher bee species richness in urban landscapes compared to farmland, driven by higher urban plant species richness and depauperate floral resources in rural landscapes, especially those areas with high-intensity agriculture (14). Conversely Bates *et al.* (2011) found higher species richness and a greater abundance of bees in rural sites compared to urban sites (15). These differences suggest that urban areas can in some instances benefit bees, but the benefits are likely affected by local habitat quality.

Modifications in land-use can affect the botanical origin and quantity of nectar collected by honey bees and thus the honey produced. Floral resource abundance and diversity are important for honey bees in terms of bee health (16), as well as honey production. Some of the most important components in honey from a human health point of view are phenols (17). More than 150 different phenols have been detected in honey from many countries around the world (18–21). Phenols are secondary metabolites of plants and are generally involved in defence against ultraviolet radiation, predators or pathogens (22), but can also positively affect bee health (23). Many plants contain hundreds of phenols and each species has a distinctive profile (24). Many phenols have potential antioxidant (25,26), antimicrobial (27), antidiabetic (28) and anti-inflammatory (29) properties and have the potential to be beneficial in preventing disease and protecting the stability of the human genome (30). The efficacy of a number of phenolic compounds is currently being investigated at various phases in clinical trials, including, caffeic acid (phase 4), ellagic acid (phase 1 & 2), kaempferol (phase 2), luteolin (phase 1), quercetin (phase 1, 2 & 3) and rutin (phase 1) (31). Phenols are present in the nectar and pollen collected by bees, and thus are incorporated into honey, but they can also be found in honey as a result of contact with propolis (bee glue made from tree sap, thus containing botanical exudates) in the hive (32). Phenolic acids and flavonoids are two classes of phenols that have been comprehensively researched in honey (18,21,33–36) and both compound classes are used to assess honey quality and authenticity for commercial purposes. The extent of these constituents are known to vary depending on the floral and geographical origin of the honey (19). In addition, processing, handling, and storage of the honey can influence the composition of these phenols (37).

A preliminary assessment of the total phenolic content (TPC) of Irish honey revealed that honey from urban hives had a higher TPC compared to honey from rural ones, and suggested that variation in TPC is linked to landscape context (38). Since honey bees can travel relatively long distances from their hives to forage (up to 15 km (2)), phenolic composition of honey is not only influenced by local floral availability, but also by the landscape composition and floral resources up to several kilometres around a hive site. Thus even if some urban habitats offer more floral resources compared to rural ones, small scale local effects and large scale landscape effects (the composition and configuration of landscape surrounding bee hives) will affect the composition of the nectar and pollen collected by the bees and subsequently the honey produced. To our knowledge no study

has reported on the possible link between surrounding landscape composition and honey quality.

Honey composition will also vary throughout the season as floral resources change due to flowering phenology. Bees respond by varying the distance travelled to reach floral resources (2). Beekeepers sometimes respond to this by moving hives to desired locations and extracting honey at particular times following blooming of particular species. In less productive regions, such as the cooler parts of the Atlantic region of north-west Europe, beekeepers harvest honey throughout the honey harvest season when possible, but the majority of Irish beekeepers will only harvest once a year at the end of the season. This also has the potential to influence phenolic composition of honey, as phenols can accumulate with time.

We investigated the relationship between phenol composition and land-use to determine the relationship between landscape composition and the presence and concentration of phenols that have potential human and bee health benefits. Specifically, we tested the following hypotheses: Phenolic composition (presence/absence of phenolic compounds and their concentrations) of honey varies according to:

i) Hive location (degree of urbanisation, classified as urban, intermediate or rural)
ii) Landscape composition up to 5km surrounding hive site
iii) Harvest season (when samples were taken)

3.2 Materials and Methods

3.2.1 Honey sampling

One hundred and three multi-floral Irish honey samples from 76 hive sites across the island of Ireland were collected directly from beekeepers between 2013 and 2015 (Appendix B3, Fig. B3-1). Once collected, all honey samples were stored in amber containers in the fridge between 0° and 4° C. The sites were categorised by the degree of urbanisation: urban centres (n=56), intermediate (towns and suburbs) (n=9) or rural areas (n=38), according to the European Commission's most recent working paper on the degree of urbanisation (39). The harvest date of each honey sample was recorded and a harvest season was assigned to all samples (except one, for which no harvest date was available and this sample was excluded from the analysis): early (May harvest n=18), mid (June-July harvest, n=26), late (August-September harvest, n=50) and later (October harvest, n=8).

3.2.2 Phenol analysis

Phenol identification and quantification was determined by high-performance liquid chromatography (HPLC), as described previously (40). Estimation of TPC was achieved using a modified Folin-Ciocalteu method (41,42). Further details are given in the Appendices, Section B1 and B2.

3.2.3 Landscape analysis

Landscape composition was quantified to a 5 km radius around each of the 76 hive sites using the CORINE (Co-ordinated Information of the Environment) 2012 land cover classification system. The 2012 data was selected for use because it was the dataset that was closest to the honey harvest dates. Land-use was classified to Level 3 of the hierarchal classes (1-3) (Appendix B3, Table B3-1).

3.2.4 Statistical analysis

For correlations of phenol concentration and phenol presence / absence with land cover, data were analysed by hive site (n=76). For some sites, honey samples were available for two or more different time points and so the mean concentration of these samples was used in these analyses. For the seasonal analysis of honey samples, 102 honey samples were used, (no harvest date was available for one honey sample and so it was excluded from this analysis). From the limits of detection (LOD) and limits of quantification (LOQ) calculations, not all phenols that were identified were quantifiable and thus separate analyses were carried out for (a) phenol presence/absence and (b) phenol concentration. The relationships between 17 phenols and 26 land covers were assessed.

Direct logistic regression was performed to assess the relationship between land cover and the likelihood that a phenol would be present in honey samples. Correlations between land cover and phenol concentration were investigated using Spearman's Rank Order Correlation because data were not normally distributed. A Bonferroni correction for multiple comparisons was applied to p-values when evaluating the relationship between phenol concentration and land cover. Kruskal Wallis analyses were used to assess the differences between phenol concentration according to harvest season and, separately, level of urbanisation. Any phenol that was quantified in fewer than five samples was removed prior to statistical analysis. This included chrysin (n=3) ferulic acid (n=4), galangin (n=3), homogentisic acid (n=2) and salicylic acid (n=4). Post-hoc Wilcoxon

signed rank tests were used to determine where significant differences lay. All analyses were carried out in R-3.2.5.

3.3 Results

3.3.1 Phenol identification and quantification

Seventeen different compounds were identified and quantified in Irish honey samples (Appendix B3, Table B3-2). Some peaks from the chromatograms were not identified / quantified due to the lack of reference standards. P-coumaric acid was the most frequent phenol and was detected in 100 samples, followed by rutin which was detected in 92 (Appendix B3, Table B3-2). Rutin had the highest concentration overall (96.64 mg/L) followed by benzoic acid (72.42 mg/L). HBA was detected in 84 honey samples and quantified in 43. Myricetin was detected in 68 honey samples and quantified in 36. Quercetin was detected in 41 Irish honey samples with a concentration range of 1.06 – 7.86 mg/L (n=13). Ellagic acid was detected in 44 honey samples with a concentration range of 3.73 – 18.95 mg/L (n=11). Pinocembrin, a rare phenol, said to originate from propolis (43), was detected in 53 samples but only quantified in five honey samples. Luteolin was detected in one third of the honey samples. Trans-cinnamic, salicylic, o-coumaric and chlorogenic acid were detected in less than a third of the honey samples. Homogentisic acid and galangin were the rarest phenols and were detected in two samples and three samples respectively. Ferulic acid and chrysin could not be quantified in 97% of the samples but were detected in 4 and 8 honey samples respectively. The LOD and LOQ varied for each phenol. Chrysin had the lowest LOD and LOQ, 0.08 mg/L and 0.24 mg/L respectively (Appendix B3, Table B3-3). Method validation parameters are shown in Table B3-3. Good inter day precision was achieved. At all levels, inter day precision was lower than 5% except for galangin (5.54%). An example of a chromatogram representing a standard mix (Fig. 3-1) and a chromatogram of a honey sample overlaid (Fig. 3-2) as well as chromatograms from four different honey samples (Fig. 3-3) are shown below.

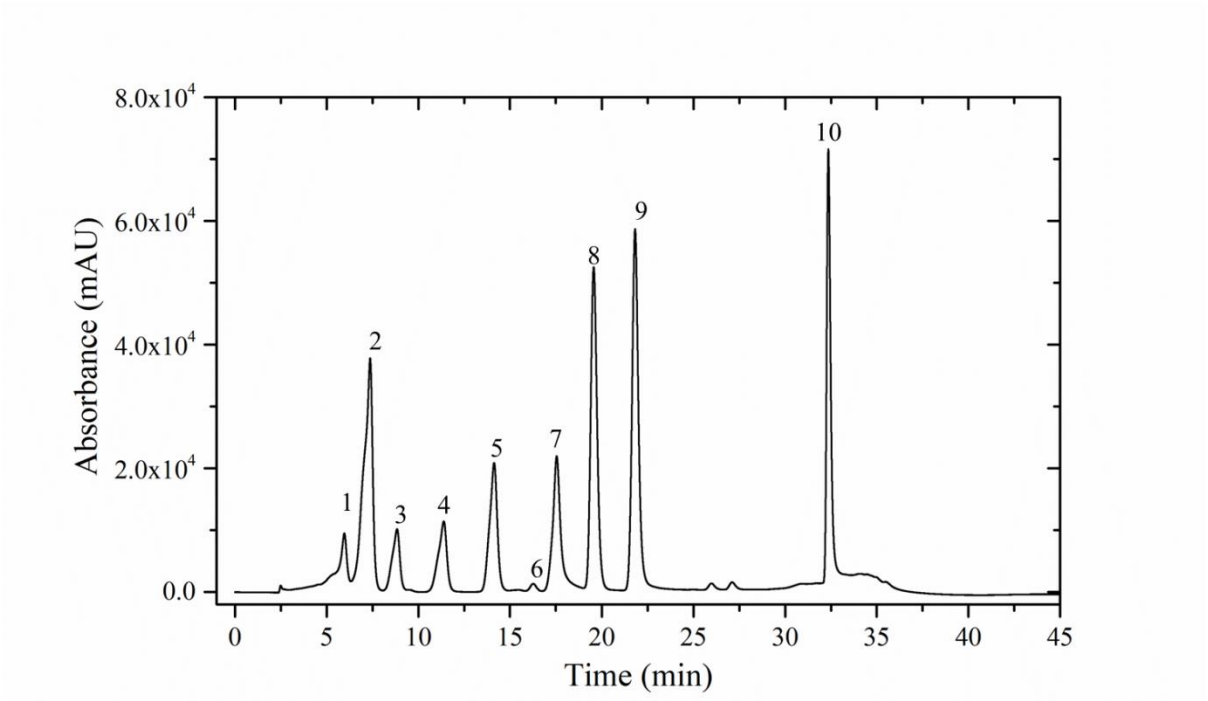

Figure 3-1 Chromatogram of phenol standard mix (PS101), (separation at 265 nm). Peak 1 = chlorogenic acid, 2 = syringic acid 3 = vanillin, 4 = ferulic acid and 5 = o-coumaric acid, 6 = salicylic acid, 7 = myricetin, 8 = trans-cinnamic acid, 9 = quercetin and 10 = chrysin.

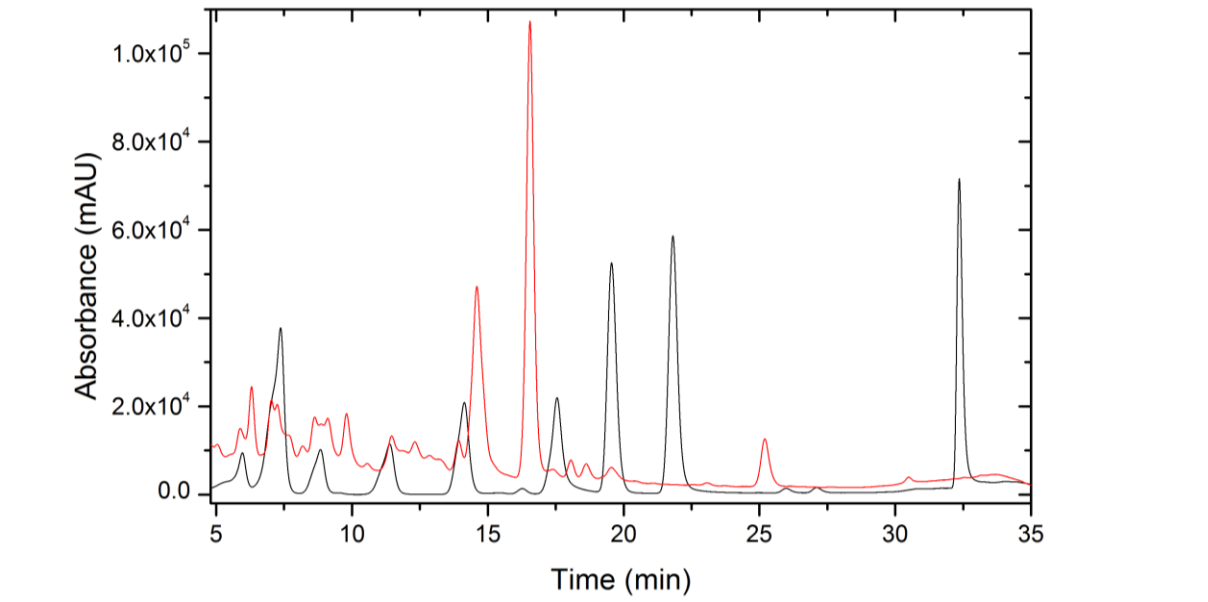

Figure 3-2 Chromatogram of phenol standard mix in black (Fig 3-1) and honey sample (HS94) (Fig. 3-3c) overlaid in red (separation at 265 nm). Identified peaks are listed below in Fig. 3-3.

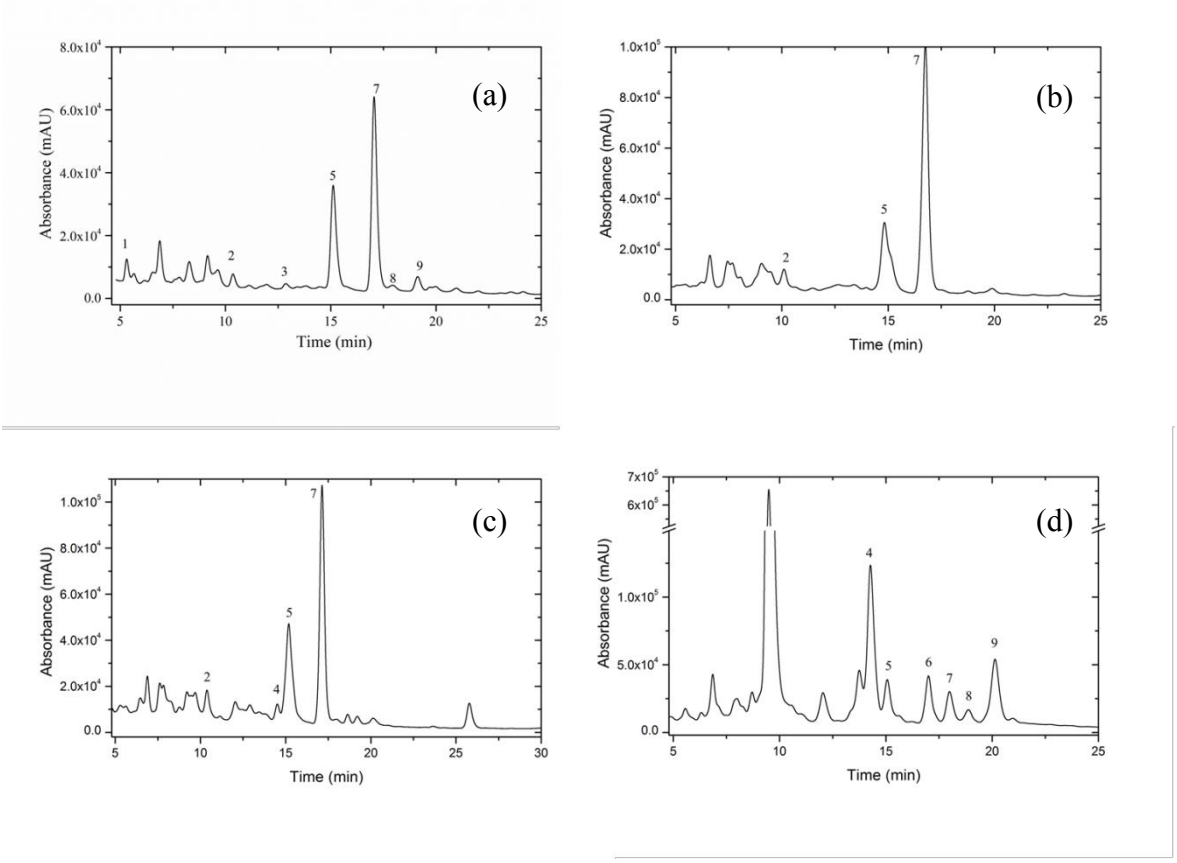

Figure 3-3 Chromatogram of four different honey samples (a) – (d) (HS70, 101, 194, 156) Peak 1 = HBA, 2 = p-coumaric acid, 3 = benzoic acid, 4 = o-courmaric acid, 5 = rutin, 6 = salicylic acid, 7 = ellagic acid, 8 = myricetin, 9 = trans-cinnamic acid.

3.3.2 Urbanisation

3.3.2.1 Phenol presence / absence

The presence or absence of phenols varied across the three levels of urbanisation (Fig. 3-4). Overall, the highest percentage of samples containing phenols were those harvested late in the season for urban centres, and later in the season for rural honeys. Honeys from the intermediate degree of urbanisation category had a greater number of phenols when honey was harvested early in the season. With respect to presence/absence of an individual phenol, a higher percentage of four phenols (HBA, luteolin, o-coumaric acid and salicylic acid) were detected in samples from rural areas compared to honeys from intermediate or urban centres (Fig. 3-4). A higher percentage of five phenols were detected in honeys from intermediate areas and a higher percentage of seven phenols were detected in honeys from

urban areas. Chlorogenic acid and ferulic acid were only detected in honeys from urban centres (Fig. 3-4).

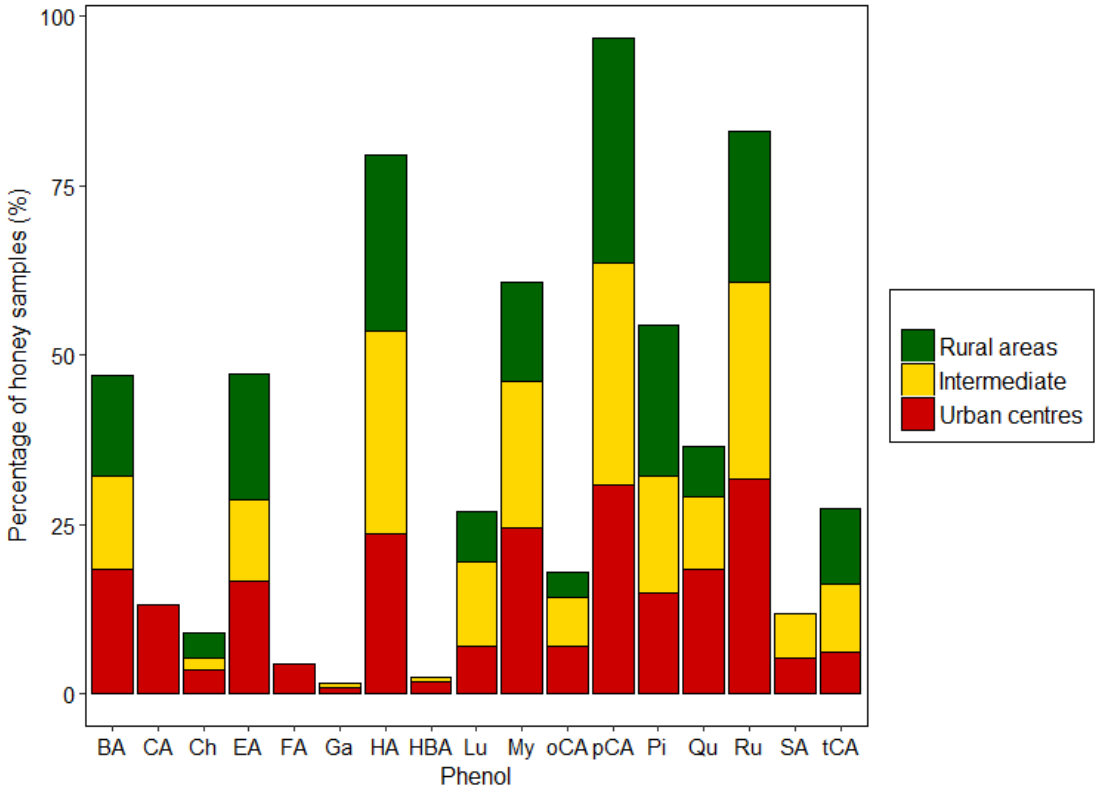

Figure 3-4 The percentage of samples in which each phenol was detected according to the degree of urbanisation, green = rural areas (n=56), yellow = intermediate (n=9) and red = urban centres (n=38). BA = benzoic acid, CA = chlorogenic acid, Ch = chrysin, EA = ellagic acid, Ga = galangin, HA = homogentisic acid, HBA = 2,4 hydroxybenzoic acid, Lu = luteolin, MY = myricetin, oCA = o-coumaric acid, pCA = p-coumaric acid, Pi = pinocembrin, Qu = quercetin, Ru = rutin, SA = salicylic acid and tCA = trans-cinnamic acid.

3.3.2.2 Phenol concentration

High phenol concentration was detected in honeys from both rural areas and urban centres (Appendix B3, Table B3-4). The concentration of most (13/17) phenols did not vary across the three levels of urbanisation, but significant differences in the concentrations of benzoic

acid, HBA, myricetin as well as TPC were found (Appendix B3, Table B3-7). Posthoc Mann Whitney tests showed significantly more HBA in honeys from intermediate degree of urbanisation compared to honey from urban centres (Appendix B3, Table B3-8). Higher median concentrations of benzoic acid, HBA, myricetin and TPC were found in honeys from urban centres compared to rural areas (Fig. 3-5; Appendix B3, Fig. B3-4).

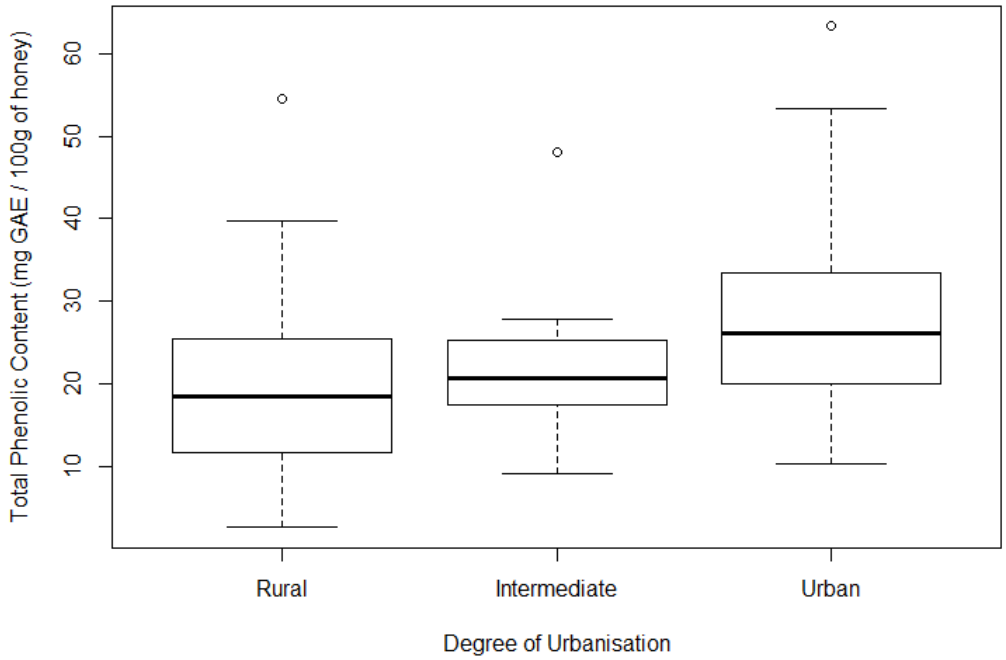

Figure 3-5 Total phenolic content of honey across the three levels of urbanisation, rural (n=56), intermediate (n=9) and Urban (n=38). Box plots represent the median (horizontal line in the box), 1st and 3rd quartiles (bottom and top box lines), data range (the upper and lower whiskers) and the outliers (dots representing any data points >1.5x outside the interquartile range).

3.3.3 Land cover

Agricultural land was the main land cover class surrounding the 76 hive sites. The most predominant land cover was pasture which made up 41% of the total land cover. Discontinuous urban fabric was the second largest at 14% of total land cover, followed by arable land which comprised 7% of the total land cover. There was a positive correlation

between green urban areas (GUA) and continuous and discontinuous urban fabric. All hive sites that contained GUA also contained discontinuous urban fabric. The percentages of pasture and GUA in landscapes surrounding hive sites were negatively correlated. The amount of pasture and the amount of GUA had the greatest effect on the phenol composition of honey.

3.3.3.1 Agricultural pastures

High concentrations of galangin, luteolin, rutin, salicylic acid and trans-cinnamic acid came from sites dominated by agricultural areas, specifically pastures. A moderate, but statistically significant, negative correlation was observed between TPC and the area of pasture and between pasture and the area of pasture, in the surrounding landscape, even when the Bonferroni correction was applied (Fig. 3-6, Table 3-1). There was a negative statistically significant relationship between pasture and 4 phenols (trans-cinnamic acid, HBA, quercetin and myricetin).

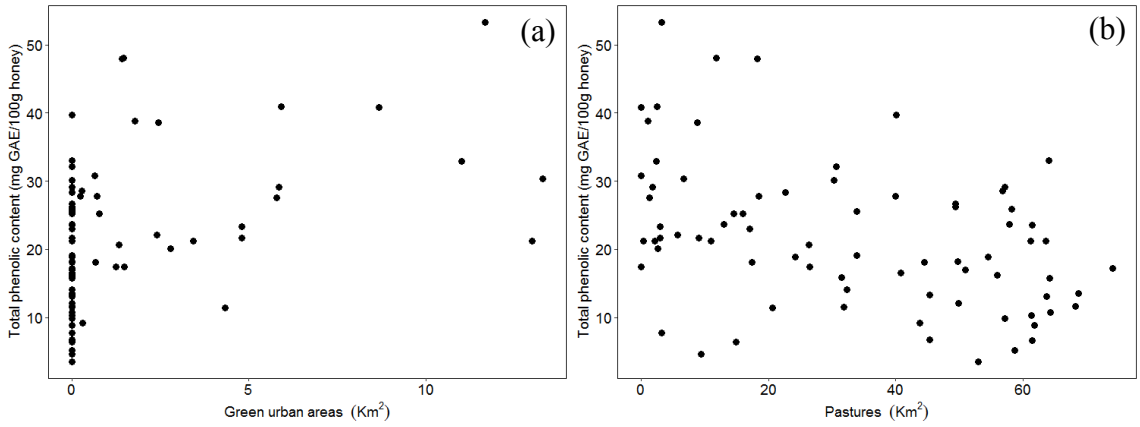

Figure 3-6 Relationship between TPC of honey and (a) area of green urban areas and (b) pastures surrounding the hive (n=76).

Table 3-1 Spearman's correlation coefficient (rho) of each phenol concentration that showed a statistically significant relationship with land cover (n=76).

Land cover	Phenol	rho	p	Bonf
Pasture	HBA	-0.317	0.005	0.125
	Quercetin	-0.263	0.022	0.55
	trans-cinnamic acid	-0.306	0.007	0.175
	Myricetin	-0.391	0.000	**0.000**
	TPC	-0.427	0.000	**0.000**
Green urban areas	Benzoic acid	0.320	0.005	0.125
	HBA	0.260	0.023	0.575
	Myricetin	0.409	0.000	**0.000**
	Quercetin	0.242	0.035	0.875
	TPC	0.467	0.000	**0.000**
Continuous urban fabric	Myricetin	0.428	0.000	**0.000**
	TPC	0.406	0.000	**0.000**
Discontinuous urban fabric	Benzoic acid	0.340	0.003	0.075
	Myricetin	0.437	0.000	**0.000**
	TPC	0.398	0.001	**0.025**
Road and rail networks and associated land	Ferulic acid	0.254	0.027	0.675
	TPC	0.364	0.001	**0.025**
Sport and leisure facilities	Benzoic acid	-0.328	0.004	0.1
	Chlorogenic acid	-0.227	0.048	1
	Myricetin	0.252	0.028	0.7
	TPC	0.400	0.000	0.125
Non-irrigated arable land	HBA	-0.354	0.002	0.55
	Quercetin	-0.303	0.008	0.175
Inland marshes	Benzoic acid	-0.262	0.022	**0.000**
Coniferous forests	Myricetin	-0.246	0.033	**0.000**

Abbreviations: Bonf: Bonferroni correction (significant values in bold).

3.3.3.2 Urban land-use

GUA increased the likelihood of three phenols being present in honey; specifically chlorogenic acid, ferulic acid and quercetin (Appendix B3, Table B3-9). Chlorogenic acid was detected in 15 honey samples from sites with predominantly artificial surfaces within a 1 km distance from each other in north Dublin. The odds ratio associated with chlorogenic acid was 1.54, indicating for every 1 km^2 increase in GUA surrounding a hive site, honey samples were 1.54 times more likely to contain chlorogenic acid ($p<0.0005$; Cox and Snell R squared = 22%, Nagelkerke R squared = 47%, AIC=32.36). For every 1 km^2 increase in GUA surrounding a hive site honey samples were 1.31 times more likely to contain ferulic acid and 1.48 times more likely to contain quercetin (Table 3-2).

Table 3-2 Statistically significant results from the logistic regression analysis for the presence / absence of each phenol according to land cover. Odds ratios are reported along with, Cox and Snell R squared (%), Nagelkerke R squared (%), p values (p) and Akaike information criterion (AIC). Degrees of freedom = 1.

Land cover	Analytes	Odds ratio	Cox and Snell R squared (%)	Nagelkerke R squared (%)	p	AIC
Pasture	Chlorogenic acid	0.81	22	48	0.04	31.634
	Ellagic acid	0.98	6	8	0.04	104.650
	Quercetin	0.98	6	8	0.04	100.700
Green urban areas	Chlorogenic acid	1.54	22	47	0.00	32.360
	Ferulic acid	1.31	8	22	0.01	29.400
	Quercetin	1.48	17	23	0.01	90.76
Continuous urban fabric	Chlorogenic acid	1.43	19	42	0.00	34.58
	Ferulic acid	1.35	10	29	0.01	27.54
	Quercetin	1.26	11	15	0.01	96.12
Discontinuous urban fabric	Chlorogenic acid	1.11	21	46	0.00	32.82
	Ellagic acid	1.04	8	11	0.02	102.89
	Ferulic acid	1.08	9	28	0.02	27.86
	HBA	0.95	12	21	0.00	57.35
	Quercetin	1.04	10	14	0.01	97.07
Road and rail networks and associated land	Chlorogenic acid	3.58	7	15	0.02	45.36
	Ellagic acid	3.44	12	16	0.01	99.91
	Ferulic acid	4.29	6	16	0.04	31.01
Sport and leisure facilities	Ellagic acid	1.82	9	13	0.02	101.82
	Quercetin	1.63	7	10	0.03	99.20

The highest concentrations of chlorogenic acid, ferulic acid, HBA and pinocembrin came from sites dominated by urban fabric, specifically discontinuous urban fabric. Conversely, a positive relationship was observed between GUA and four phenols (benzoic acid, HBA, myricetin and quercetin). Both continuous and discontinuous urban fabrics had a positive relationship with the concentration of myricetin in honey. Sports and leisure land-use had a positive relationship with one phenol (myricetin), and a negative relationship with two phenols (benzoic acid and chlorogenic acid). A statistically significant moderate positive correlation was observed between TPC and the amount of GUA (Fig. 3-6, Table 3-2).

3.3.3.3 Other land covers

The highest concentration of benzoic acid was quantified in honey originating from a site with a high area of land containing peat bogs surrounding the hive. Inland marshes and coniferous forests had a negative relationship with benzoic acid and myricetin respectively. The relationship was still significant after the Bonferroni correction was applied (Table 3-1). The highest odds for the likelihood of a phenol being present were produced with road and rail networks and associated land. Chlorogenic, ferulic and ellagic acids were most likely to be present in honey produced in hives with high surrounding areas of road and rail networks and associated land. However, the model as a whole explained less than 20% of the variance (the more variability explained, the better the model) (Table 3-2). The small sample size of ferulic acid is a limitation in this study but that is not the case for chlorogenic acid and ellagic acid (detected in 15 and 44 samples respectively). Hive sites with high areas of forest and semi natural habitat tended to contain honey samples with high concentrations of ellagic acid, myricetin, o-coumaric acid, p-coumaric acid and rutin (Table B3-4).

3.3.4 Influence of harvest season

3.3.4.1 Phenol presence / absence

Some phenols were completely absent from honeys harvested at different times of the year, however all phenols were detected in honeys harvested between August and September (late harvest) (Fig. 3-7).

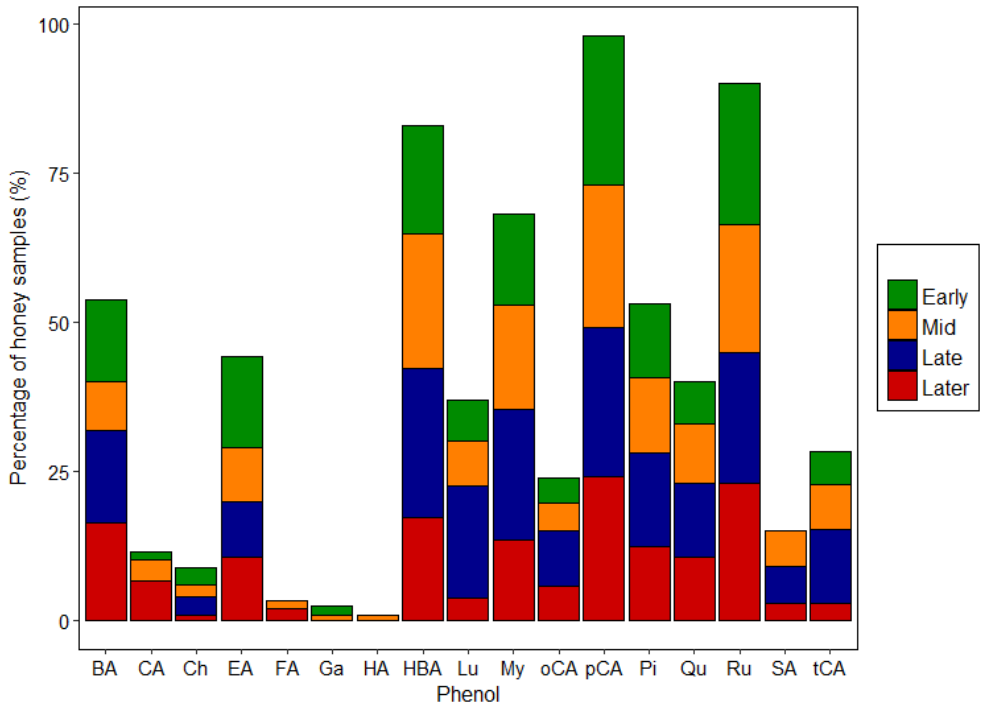

Figure 3-7 The percentage of honey samples in which each phenol was detected according to harvest season: green = early (May, n=18), orange = mid (June-July, n=26), blue = late (August-September, n= 50) and red = later (October, n=8). BA = benzoic acid, CA = chlorogenic acid, Ch = chrysin, EA = ellagic acid, Ga = galangin, HA = homogentisic acid, HBA = 2,4 hydroxybenzoic acid, Lu = luteolin, MY = myricetin, oCA = o-coumaric acid, pCA = p-coumaric acid, Pi = pinocembrin, Qu = quercetin, Ru = rutin, SA = salicylic acid and tCA = trans-cinnamic acid.

Ferulic and salicylic acids were completely absent from honeys harvested early in the season (Fig. 3-7). Homogentisic acid and galangin were completely absent from honeys harvested mid-season (June). Chlorogenic acid, ferulic acid, homogentisic acid and galangin were completely absent from honeys harvested later in the season (October).

3.3.4.2 Phenol concentration

High phenol concentration was detected in honeys harvested throughout the four seasons (Early – Later, Table B3-4). The median concentrations of six of the 17 phenols were statistically significantly different across the four harvest seasons: benzoic acid, ellagic acid, HBA, luteolin, o-coumaric acid and quercetin (Appendix B3, Table B3-5). There was

significantly less benzoic acid in honeys harvested mid-season compared to honeys harvested late in the season (Appendix B3, Fig. B3-2 and Table B3-6), and more HBA and rutin in honey harvested early in the season (May) compared to honey harvested late in the season (August-September). The TPC also varied according to honey harvest season (Table B3-5): there was less TPC in honey harvested early in the season compared to honey harvested late in the season (Fig. 3-8, Table B3-6). The late harvested honey (August-September) had the highest median and the widest range of TPC overall.

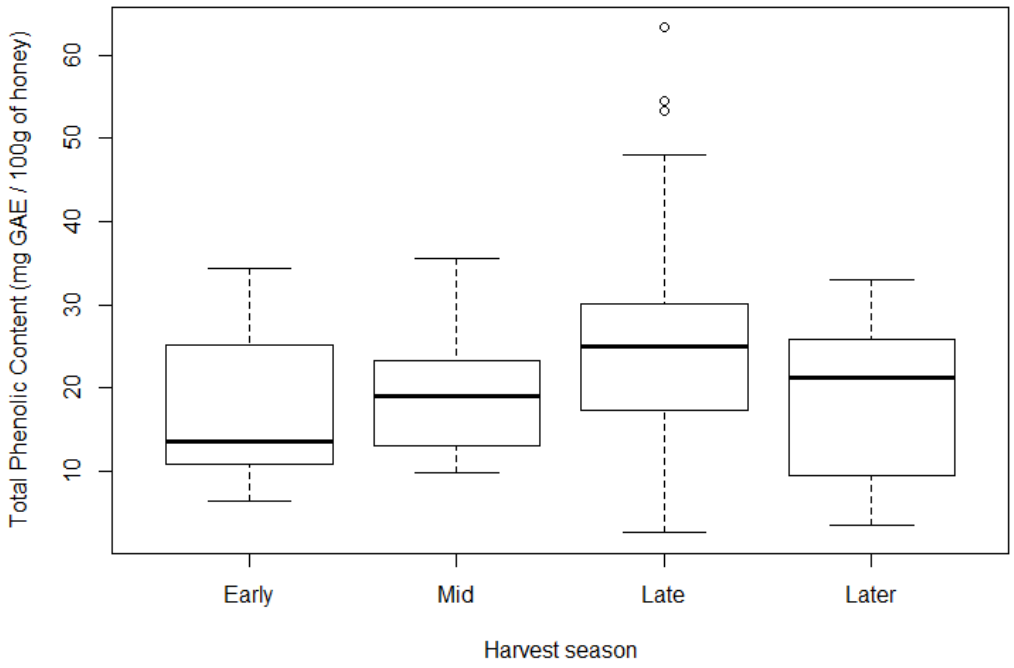

Figure 3-8 Honey TPC across four harvest seasons, early (May, n=18), mid (June-July, n=26), late (August-September, n= 50) and later (October, n=8).

3.4 Discussion

3.4.1 Phenol identification and quantification

A number of phenolic compounds that have been identified in Irish honeys are associated with promoting human and bee health. Those detected most frequently in below.

p-Coumaric acid and rutin: a number of studies have detected p-coumaric acid and rutin in Polish honeys (40,44,45), and these phenols were the most frequent phenols detected in Irish honey, detected in 90% of honeys analysed. p-Coumaric acid is a common constituent of honeys from various floral origins including chestnut (*Castanea sativa*), rosemary (*Rosmarinus officinalis*) and sunflower (*Heliantus annuus*) (43). The concentrations of p-coumaric acid shown here are similar to those previously published for lime tree, heather (18) and fireweed (*Epilobium angustifolium*) honeys (19). Rutin has been has been detected in rape and heather honeys (40). The concentration of rutin found here is similar to that of linden (*Tillia spp.*) honey (45).

Hydroxybenzoic acid (HBA): HBA was the third most frequent phenol, detected in more than 80% of honeys. This phenol has been previously detected in buckwheat and heather honeys from Poland and the concentrations reported here are similar to those reported for buckwheat (44) and acacia (18) honeys.

Myricetin: myricetin was detected in 66% and quantified in 35% of honeys with an average concentration of 2.37 mg/L. Myricetin has potential anti-cancer effects and has been shown to suppress the invasion of, and promote cell death in human placental choriocarcinoma cells (46). The concentrations found here are similar to those reported for heather honey (44).

Benzoic acid: benzoic acid (detected in nearly half of Irish honey samples) is used as an active ingredient in anti-fungal cream. The presence of benzoic acid in honey can indicate a floral origin within the Ericaceae family and has been previously recorded as a floral marker of heather honeys (43). Benzoic acid is commonly used as a food preservative and EU regulations control the maximum levels of benzoic acid and its salts for use in food stuffs. The levels found in Irish honeys are lower than the EUs maximum levels and similar to those previously reported for European honeys (18).

Ellagic acid: ellagic acid was also detected in nearly half the Irish honeys analysed. Ellagic acid and its metabolites have preventative and therapeutic potential against human cancers (31). Ellagic acid has been previously isolated from many fruits including blackberries and strawberries (47) and detected in *Calluna* (heather) (48) and *Robinia pseudoacacia* (acacia) honeys (43). The concentration of ellagic acid found in this study is similar to those previously reported in buckwheat honey (44).

Quercetin: quercetin has been found in many honeys across the world (19,44,45) and its efficacy in human medicinal properties is currently being investigated in phase 1, 2, 3 and 4 clinical trials (31). Quercetin was detected in 40% of Irish honey samples. The concentrations reported in this study are similar to those reported for fireweed honey (19).

Pinocembrin: pinocembrin originates from many plant species in a number of genera including, *Eucalyptus,* pine (*Pinus*) and lime tree (*Tilia*) (49). This phenol was detected in more than half of the honey samples and the concentrations reported here are similar to those reported for fireweed (19) *Eucalyptus* (50) and heather (45) honeys.

Luteolin: luteolin was detected in a third of Irish honey samples and the concentrations reported here are similar to those of Australian Jelly bush (*Leptospermum polygalifolium*) and New Zealand manuka (*Leptospermum scoparium*) honeys (51).

Phenolic compounds detected in a minority of samples: Trans-cinnamic and o-coumaric acids were also detected and quantified in Irish honeys and the concentrations reported here are similar to those of Mattonai *et al.* (2016) (52). Salicylic acid is a common phenol in willow trees *(Salix spp.*) and was detected in 17 honey samples. The concentrations recorded here are similar to those of chestnut honey (18). Salicylic acid is a widely used anti-inflammatory, analgesic and antipyretic agent (53) and has also been isolated from meadowsweet (*Filipendula ulmaria*) (54), another common plant species in Ireland. Chrysin is present in many fruits and vegetables and was detected in eight honeys samples. Chrysin was quantified in 3 honeys and had a low mean concentration (0.02 mg/L). The small number of quantified samples may suggest that the concentration of this phenol in honey was too low to detect with the method used. Homogentisic acid and galangin were detected in two and three honey samples respectively. The absence of these phenols may be due to the low number of plant species in Ireland that are likely to contain these phenols. Chlorogenic acid was detected in 15 Irish honeys from hives sites within 1.1 km

of each other. Chlorogenic acid has been isolated from dandelions (*Taraxacum officinale)* (55) and was found in a high number of raspberry and forest honeys from Lithuania (56), both of which are common plants across the island of Ireland. The low number of honeys where ferulic acid was detected (n=4) may be a limitation of this research in terms of the limits of detection or it may be due to a limiting number of plant species containing this phenol in the Irish landscape.

Overall, a unique phenol composition was found for each honey sample analysed, highlighting the extent to which phenol profiles vary between honeys. Additionally, while each phenol had a distinctive relationship with land cover, a number of patterns were observed. The patterns observed between phenol composition (detection and quantification) in terms of harvest season, degree of urbanisation and land cover are discussed in the next section.

3.4.2 Urbanisation

3.4.2.1 Phenol presence / absence

Overall, more phenols were detected in honeys from urban environments suggesting that a wider variety of phenols can be found in honeys from urban areas. Floral composition tends to be different in rural vs urban environments, with higher plant species richness often recorded in the latter (14). Most of the urban sites in our study were located in Dublin, a relatively large area that supports a high diversity of plants (57). Since we did not have honey samples from other urban areas, it is not possible to generalise too broadly from our findings. However, studies elsewhere have found differences between plant pollination (in terms of visitation rates of bumble bees) and the landscape, where pollination was positively related to urban areas (58) and other studies have reported positive effects of urbanisation on honey bee colony growth (59–61).

Of all the phenols detected in honeys originating from urban centres, only chlorogenic and ferulic acids were exclusively found in these environments and absent in honeys originating from rural areas. Based on the flora of Ireland it is not surprising that ferulic acid, which has been considered a floral marker of chestnut honey (18), was predominantly detected in honeys from urban landscapes. This phenol originates from the sweet chestnut (*Castanea sativa*), a non-native tree commonly planted in urban landscapes. However, since it was only detected in four samples, this relationship needs further investigation.

3.4.2.2 Phenol concentration

The concentration of most (14) phenols did not vary across the three levels of urbanisation, However, the concentration of benzoic acid, HBA, myricetin and TPC was higher when honeys originated from urban centres compared to rural areas. No phenol had a higher concentration when honeys originated from rural areas. The concentrations of phenols will vary depending on the plant species with some plants having higher concentrations than others. For example, seven times more chlorogenic acid was isolated from the leaves of *Vaccinium arctostapylos* compared to *Taraxacum officinale* (55) both of which are found in urban areas. Although recent studies show that nurse bees do not consume pollen based on nutritional quality (62) worker bees have been shown to have foraging preferences according to the nutritional requirements of the hive (63). The higher phenol concentrations found in honey from urban areas may be a result of honey bees choosing plant species that are potentially more nutritional and this choice may be greater in urban environments.

3.4.3 Land cover

The analyses of surrounding land-use with phenol composition allowed greater insight into the wider scale effects of agriculture and urbanisation on the phenols in honey. Trans-cinnamic acid, p-coumaric acid, luteolin, pinocembrin and rutin showed no statistically significant relationship with land cover. It is likely that this is due to the wide distribution of these phenols across the plant kingdom, and therefore, it is expected that they are found in many land cover types. This is most likely the case for p-coumaric acid and rutin as these phenols were present in >90% of honey samples and are known to have a widespread occurrence in plants (64). However, for other phenols, significant relationships with land cover were found.

3.4.3.1 Agricultural pastures

Pastures decreased the likelihood of four phenols being present in the honey; namely chlorogenic acid, ferulic acid, ellagic acid and quercetin. The decreased likelihood of phenol detection with amount of pasture surrounding the hive suggests that this land cover has negative effects on honey quality. This is likely due to lower floral resource diversity for bees in landscapes with high quantities of pasture (8). Similar to the presence / absence analysis, pasture had negative relationships with the concentration of a number of phenols in honey as well as the TPC.

3.4.3.2 Urban

For Irish honeys, the more green urban area (GUA) surrounding the hive, the higher the likelihood of detecting phenols in honey with a potential human health benefit. Although the potential human health benefits of consuming foods high in phenols, like honey, are recognised, few studies have assessed the potential health benefits of phenols for bees. p-Coumaric acid and quercetin have been shown to activate immune and detoxification signalling pathways in honey bees (23,65). Quercetin has been shown to enhance the longevity of honey bee workers exposed to pyrethroids (66). GUA increased the likelihood of quercetin being present in honey. Like GUA, discontinuous urban fabric also increased the likelihood of quercetin being present in honey however, this land-use decreased the likelihood of HBA being present in honey. GUA and discontinuous urban fabric (associated with vegetated areas such as private gardens and other green spaces smaller than 25 ha (67)) also increased the likelihood of chlorogenic and ferulic acids being present in honey, however these phenols were only detected in four and five honey samples respectively. Despite the relationship between land cover and the presence or absence or certain phenols, different relationships were observed when individual phenol concentrations were analysed. The higher the area of GUA surrounding the hive sites the higher the concentration of myricetin. The results also show that the higher the area of GUA the higher the TPC of honey. Given that honeys with a higher TPC have a higher antioxidant capacity, it could be beneficial for beekeepers to place their hives in urban environments as the higher TPC may infer a higher market value for the honey, however this may have negative implications for wild bees. As there is higher floral resource diversity in some urban landscapes (68) it is possible that this is the reason for the higher TPC in honey samples with large areas of GUA surrounding the hive. Additionally, there may be plant species in the urban landscape that contribute to a higher TPC in honey, which are less abundant or not present in most rural landscapes.

Given that air quality improves as areas become more rural (69), honey originating from urban areas may contain more contaminants. Urban environments have been linked to a reduction in the honey bee's ability to recognise floral odours by altering floral volatiles (70,71) and various studies have detected environmental pollutants in bees, honey and pollen (72). To our knowledge there are no data available on the environmental pollutants in nectar, however, nectar chemical composition has been shown to alter the foraging behaviour of *Bombus koreanus* and *Apis mellifera* (73).

3.4.3.3 Other land covers

Other notable land covers that showed statistical significant relationships with phenol composition in honey include; coniferous forest, continuous and discontinuous urban fabric, inland marshes, non-irrigated arable land, road and rail networks and associated land and sport and leisure facilities. However non-irrigated arable land and sport and leisure facilities were not significant after the Bonferroni correction. Each of these land covers have a different assemblage of plant species and therefore the honey produced in areas containing these land covers are likely to have a unique phenol composition.

Rutin had the highest phenol concentration for all honey samples, followed by benzoic acid. High concentrations of benzoic acid have been reported in the berries of *Vaccinium* species (74), a plant commonly found in bogs and in heather honey. Guyot *et al.* (1999) suggest that heather honeys can be distinguished from non-heather honeys on the basis of their benzoic acid content (75). As benzoic acid is commonly found in *Vaccinium* species and other ericaceous plants, and has been suggested as a potential marker for heather honey, it was hypothesised that there is likely to be a positive relationship with the presence and concentration of benzoic acid with specific land covers that are likely to contain ericaceous plants (e.g. peat bogs). Surprisingly, this was not the case, as we found that inland wetlands negatively impact the presence of benzoic acid in honey. Given that the CORINE 2012 data series classifies all peat bogs together, whether peat is harvested for fuel or not, it may be possible that honey samples were from areas of exploited peat bogs with a low abundance of Ericaceous plants. Peatlands currently account for more than 20% of the land area in Ireland (76). The harvest date of these honeys may also be a contributing factor as some of these honeys were harvested outside of the heather flowering season.

3.4.4 Influence of harvest season

3.4.4.1 Phenol presence / absence

Some phenols were completely absent from certain harvest seasons, however all 17 phenols were detected in honeys harvested in August/September. This was not unexpected, as it is likely that the flowering time of specific plant species will influence the prevalence or absence of certain phenols at different times of the year. For example, the floral origin of ferulic acid (*Castanea sativa*) may explain why this phenol is not present in early harvested honey as this plant is known to flower in June/July in Ireland. It is also likely

that the number of honey harvests per hive will affect the presence or absence of certain phenols in honey. Honey harvests taken later in the year may be the last in a number of harvests over a season; however, they are sometimes an accumulation of the whole season (May to October) because in Ireland beekeepers may only harvest once. The absence of chlorogenic acid, ferulic acid, homogentisic acid and galangin from honeys harvested later in the season illustrate the complex nature of potential relationships. If the samples are not entire season amalgamations (this information was not provided by the beekeepers), the specific phenol absences may be due to the flowering period of the floral origin of these compounds. However, the absences may also be an artefact of the small sample size of honey harvested in October (n=8). In fact, as all October harvested honeys originated from rural landscapes, the absence of these phenols is more likely due to the absence of flowers in the landscape that contain these phenols.

3.4.4.2 Phenol concentration

The varying concentration of phenols throughout the season may indicate that the abundance of plants (i.e. the number of plants from the same species in a population or area), may play an important role in honey's phenol composition. No variation in p-coumaric acid concentration was observed across the four harvest seasons. The low variation in p-coumaric acid concentration across the four seasons supports the finding that no association was observed between phenol composition and land cover, due to the wide distribution of this phenol in plants (77). The large variation in the TPC of late harvested honey (August-September) may be due to the beekeeping management practices in Ireland (one honey harvest per year).

3.5 Conclusion

This research for the first time has identified what phenols are present in Irish honey. It has also assessed the relationship between honey's phenol composition and the degree of urbanisation, land cover composition and honey harvest season. The higher proportion and concentration of phenols in honeys from urban centres suggests that these environments may provide more diverse forage material for bees, and may influence commercial honey production for human consumption. However, the higher proportions and concentrations of phenols in honey from urban centres may also indicate a scarcity or reduced quality of floral resources for bees in rural areas. GUA had the most positive effect on honeys phenol

composition in terms of the likelihood of a phenol being present and in terms of increased phenol concentration and total phenolic content. Although our results show that urban environments have a positive relationship with honey quality in terms of phenols, there may be negative impacts on honey that were not assessed here.

Land cover and harvest season also affects the phenol composition of honey, with some phenols being completely absent from honey originating in areas with high quantities of pasture and from honeys harvested very early or late in the season. In terms of floral abundance and diversity, each land-use has a unique assemblage of floral resources for bees (7,8) and some land-uses are considered more beneficial than others. This study has identified for the first time landscapes and harvest seasons that can be beneficial (in terms of honey quality) for honey production, for example green urban areas. Given the range of habitats associated with GUA within the CORINE data set, we recommend a more in-depth analysis of areas classified as GUA. This analysis could provide additional insight into the potential correlations between landscape and phenol content. The results support the growing evidence that some types of agricultural land provide insufficient floral resources for bees.

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Chapter 4 Neonicotinoid pesticides in Irish honey

Abstract

The presence and concentration of pesticide residues in honey can influence how potentially harmful it may be to bee health. Although recent studies have detected neonicotinoid residues in honeys from around the world, little is known about pesticide residues in Irish honey. Variation in land use and vegetation composition, and thus exposure to pesticides, as well as, the availability of forage sources for bees, are likely to influence honey properties. We sampled multi-floral honey from 30 *Apis mellifera* hives from urban, rural and semi-natural habitats (SNH), across Ireland, identified and quantified three neonicotinoids (clothianidin, imidacloprid and thiacloprid) using UHPLC-MS, and classified surrounding land use, up to 5 km around hive sites. We evaluated the relationship between each of the neonicotinoids and a) habitat type (agricultural, SNH or urban) and b) landscape composition surrounding the hive. Neonicotinoids were most frequently detected in honeys from agricultural habitats, and 70% of samples contained at least one of the three neonicotinoid compounds. In addition, we found that clothianidin and thiacloprid were more frequently detected in honeys from urban habitats. Our results add to the growing body of evidence that bees are exposed to neonicotinoids in their food.

4.1 Introduction

Neonicotinoids are the most widely used group of insecticides in the world and can be classified into one of three chemical groups based on their pharmacophore moieties (1) N-nitroguanidines (imidacloprid, thiamethoxam, clothianidin, and dinotefuran), nitromethylenes (nitenpyram), and N-cyanoamidines (acetamiprid and thiacloprid) (2,3). They are neuro active insecticides causing nervous stimulation at low concentrations and receptor blockage, paralysis and death at higher concentrations (4). They act systemically, travelling through the plant tissue, protecting all plant parts, and provide effective pest control.

Neonicotinoids are the fastest growing class of insecticides in modern crop protection (1) and within 20 years of their introduction (late 1980's) they dominated 25% of the insecticide market (5). These pesticides have been demonstrated to cause high acute and chronic toxicity to bees. For example, non-lethal exposure of thiamethoxam has been found to decrease the foraging success of the Western honey bee (*Apis mellifera*) (6) and has been found to induce a variety of behavioural changes (7). Clothianidin has also been found to induce a variety of behavioural changes (7) and sub-lethal doses of clothianidin have been shown to alter the navigation of the honey bee (8). Imidacloprid has been found to impair memory and brain metabolism in the honey bee (9) and induce a variety of behavioural changes (7) including changing the navigation behaviour of the honey bee (8). Thiacloprid has also been shown to alter the navigation of the honey bee (8). The LD_{50} value (lethal dose 50%; the dose required to kill half the members of a tested population after a specified test duration) for honey bees ranges from 3.8 to 39,000 mg/kg (10) and varies depending on the neonicotinoid and method of application. Although thiacloprid is reported as being several orders of magnitude less toxic to honey bees (thiacloprid acute oral LD_{50} 8.1–39 μg per bee) compared to the other neonicotinoids (imidacloprid, thiacloprid and thiamethoxam acute oral LD_{50} = 0.004-0.005 μg per bee), the method of application of thiacloprid may lead to higher residues in the honey bee's food sources (11). Concentrations as low as 0.003 mg/kg (acetamiprid) (12), 0.08 mg/kg (clothianidin) (13), 0.001 mg/kg (imidacloprid) (13), 0.29 mg/kg (thiacloprid) and 1.34 ng/bee (0.118 mg/kg) thiamethoxin (14), have been shown to have negative effects on honey bees (when given orally). There is a knowledge gap regarding the impacts of neonicotinoids on other organisms; however, the evidence suggests that it is likely that these pesticides have

negative biological and ecological impacts on a wide range of non-target invertebrates in terrestrial and aquatic habitats (15,16).

Five neonicotinoids are currently approved as active substances in the EU for the use in plant protection products (PPP), namely acetamiprid, clothianidin, imidacloprid, thiacloprid and thiamethoxam (17) four of which are currently approved for use in Ireland (all except thiamethoxam) (18). Details regarding the off-label approvals (product not originally designed for the said specific use) for use of neonicotinoids on food and non-food crops as of 18th July 2018 are presented in Table 4-1. Clothianidin is not on the list of off-label approvals; but it is the active ingredient of Redigo Deter, a product used in Ireland since 2012 as a seed dressing. Between 2011 and 2014 (the years prior to the harvest of the honeys used in this study) all five neonicotinoids were used in Ireland (19–21). The quantities applied (kg) and area (spray hectares) were higher for clothianidin, imidacloprid, thiacloprid and thiamethoxam compared to acetamiprid (Table 4-1) and for this reason acetamiprid was not included in this study. Although thiamethoxam was one of the top 10 active ingredients most extensively used on oilseed rape in Ireland in 2012 (22), its approval was withdrawn from February 2014 (21) (the first year of the honey harvest) and for this reason thiamethoxam was not used in the analysis. The information regarding PPP usage in Ireland is sparse in terms of the crop the product was used on, the quantity used, and the area to which the product was applied. For example, thiacloprid is used on both soft fruit and vegetable crops in Ireland, however only one report exists for PPP usage on soft fruit, published in 2014, and only one report exists for its use on vegetable crops, published in 2011.

Table 4-1 Neonicotinoids approved for use in Ireland as of 18th July 2018; their main usage in terms of crop type between 2012- 2018 and the quantity of product applied and spray area between 2011-2014.

Compound	Approved for use in Ireland (18)	Off-label approval (18)	Main usage	Quantity applied (kg)	Spray area (spray hectares)
Acetamiprid	Yes	used on 11 fruit and vegetable crops and one non-food crop	two vegetable crops; ranked 37 in the 50 active ingredients most extensively used on vegetable crops in Ireland in 2011 (23)	8.91 (23)	178 (23)
Clothianidin	Yes	No	used on arable crops (22)	418 (22)	4749 (22)
Imidacloprid	Yes	one non-food crop	top 10 active ingredients most extensively used on fodder turnip & fodder swede crops in 2013(24)	103 (24)	254 (24)
Thiacloprid	Yes	used on 29 fruit and vegetable crops and 12 non-food crops	ranked 14 in the 50 active ingredients most extensively used on vegetable crops in Ireland in 2011; was used on 16 different vegetable crops (23); used on at least 7 different soft fruit crops in Ireland in 2014 (25)	> 100.9* (23,26)	> 929.27* (23,26)
Thiamethoxam	No	No crops	one of the top 10 active ingredients most extensively used on oilseed rape (22)	203 (22)	16,803 (22)

* The quantity and area for some crops are not given and the exact figures cannot be presented here.

These neonicotinoids can persist and accumulate in the environment (4) and each neonicotinoid has specific properties concerning their environmental effects (persistence, biodegradability and accumulation) and specific toxicity levels. For example, the reported half-lives of seed treated neonicotinoids range from 28–1250 days for imidacloprid (4,11,27) and 148–6931 days for clothianidin (4,11,28). Thiacloprid is not used in seed treatments but common in spray application and has a shorter half-life (3-74 days) (4,11,28) compared to clothianidin and imidacloprid.

Due to the wide application in both agriculture and horticulture, persistence in soil and water and potential for uptake by non-target plants (e.g. wild plants and succeeding crops), neonicotinoids are bioavailable to bees for most of the year (29,30). This can result in the presence of neonicotinoids in honey. Residues of neonicotinoids have been found in honey samples from many countries around the world (Austria (31), France (32,33), Poland [114], USA (35)). A recent worldwide survey has shown that these pesticides can be found in honeys from all continents (except Antarctica) around the world, indicating their global prevalence (36). Different national regulations have established maximum levels of pesticide residues (MRLs) permitted in honey [136]. There is however a lack of homogeneity that causes problems in international marketing and trade (37). In Europe, current MRLs for clothianidin and imidacloprid are 0.05 mg/kg and the MRL for thiacloprid is 0.2 mg/kg. The pesticide levels found in honey samples vary considerably and are usually below the MRLs authorised for human consumption (36,38). However, the levels found in honey can be higher than the concentrations which have been found to adversely impact bee health (36).

Clothianidin, imidacloprid and thiamethoxam were initially identified by EFSA (European Food Safety Authority) as a risk to bees in 2013, and more recently, in 2018, the risks were reviewed and confirmed (39,40). Consequently, their use was restricted in the EU: since 2013, these compounds cannot be used as seed or soil treatment for crops attractive to bees and for cereals, except for uses in greenhouses and for winter cereals. In addition, they cannot be used in foliar application for crops attractive to bees and for cereals, with the exception of uses in greenhouses and uses after flowering (EC Regulation 485/2013). Since May 2018, these compounds cannot be used outdoors and their use is restricted to permanent greenhouses once the crop stays within the greenhouse during its entire life cycle (EU Regulations 2018/783, 2018/784, 2018/785) (17). Of the other two compounds approved for use in the EU, thiacloprid is currently considered by the EC as a ‘candidate

for substitution' (replaced by an alternative which is safer for humans and the environment), based on its endocrine disrupting properties that may cause adverse human health effects (17). EFSA established that acetamiprid is a low risk to bees, and the EC has deemed it neither scientifically nor legally appropriate to restrict its use.

Despite the sub-lethal effects clothianidin, imidacloprid and thiamethoxam have on honey bees, these three active ingredients were, at the time of this study, approved for use on crops grown in enclosed systems, winter cereals and in grass management in Ireland, as discussed in Chapter 1. Since these compounds were not used on crops attractive to bees, we would expect residues in honey to be absent or very low. The aims of the present study were therefore to;

(i) Identify and quantify three neonicotinoids (clothianidin, imidacloprid and thiacloprid) in Irish honeys.
(ii) Determine if there is an association between their occurrence and the land use composition of the landscape surrounding the hive.

4.2 Methods

4.2.1 Honey Sampling

Thirty Irish honey samples from 30 hive sites across the island of Ireland were collected directly from beekeepers in 2014 (Fig. 4-1). Once collected, all honey samples were stored in amber containers in the fridge between 0° and 4° C. The sites were grouped into one of three habitat categories (agriculture (n=10), SNH (n=10) and urban (n=10)) based on the proportions of land cover surrounding each of the hives, according to the CORINE 2012 data series. Where possible, sites with the most extreme land use patterns (i.e. highest proportions of agriculture, SNH and urban) from Chapter 3 were selected. The percentage of each habitat surrounding the hive sites to a 5 km radius ranged from 29-97% for agriculture, 4-54% for SNH and 40-95% for urban around. Honeys were harvested either in June or July.

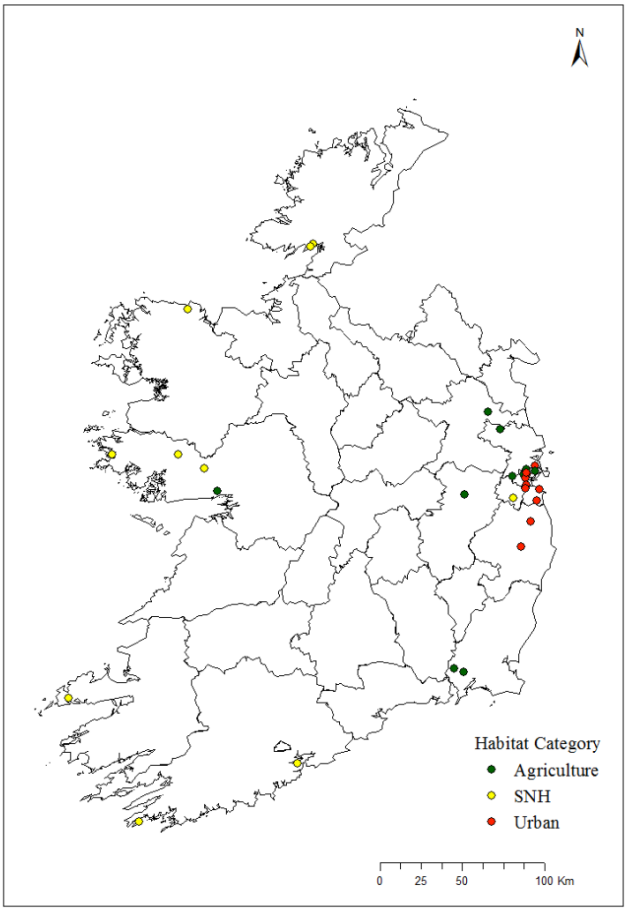

Figure 4-1 Location of honey sampling sites across Ireland (n=30). Habitat categories: agriculture (green dot), SNH (yellow dot) and urban (red dot).

4.2.2 Neonicotinoid analysis

Neonicotinoid identification and quantification was determined by ultra-high performance liquid chromatography (UHPLC), coupled with mass spectrometry detection (hereafter referred to as LC-MS) as described previously (41). Further details are given below.

4.2.2.1 Reagents and Standards

All salts, solvents and standards were analytical or LC-MS grade. Water, ACN, formic acid, ammonium formate, all salts used for QuEChERS and each of the standards (clothianidin, imidacloprid and thiacloprid) were obtained from Sigma Aldrich, Ireland. Isolute PSA (primary secondary amine) bulk phase was purchased from Biotage, Sweden. Isotopically labelled standards (clothianidin-D3, imidacloprid-D4, and thiacloprid-D4) were obtained from QMX Laboratories, UK. Millipore Millex syringe filters with

hydrophobic PTFE membrane (pore size 0.22 μm and 20 mm diameter) and low-adsorption LC-MS certified vials and conical inserts were purchased from Sigma Aldrich. XBridge UPLC BEH column 4.6x100 mm i.d., 3.5 μm particle size using Xbridge BEH C18 VanGuard Cartridge (3.5μL 3.9 X 5 mm) and VanGuard cartridge holder were all purchased from Waters Chromatography Ireland.

4.2.2.2 Sample preparation

Honey samples were prepared using a QuEChERS protocol described previously (32,41). Specifically, 2.5 g of honey was weighed in a 15 mL polypropylene tube (Sparks Lab Supplies Ltd., Ireland) to which 9 mL of H_2O: ACN (50:50, v/v) and 20 μL of a 500 ng/mL internal standard (IS) methanolic solution containing the 3 labelled neonicotinoids were added. Honey was dissolved by manual agitation and ultrasonication for 10 min and the resulting solution was transferred into a 15 mL tube containing the extraction salts (2 g magnesium sulphate ($MgSO_4$), 0.5 g sodium chloride, 0.5 g sodium citrate hydrate and 0.25 g sodium citrate sesquihydrate). One mL of H_2O:ACN (50:50, v/v) was added to the first tube and after a brief agitation period the remaining solution was also transferred to the extraction salts tube. The mixture was vigorously shaken by hand for approximately 2 min and centrifuged at 4000 g for 10 min. The upper phase (4.5 mL) was collected in a second 15 mL tube containing the purification salts (0.15 g $MgSO_4$ and 0.1 g PSA). After vigorous shaking for 1 min, the tube was centrifuged at 4000 g for 10 min and the supernatant (4.5 mL) recovered and dispensed into three 1.5 mL Eppendorf tubes (Lennox Laboratory Supplies, Ireland). The solution was evaporated until dry in a CentriVap centrifugal evaporator (Labconco, Thermo Scientific, Ireland) thermostated at 40 °C and the dried residue that remained was re-suspended in 0.25 mL of MeOH 25%. The tubes were vortexed and filtered through 20 mm PTFE hydrophilic syringe filters (Sigma Aldrich) into LC-MS vials containing 250 μL conical inserts. The final concentration of IS was 20 ng/mL.

4.2.2.3 UHPLC-MS/MS analysis

The analysis of neonicotinoids in honey was performed on a Dionex Ultimate 3000 RSLC (Thermo Scientific) coupled to a LTQ XL mass spectrometer (Thermo Scientific). Neonicotinoid separation was achieved on an Xbridge UPLC BEH column 4.6x100 mm i.d., 3.5 um particle size, Waters) using a temperature of 25°C and a flow rate of 0.3 mL/min. Mobile phase A consisted of 5mM ammonium formate with 0.05% formic acid and mobile phase B comprised ACN with 0.05% formic acid. The following gradient

program was used: linear increase from 10 to 80% B in 15.0 min, 80-98% B in 0.1 min, holding at 98% B for 2 min and returning to initial conditions at 10% B in 2.0 min. The injection volume was 5 µL. The flow was deviated from the mass spectrometer from 0.0-5.0 min using a 6 port diverter Valco valve.

These experiments were accomplished by means of an "inclusion list" in the mass spectrometry instrument method. The inclusion list on the LTQ XL (Xcalibur 2.1 software release 2.1, March 31, 2011) consisted of *m/z*, collision-induced dissociation (CID) fragmentation energy and retention time values for the three neonicotinoids and their associated labelled standard (retention times were determined from previous experiments using standards) (Table 4-2).

Table 4-2 Inclusion list showing the mass, retention times and CID energy for each of the six standards.

MS (*m/z*)	Start time (min)	End time (min)	MS CID energy	Name
250	10.7	11.2	32	Clothianidin
253	10.7	11.2	32	Clothianidin Label
253.1	12.4	12.8	32	Thiacloprid
256.1	11.2	11.8	32	Imidacloprid
256.1	12.4	12.8	32	Thiacloprid Label
259.1	11.2	11.8	32	Imidacloprid Label

The following parameters were used for inclusion list-dependent acquisition on the LTQ XL mass spectrometer. MS detection was performed in positive electrospray mode using the following parameters: capillary voltage 5 kV, capillary temperature 275 °C, sheath and auxiliary gas flow (N_2) ten and three (arbitrary units), tube lens voltage 55 V. The analysis was carried out in the ultra-zoom single ion monitoring (SIM) mode in positive mode profile. The intensity threshold for triggering of detected peaks was set to ten, isolation width (*m/z*) 1.6, activation time of 90 ms and collision energy was specified at 32% for all list members. The repeat count was set to six. Neonicotinoids were identified and quantified according to the corresponding spectral characteristics: mass spectra, mass, characteristic fragmentation, and characteristic retention time.

4.2.2.4 Detection, quantification and validation

The identification of eluted components was achieved by comparing the retention time of reference standards with the honey samples. Neonicotinoids were detected based on a) the characteristic fragmentation pattern and b) calibration. For detection based on fragmentation, quantitation was carried out in scan mode by monitoring the response for a specific ion in an analyte's mass spectrum. In many cases, this ion, termed the "quantifier" ion, is the most abundant in the spectrum. Other lesser abundant ions may also be monitored to aid in proper identification of the analyte. These are often termed "qualifier" ions. Neonicotinoids were quantified by internal calibration using calibration solutions prepared in MeOH 25% at 0.25, 0.5, 1, 10, 50 and 200 µg/L. The linearity range of the analytes, the limit of detection (LOD), the limit of quantification (LOQ), and the recoveries for three concentration levels were determined using a three point calibration curve (0.25, 0.5, 1 µg/L). Although initially the calibration range was 0.25 – 200 µg/L (7 data points) it was noticed the sensitivity of the calibration curve was lower when > 1 ng/L standards were included. A more accurate calibration curve for the target analytes was found using the lower range (0-1 µg/L) and hence this range was used. A validation assay was performed using methanol blanks (n=2) before each batch was analysed. Validation parameters are presented in Table 4-5. Three replicates of each honey sample were analysed. Thermo Xcaliber 2.2.0 (Thermo Scientific) was employed to process the data.

4.2.3 Landscape analysis

Landscape composition was quantified to a 5 km radius around each of the 30 hive sites using the CORINE (Co-ordinated Information of the Environment) 2012 land cover classification system. The 2012 data were selected for use because this dataset was closest to the honey harvest dates. Land cover was classified to Level 1, 2 and 3 of the hierarchal classes (1-3) (see Appendix A1). A total of 11 land cover types were determined in the areas surrounding the 30 hive sites.

4.2.4 Statistical analysis

Fishers exact tests were used to compare the proportions of honey samples containing clothianidin, imidacloprid and thiacloprid and the proportions where at least one, two or all three neonicotinoids were detected according to habitat type (agriculture, SNH and urban). Mann-Whitney U tests were used to compare differences in the area of each of the 11 land covers according to whether each of the three neonicotinoids were present or not. Eleven

land covers were selected for analysis on the basis of their presence in at least 60% of the hive sites. Only imidacloprid was quantifiable, as the concentrations of clothianidin and thiacloprid fell below the limits of quantification (LOQ). The relationships between the concentration of imidacloprid and percentage of 11 land covers were assessed where possible (8 samples). Correlations between land cover and imidacloprid concentration were investigated using Spearman's Rank Order Correlation because data were not normally distributed. All analyses were carried out in R-3.2.5.

4.3 Results

Separation of the three neonicotinoids can be seen in Fig. 4-2. The results presented here have been generated using detection based on fragmentation (discussed in Section 4.3.1) and detection and quantification based on calibration (discussed in Section 4.3.2). Subsequently in Section 4.3.3 the relationship between the presence and / or concentration of each of the three neonicotinoids with habitat type and separately, with land cover, are assessed.

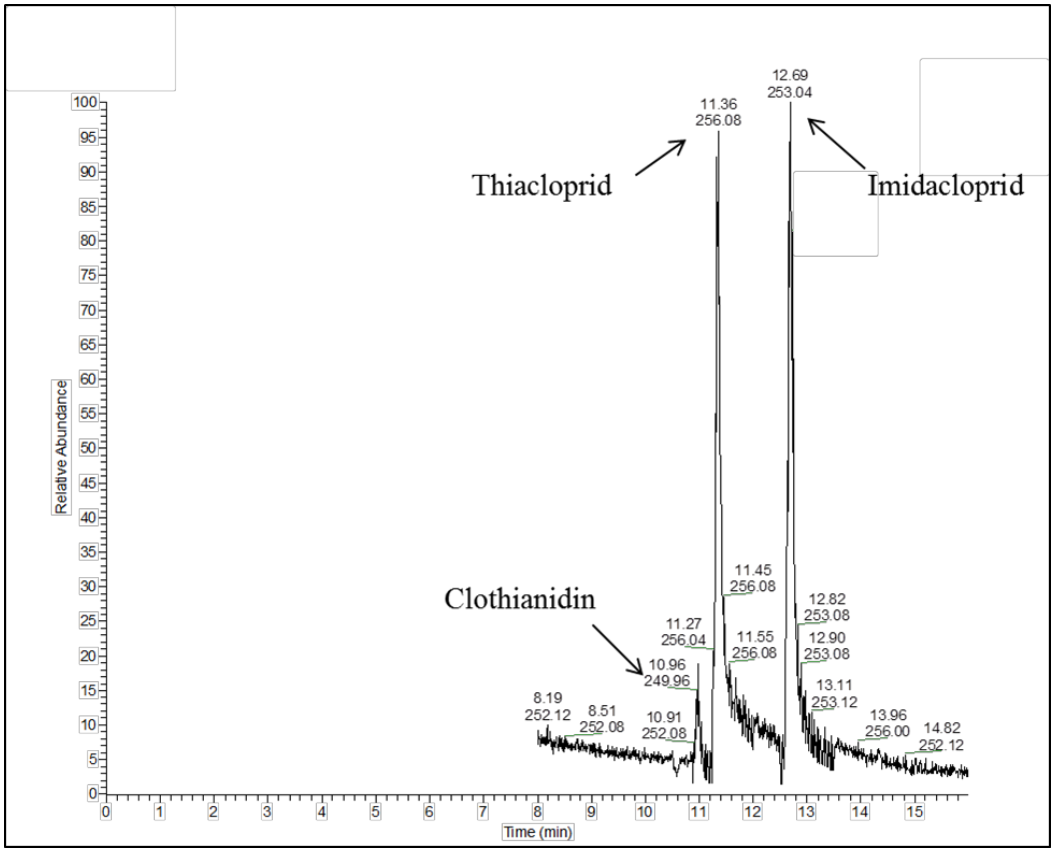

Figure 4-2 Separation of three neonicotinoids (clothianidin, imidacloprid and thiacloprid). Chromatographic methods as per Section 4.2.2.3.

4.3.1 Detection based on fragmentation

Analysis of blank samples revealed no traces of the pesticides studied. Three neonicotinoid peaks were structurally identified by comparing retention times, high resolution mass spectrometry data, and fragment ions with those of the reference substances. The mass spectra and characteristic fragmentation pattern of each of the three neonicotinoids are illustrated in Figs. 4-3, 4-4 & 4-5 and documented in Table 4-3. The protonated molecular ion $[M+H]^+$ was the base peak in the spectra of the three compounds in the positive ion mode (Figs. 4-3a, 4-4a and 4-5a). In Fig. 4-3b (clothianidin), fragments at *m/z* 250, 169 and 132 were observed. The quantifier ion (169 *m/z*) derives from, $[M+H–NO_2–Cl]$, and the qualifier ion (132 *m/z*) might derive from $[M+H–NH–C–NH–NH–NO_2–CH_3]$ (42). In Fig. 4-4b (imidacloprid) fragments at *m/z* 256.1, 209 and 175 were observed. The quantifying ion (209 *m/z*) derives from $[M+H–HNO_2]$ and the qualifier ion (175 *m/z*) might derive from $[M+H–NO_2–Cl]$ (42). In Fig. 4-5b (thiacloprid), fragments at *m/z* 253.1, 126.1 and 226.1 were observed. The quantifying ion (126.1 *m/z*) derives from $[M+H–CN–N–C–S–CH_2–CH_2–NH]$ and the qualifier ion (226.1 *m/z*) might derive from $[M+H–CN–N–C–S–CH_2–CH_2–NH–HCl]$ (42). The fragmentation pattern proposed here is similar with that reported previously (41–44).

Table 4-3 Compound specific UHPLC-MS/MS retention times (Rt), quantifying transition ions (Q) and qualifier transition ions (q) for the three neonicotinoids analysed.

Analytes	Rt (min)	Transitions (*m/z*)
Clothianidin (Q)	10.96	250/169
Clothianidin (q)		250/132
Clothianidin-D3(Q)	10.96	253/172
Clothianidin-D3(q)		253/132
Imidacloprid (Q)	12.69	256/209
Imidacloprid (q)		256/175
Imidacloprid-D3 (Q)	12.69	259/213
Imidacloprid-D3 (q)		259/178
Thiacloprid (Q)	11.36	253/126
Thiacloprid (q)		253/226
Thiacloprid-D3 (Q)	11.36	256/126
Thiacloprid-D3 (q)		256/229

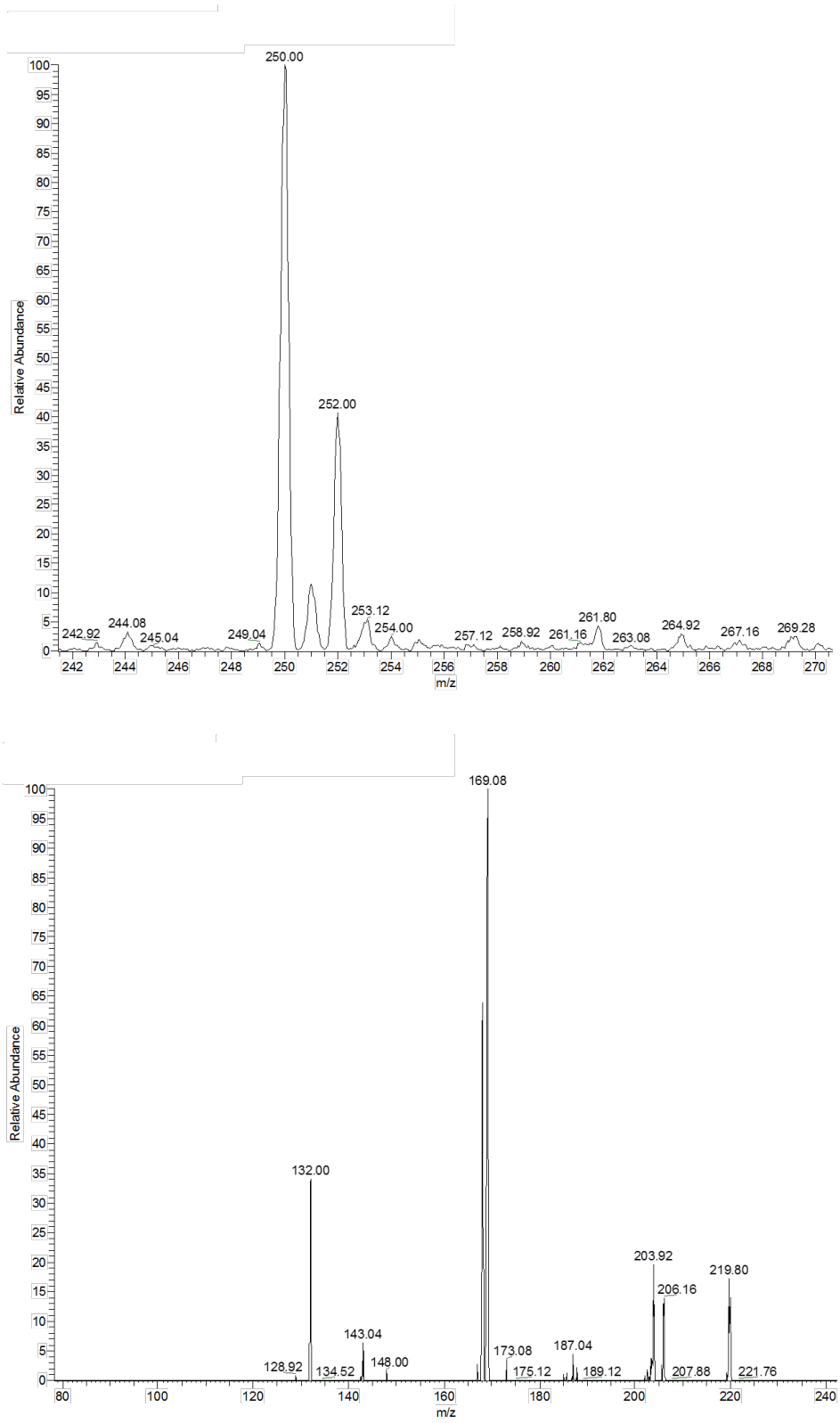

Figure 4-3 Mass spectra and characteristic fragment pattern of clothianidin at *m/z* 250.

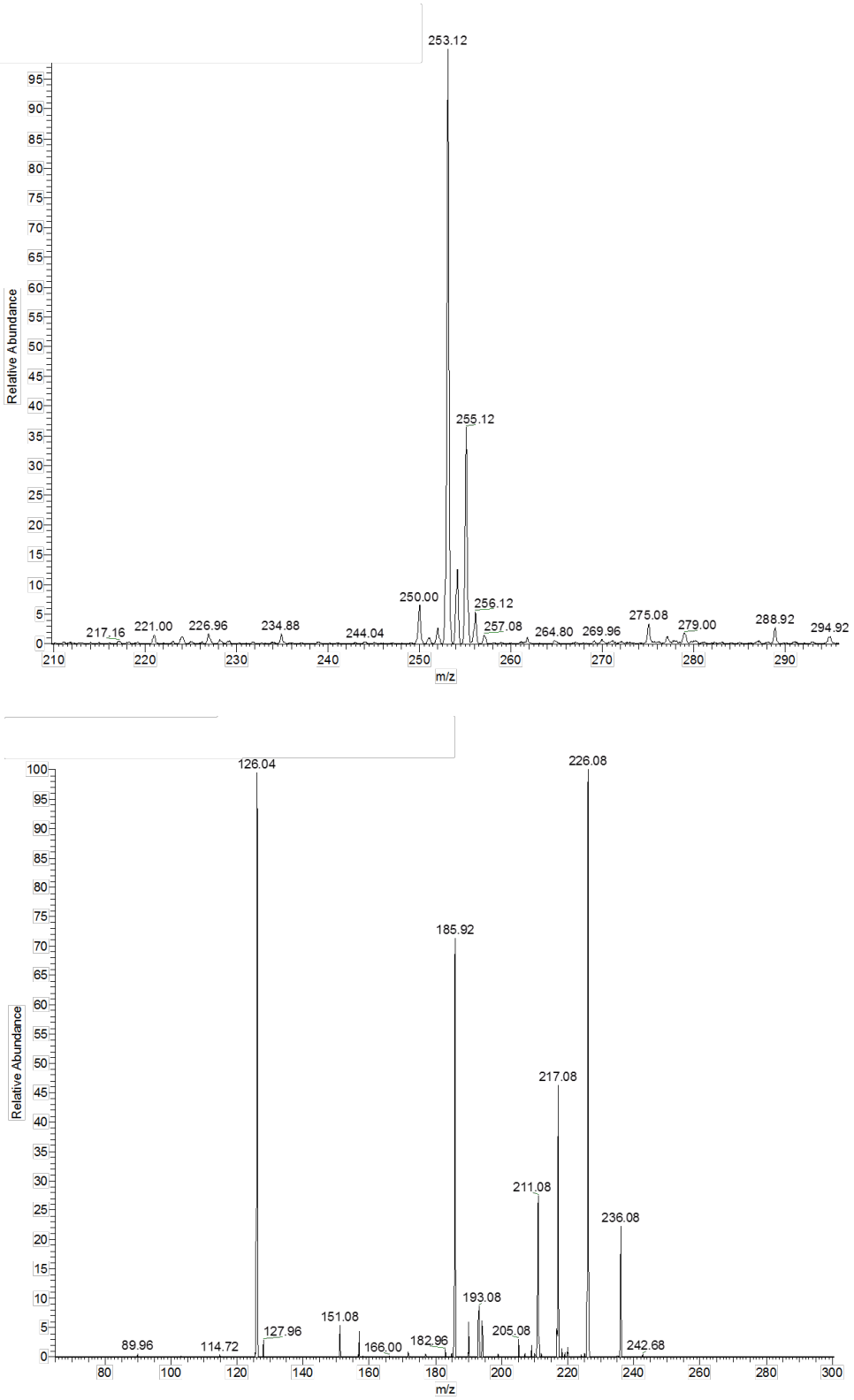

Figure 4-4 Mass spectra and characteristic fragment pattern of thiacloprid at *m/z* 253.

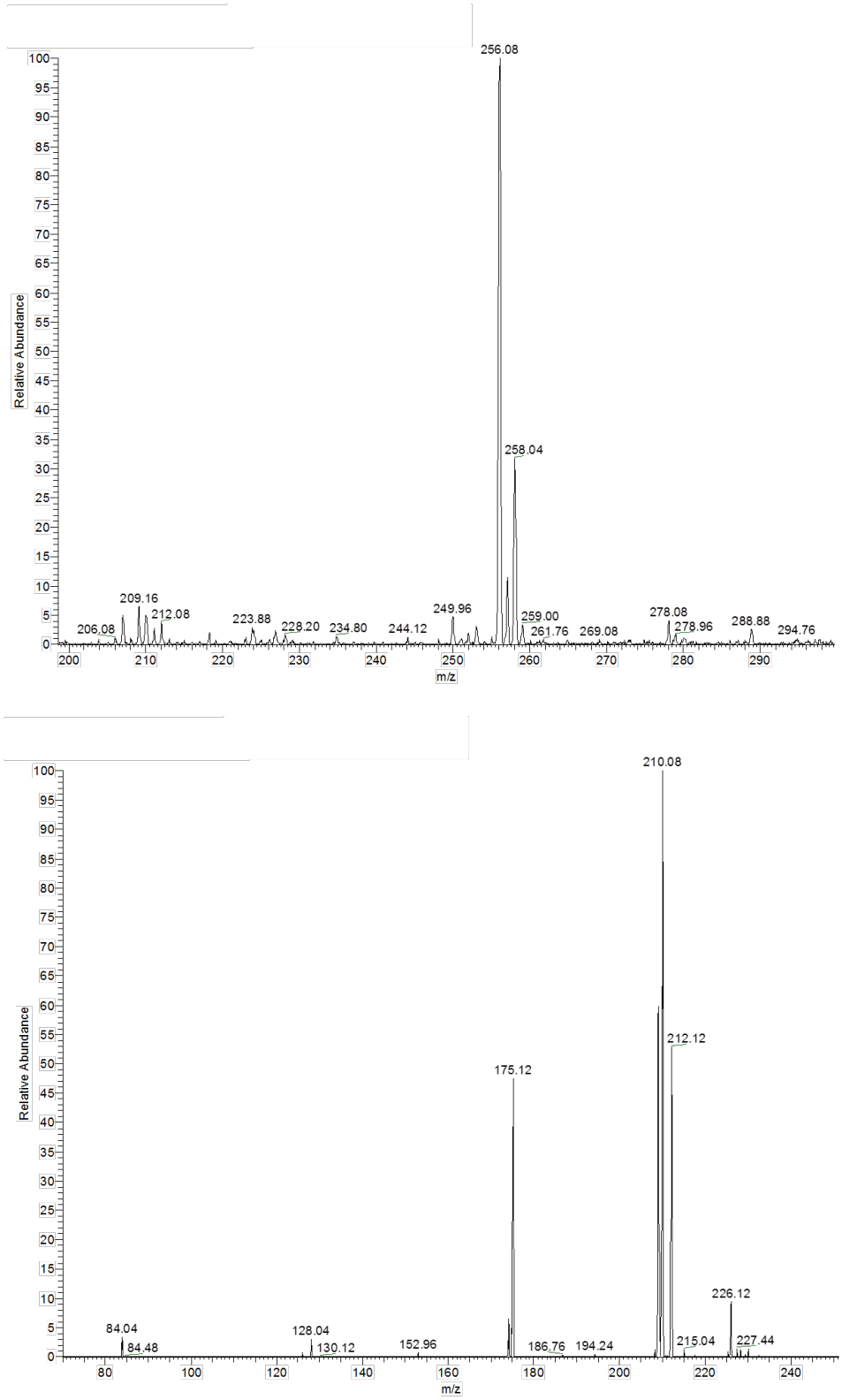

Figure 4-5 Mass spectra and characteristic fragment pattern of imidacloprid at *m/z* 256.

Imidacloprid was the most frequently detected neonicotinoid (found in 13, 43% of honey samples) followed by clothianidin (12, 40%) and thiacloprid (11, 37%). The results show that 70% of all analysed samples contained at least one neonicotinoid (clothianidin, imidacloprid and thiacloprid). At least two neonicotinoids were detected in 48% of the samples and two of the 30 samples contained all three neonicotinoids.

4.3.2 Detection and quantification based on calibration

Using detection based on calibration (i.*e.* LODs), the number of honey samples where imidacloprid was detected was equal to the number detected using identification based on fragmentation (i.e. 13). However, using the calibration method to determine LODs, clothianidin was only detected in four samples and thiacloprid was not detected in any honey sample (Table 4-4).

Table 4-4 Summary statistics associated with each neonicotinoid detected and/or quantified in honey samples. The maximum concentration (Max. conc.) is given in mg/kg together with mean, the standard deviation, LOD and LOQ. Number Detected = number of honey samples in which the pesticide was detected; Number Quantified = number of honey samples in which the pesticide could be quantified.

Neonicotinoid	Number Detected	Number Quantified	Max. conc. (mg/kg)	Mean (mg/kg)	SD (mg/kg)	LOD (mg/kg)	LOQ (mg/kg)
Clothianidin	4	0	<LOQ	<LOQ	<LOQ	0.104	0.312
Imidacloprid	13	8	0.018	0.011	0.004	0.003	0.008
Thiacloprid	0	0	<LOQ	<LOQ	<LOQ	0.018	0.055

The LOD and LOQ varied for each neonicotinoid. Imidacloprid had the lowest LOD and LOQ, 0.006 mg/kg and 0.017 mg/kg respectively. Overall, only 27% of honeys contained quantifiable amounts of neonicotinoids and imidacloprid was the only neonicotinoid that could be quantified (n=8) (Table 4-4). The concentration of imidacloprid ranged from 0.007 to 0.018 mg/kg. Clothianidin and thiacloprid could not be quantified in honey samples as concentrations were <LOQ. Method validation parameters are shown in Table 4-5. At all levels, inter day precision was lower than 25%.

Table 4-5 Validation parameters for the quantification of neonicotinoids in Irish honey: calibration range (linearity), limits of detection (LOD) and quantification (LOQ) and precision (%RSD).

Neonicotinoid	Linearity (mg/L)	LOD (mg/L)	LOQ (mg/L)	Precision (%RSD)
Clothianidin	0.025-1	0.073	0.220	23.51
Imidacloprid	0.025-1	0.004	0.011	20.2
Thiacloprid	0.025-1	0.026	0.078	5.66

4.3.3 Relationship with landscape

Of the 70% of samples where at least 1 neonicotinoid was detected, 14% came from SNH, 43% came from agricultural habitats and another 43% came from urban habitats (Table 4-6). Of the two honey samples that contained all 3 neonicotinoids, one of these came from the agricultural habitat category and one came from the urban habitat category (Table 4-6).

Honeys from urban habitats had the highest number of samples where clothianidin and thiacloprid were detected. The highest number of honey samples where imidacloprid was detected came from the agricultural habitat category (Table 4-6). Clothianidin and thiacloprid were least frequently detected in honey samples from SNH, while imidacloprid was detected in two samples from SNH and two from urban habitats.

Table 4-6 Number of honey samples where at least one, two, all three and each neonicotinoid was detected according to habitat group.

	Agriculture	SNH	Urban
At least 1 neonicotinoid	9	3	9
At least 2 neonicotinoids	6	2	5
All three	1	0	1
Clothianidin	4	1	7
Imidacloprid	9	2	2
Thiacloprid	3	2	6

Clothianidin was more frequently detected in honeys from urban habitats compared to SNH ($p=0.02$) (Fig. 4-6a, Table 4-7)). Although clothianidin was more frequently detected in honey samples from agricultural habitats compared to SNH, this difference was not statistically significant. Imidacloprid was more frequently detected in honeys from

agriculture habitats compared to both SNH and urban habitats (p= 0.005, Fig. 4-6a, Table 4-7). There was no statistically significant association between the detection of thiacloprid and habitat category (Table 4-7), however the number of detected samples was highest in urban habitats (Fig. 4-6a). The detection of at least 1 neonicotinoid was significantly higher in samples from both agricultural and urban habitats compared to SNH (p=0.02 Fig. 4-6b). Finally, no statistically significant difference was observed between habitats when at least 2 neonicotinoids were detected (Table 4-7).

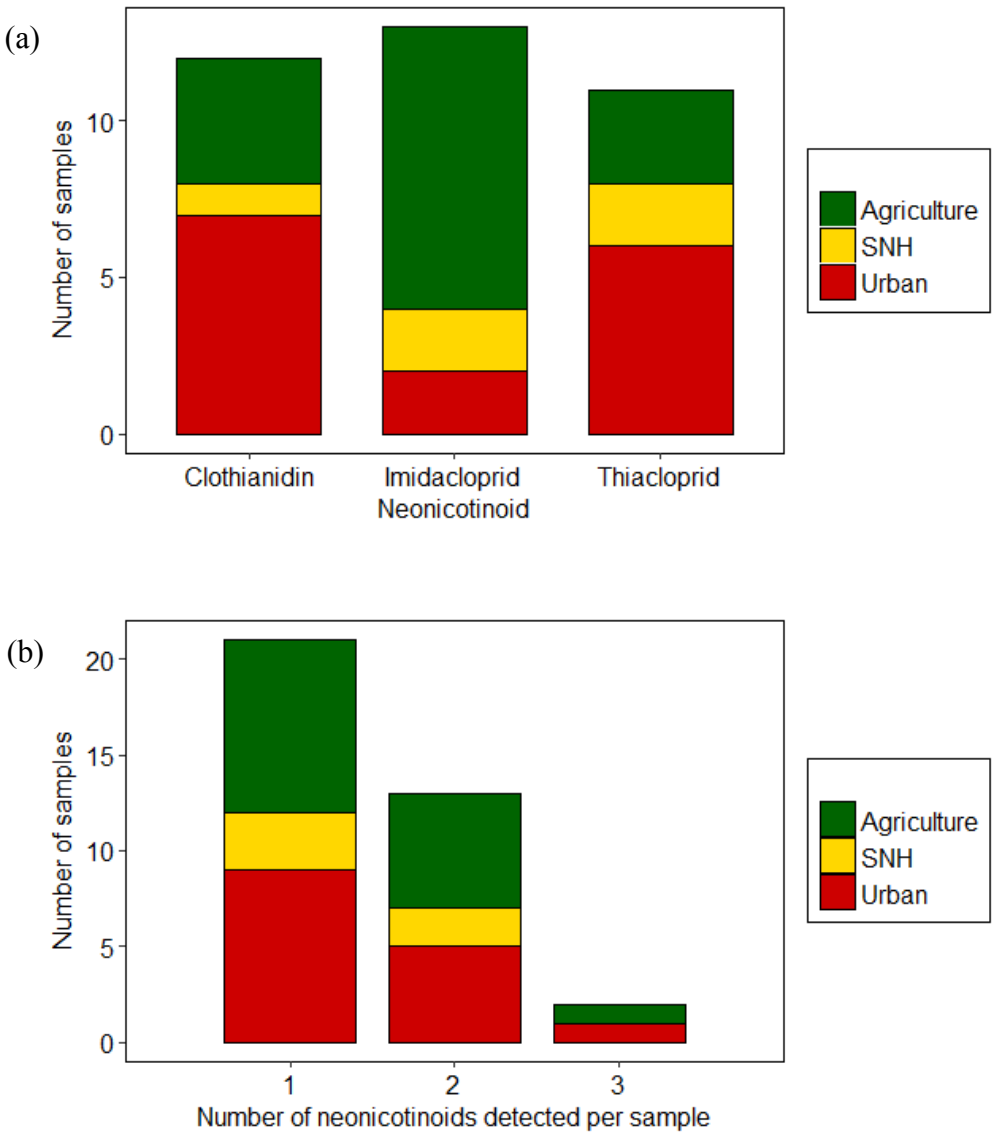

Figure 4-6 Number of honey samples where (a) each the three neonicotinoids (clothianidin, imidacloprid and thiacloprid) were detected and (b) at least one, two or all three neonicotinoids were detected, according to habitat type (agriculture SNH and urban).

Table 4-7 Associations between habitat type (Agricultural (Ag), SNH and Urban) and the presence of at least one pesticide, and between habitat type and each of the three pesticides individually (clothianidin, imidacloprid and thiacloprid). Odds ratio and p values were calculated using Fisher's exact test, n= 10 for each habitat group. Significant p-values are in bold.

	Number of samples with compound(s) detected	Odds ratio	p-value
At least 2 neonicotinoids			
Ag / Urban	6 / 5	0.68	1.00
Ag / SNH	6 / 2	0.18	0.17
SNH / Urban	2 / 5	0.27	0.35
At least 1 neonicotinoid			
Ag / Urban	9 / 9	1.00	1.00
Ag / SNH	9 / 3	0.06	**0.02**
SNH / Urban	3 / 9	0.06	**0.02**
Clothianidin			
Ag / Urban	4 / 7	0.30	0.37
Ag / SNH	4 / 1	0.18	0.30
SNH / Urban	1 / 7	0.06	**0.02**
Imidacloprid			
Ag / Urban	9 / 2	27.33	**0.005**
Ag / SNH	9 / 2	27.33	**0.005**
SNH / Urban	2 / 2	1.00	1.00
Thiacloprid			
Ag / Urban	3 / 6	0.31	0.37
Ag / SNH	3 / 2	0.60	1.00
SNH / Urban	2 / 6	0.06	0.17

Abbreviations: Ag: agriculture; SNH: semi-natural habitat

There were significant differences in the area of nine different land covers surrounding the hive according to the presence or absence of one of the neonicotinoids (Table 4-8). In samples where clothianidin was detected, there was significantly less coastal wetlands in the surrounding landscape (Table 4-8). Conversely, in samples where clothianidin was detected, there was a greater area of artificial surfaces and artificial non-agricultural vegetated areas surrounding the hives. For samples where thiacloprid was detected, there were significantly lower areas of heterogeneous agricultural areas and by land principally

occupied by agriculture surrounding hives. Finally, for samples in which imidacloprid was detected, there were more agricultural areas and areas of pastures surrounding the hive, and lower areas of artificial surfaces and arable land surrounding the hive (Table 4-8).

Table 4-8 Mann-Whitney U test results of each land cover that showed a statistically significant association with one of the three neonicotinoids, clothianidin, imidacloprid and thiacloprid. Mann Whitney U values (U), the number of samples containing the land cover (n), the median land cover when the neonicotinoid was present and absent, the standardised test statistic (Z), p-value and the effect size (r) are presented below.

Clothianidin	AS	UF	ANA	W
U	156.5	155.5	145	24.5
n	29	29	29	23
Median when present	31.52	25.19	6.11	1.74
Median when absent	1.69	0.79	0.33	3.95
Z	2.59	2.55	2.27	-2.5
p-value	0.008	0.009	0.039	0.01
r	0.48	0.47	0.42	0.52
Imidacloprid	AS	AA	AL	P
U		169.5	151.5	159.5
n		29	29	29
Median when present	4.21	44.6	0.44	33.83
Median when absent	7.23	17.7	9.65	7.77
p-value	0.003	0.003	0.036	0.013
Z		2.87	2.14	2.43
r		0.53	0.4	0.82
Thiacloprid	HAA	A		
U	24.5	34		
n	29	29		
Median when present	0.44	0.00		
Median when absent	4.22	2.97		
p-value	0.001	0.004		
Z	-3.24	-2.83		
r	0.6	0.6		

Abbreviations: AS: artificial surfaces; UF: urban fabric; ANA: artificial non-agricultural vegetated areas; W: wetlands; AA: agricultural areas; AL: arable land; P: pasture; HAA: heterogeneous agricultural areas; A: land principally occupied by agriculture.

Six of the eight honeys where imidacloprid was quantified came from the agricultural habitats category, one came from urban habitats and one came from SNH. The highest concentration of imidacloprid quantified came from the agricultural habitat category. A moderate negative relationship was observed between arable land and the concentration of imidacloprid in honey; however, this relationship was not statistically significant (Fig. 4-7a, Table 4-9). A moderate positive relationship was observed between forestry and the concentration of imidacloprid, however, again, this relationship was not statistically significant (Fig. 4-7b, Table 4-9).

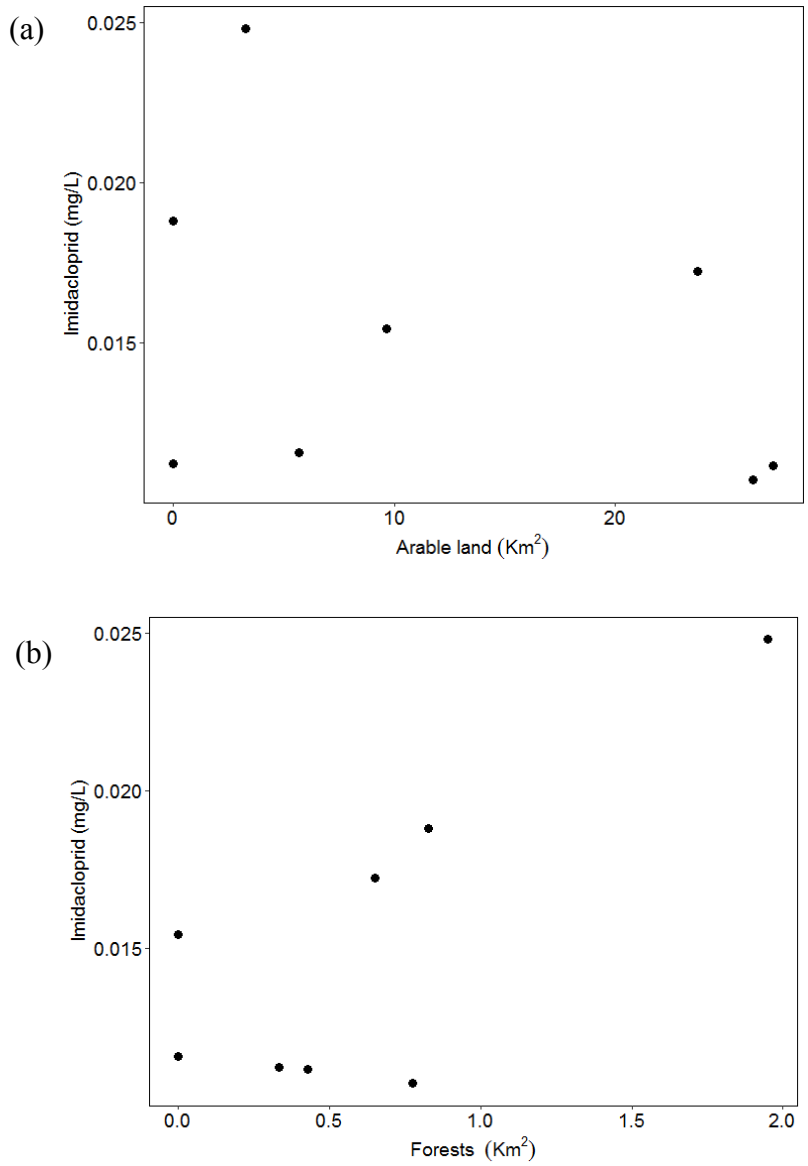

Figure 4-7 Relationship between the concentration of imidacloprid in honey (n=8) and the area of (a) arable land and (b) forestry surrounding the hive.

Table 4-9 Spearman's correlation coefficient (rho) of imidacloprid concentration in honey and land cover type (n=8).

Land cover type	rho	p-value
Artificial surfaces	-0.33	0.43
Continuous urban fabric	-0.48	0.23
Artificial non-agricultural vegetated areas	0.12	0.77
Agricultural areas	0.14	0.75
Non-irrigated arable land	-0.55	0.16
Pastures	0.33	0.43
Heterogeneous agricultural areas	-0.02	0.98
Land principally occupied by agriculture	0.06	0.88
Forest and semi-natural areas	0.30	0.47
Broad-leaved forest	0.42	0.30
Wetlands	0.00	1.00

4.4 Discussion

We have shown for the first time that Irish honeys can be contaminated with clothianidin, imidacloprid and thiacloprid. Given that these compounds have been shown to have adverse effects on both honey bees and wild bees, their detection in honey is of concern and potential contamination routes should be explored further.

Imidacloprid was the most frequently detected neonicotinoid in this study and it is currently the active ingredient of six plant protection products (PPP) approved for professional use in Ireland (18); this figure has increased from five in 2013 (20). The frequency of imidacloprid in honey samples may be due to its presence in a range of products, not limited to PPP, including ant bait and housefly bait. Pesticide application method, the quantity used and rate of usage do not indicate the exposure level of these neonicotinoids to bees. Clothianidin was the active ingredient of only one PPP approved for professional use in Ireland however a large quantity of this product was applied between 2011 and 2014 (4 times more than imidacloprid and thiacloprid (Table 4-1)). Although thiacloprid was used on a number of crops in Ireland and is available in a wide variety of products (for amateur and professional use) it was the least frequently detected neonicotinoid in our samples.

Neonicotinoids are broadly used, not only in agriculture but in plant nurseries, domestic gardens and by local authorities in public parks. Bees can be exposed to neonicotinoids

directly via contact during application to crops, and during application to amenity plants in domestic gardens and public parks and indirectly through foraging on nectar and pollen of contaminated plants and through foraging on contaminated water. Neonicotinoids are highly soluble and their uptake by non-target plants has been reported (45). A recent study reported that 70% of ornamental plants sold in the UK as "bee friendly" contained neonicotinoids (46). One plant species (*Erica carnea*) contained 10 different agrochemicals. Thiamethoxam, clothianidin and imidacloprid were quantified in pollen at concentrations between 6.9 and 81 ng/g (0.0069 and 0.081 mg/kg).

Neonicotinoids are available in many PPPs in Ireland and are used by both amateurs and professionals throughout the country. Therefore it is not surprising that honey which originates from the nectar of plants foraged by bees contains these pesticides. Exposure levels to these compounds can depend on a number of factors including but not limited to the compound's breakdown, and persistence in the environment. For example, imidacloprid is the active ingredient of Merit Turf (an insecticide control for chafer grubs and leterjackets on turf grass). This product has been discontinued in the UK but is still available in the Republic of Ireland. The half-life of imidacloprid can range from 28–1250 days (4,11) and it has been shown to accumulate in soils (47). Merit turf is a product used on golf greens in Ireland. The area of golf greens in Ireland is unknown, however there are over 430 golf courses in Ireland and the average length of a golf course is 6037 meters (48). It is likely that imidacloprid is present in Irish soils and its uptake by non-target plants is expected and this is one possible route for its presence in honey.

Differences in the number of neonicotinoids and in the number of honey samples where neonicotinoids were detected were observed between the two methods of detection used (fragmentation and calibration). Using calibration data to calculate the limits of detection is a more conservative method compared to using detection based on fragmentation. Using the latter method, the differences in the number of honey samples where neonicotinoids were detected were minor. However, the neonicotinoids identified in Irish honey have negative effects on honey bees and other non-target organisms. Brandt *et al.* (2016) showed that oral exposure of clothianidin, imidacloprid and thiacloprid affect the immunocompetance of honey bees, reducing haemocyte density, encapsulation response, and the antimicrobial activity of honey bees. The dosage concentrations of imidacloprid used in the Brandt study were lower than the average concentration quantified here (13). Imidacloprid has also been shown to have negative effects on the behaviour of bumble

bees (49) and one honey sample in this study exceeds the dosage concentration shown to negatively affect bumble bees. Although 70% of samples tested positive for at least one neonicotinoid, concentrations were below the admissible limits for human consumption according to current EU regulations (i.e. MRLs). Mitchell *et al.* detected neonicotinoids in 79% of European honey samples, most of which had multiple neonicotinoids present. The authors found that 45% of honey samples had multiple neonicotinoid contamination (36), this is similar to the value obtained here (48%). Thiacloprid dominated the European honey samples in terms of concentration. The concentration of thiacloprid found here was lower than the LOQ and cannot be compared with the European honey, however the concentration in both studies is lower than the MRL. Multiple classes of pesticides have been detected in honeys from many countries including France (33) and Poland (34). Thiamethoxam has been detected in honey from Poland (34) and Austria (31) and was quantified in 32.1 % of European honey (36). Acetamiprid has been detected in honey from Austria (31) and was quantified in 41.5 % of European honey.

The results suggest that imidacloprid is more likely to be detected in honeys from agricultural habitats including pastures; however this association was not observed for arable land. Based on the products widely in use prior to honey sampling, in which imidacloprid was the active ingredient, this finding is not surprising. These results also suggest that clothianidin is more likely to be detected in honeys originating from urban areas or landscapes containing high quantities of artificial surfaces as well as artificial non-agricultural vegetated areas. Given the high quantity of clothianidin used in Ireland it is not surprising that this compound was detected in honey from both agricultural and urban landscapes. The highest number of samples where thiacloprid was detected came from urban habitats and the results suggest that the more heterogeneous agricultural areas and land principally occupied by agriculture surrounding the hive, the less likely that thiacloprid would be detected in the honey. In 2013, thiacloprid was the active ingredient of five PPPs approved for amateur use and sold in most garden centres. The frequency of detection of this compound in honey samples from urban land covers may be due to the broad range of products available on the market.

Given the frequency of detected samples from urban areas, it is likely that a significant proportion of clothianidin and thiacloprid detected in Irish honey has originated from garden plants either applied in nurseries, garden centres or by local authorities or even by

the general public in domestic gardens. Not all garden plants sold in Ireland are grown here, and as such, the data gathered on pesticide application in Ireland may be underestimated (50). Insecticides are just one group of PPP that can be detected in honey. Glyphosate (the most widely used herbicide in the world) is the active ingredient in over 100 products approved for use in Ireland (18). It has been detected in honey from all over the world including Switzerland (51) Hawaii (52) and other US states (53) but it is not known if this chemical compound is present in Irish honey. Given the frequency of application and quantity of PPPs used in Ireland and their detection in honey from multiple countries around the world, it is highly likely that there are other contaminants in Irish honey; however, no analysis for additional PPPs was conducted in this study.

4.5 Conclusion

Our results confirm the exposure of honey bees to neonicotinoids in one of their important food stores, honey. This research for the first time has identified the presence of clothianidin, imidacloprid and thiacloprid in Irish honey. The presence of these neonicotinoids in Irish honey may have negative effects on honey bees and their presence in nectar may have negative effects on other pollinators. The concentrations detected here are below the maximum residue level authorized for human consumption. These results mirror those found in the global survey of honey by Mitchell *et al.* and therefore we can conclude that, although Irish honey can be contaminated with neonicotinoids, the concentrations are so low that it is not thought to harm human health. This research has also assessed the relationship between the three neonicotinoids and the habitat and surrounding land use in which the hive is placed. The higher proportion and concentration of neonicotinoids in honeys from both agricultural and urban habitats suggests that these environments may be more harmful for bees in terms of exposure to neonicotinoids. This land cover makes up the majority of the country. Although previous results show that urban environments have a positive relationship with honey quality in terms of phenolics, here we demonstrate that neonicotinoids are present in Irish urban honey and these pesticides may have negative impacts on bee health and thus honey production.

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Chapter 5 Conclusions and Future Work

5.1 Irish honey chemical composition

The chemical composition of honey not only affects the physical properties of the honey i.e. aroma, colour and taste but can also influence how beneficial it is to both human and bee health. This study evaluated the physiochemical properties, phenolic content and neonicotinoid composition of honeys from differing harvest seasons, floral origin, hive location, landscape composition and country of origin. This research has produced novel data on ivy honey. Honey colour correlates with electrical conductivity and with total phenolic content (TPC), although ivy honey was an exception to this. While ivy was the darkest Irish honey analysed, its TPC was less than that of heather and Manuka honeys. The Irish heather honey samples analysed were found to have the highest TPC when compared with other mono and multi-floral honeys from both Ireland and overseas. Urban multi-floral honeys contained a higher TPC than rural honeys. Physiochemical properties varied according to floral origin, and whether hives were located in urban or rural locations. Rural multi-floral honeys exhibited noticeably different physiochemical characteristics compared to the other samples analysed: rural samples had both relatively low TPC and colour. Finally, Chapter 2 confirmed that the physiochemical properties (EC, moisture, pH and colour) can be used to distinguish between floral honey types but not between multi-floral honeys from different landscapes (i.e. rural and urban honeys) and that pH is the factor that is most beneficial in differentiating between honey types. The Irish heather honeys had similar TPC and physiochemical characteristics to Manuka honeys analysed. This first examination of Irish honey confirms that TPC and physiochemical properties vary with honey type and hive location. Given the medicinal properties that have been associated with Manuka honey, these findings suggest that Irish heather honey should be examined for potential health benefits.

5.2 Relationship between land covers and honey chemistry

Human activities, including farming, forestry, housing and other industries, affect the availability of flowering plants and other forage sources for bees across landscapes. Since honey composition can vary depending on botanical origin, it was hypothesised that variation in land-use and thus vegetation composition will have an impact on the chemical composition of honey. Using honey as a tool to assess the relationship between the landscape and the potential nutritional value of the floral resources for bees within that landscape has never before been reported. This research has identified landscapes (e.g. green urban areas) that are potentially more beneficial to bees in terms of the phenol

composition and provides a greater insight into the threats facing these important pollinators, e.g. lack of floral resources for bees and the potentially reduced quality of resources, with impacts on bee nutrition.

I found that hives located in urban centres had a greater diversity of phenols present in the honey, and phenol concentration was greater in honeys from these hives compared with honeys from hives in rural areas. The area of green urban areas surrounding a hive had an overall positive relationship with the number and concentration of phenols in honey; however, this was not the case for every phenol. Agricultural land-use also influenced phenol content in that some phenols were completely absent from honey from landscapes containing a high proportion of pastures, and the area of pastures had an overall negative relationship with the concentration of a number of phenols. Temporal changes in phenolic composition of honey were also observed: some phenols were absent from honeys harvested early or late in the season. Consequently, it is tempting to conclude that urban honeys are better than rural honeys in terms of the number and concentration of phenols detected and quantified. It must be noted however that urban environments are known to contain more pollutants compared to some rural areas and there may be other substances e.g. trace metals present in urban honeys that affect the honey's composition.

I also found that Irish honey samples were contaminated with three neonicotinoids (clothianidin, imidacloprid and thiacloprid). The three neonicotinoids were detected in honeys from different habitats in Ireland (agricultural, SNH and urban). Although honeys from agricultural habitats had a higher frequency of detection of a combination of all three neonicotinoids, honey originating from urban habitats had a higher frequency of both clothianidin and thiacloprid.

The results presented here suggest that anthropogenic land-use was the main current indirect driver of the chemical composition of honey. This research has broadened the current knowledge of the chemical constituents of Irish honey.

5.3 Challenges of this research

Instrumentation: Analysis of honey is time-consuming and requires specialised instrumentation. I optimised and validated methods for analysing phenols and pesticides in honey (Chapter 3 and Chapter 4), but further research in this field was restricted by analytical sensitivity.

Honey samples: Obtaining a representative honey sample set in terms of geographical distribution was a limitation for this study. My samples were donated by Irish beekeepers, who were contacted via email, telephone and social media and agreed to provide samples for the research at no cost. However, relying on willing voluntary contributors meant I had little control over where the samples originated. As all urban honey samples originated in the greater Dublin area, this limited the interpretation of urban data.

Finally, some information relating to the honey samples was unavailable, for example, information about what plant protection products were used in the foraging environment of each of the hive sites. This was because beekeepers did not know. This was a significant limitation, particularly for the neonicotinoid analyses, as it restricted the extent to which their presence in honey samples could be correlated with local agricultural practices.

5.4 Directions for future research

Although a large body of work is available on the physiochemical properties, phenolic composition and neonicotinoid content of honey (originating outside Ireland), this is the first study to look for relationships between the Irish landscape and these chemical traits. As a result of this work some key areas which require further investigation have been identified, as discussed below.

5.4.1 Assessing bumble bee honey

A method for the extraction of bumble bee honey from managed bumble nests was developed during this research and preliminary results show that honey produced by *Bombus terrestris audax* has similar physiochemical properties and total phenolic content to honey produced by *Apis mellifera*. The mean electrical conductivity, mean sugar content and mean pH of bumble bee honey were very similar to multi-floral honey bee honey (Appendix C, Fig. C-1a - C-1c). The moisture content of bumble bee honey was lower than honey bee multi-floral honey but similar to ivy and heather honey (Appendix C, Fig. C-1d). A further evaluation of bumble bee honey in terms of the phenols present, and the relationship between phenols with the surrounding landscape may provide further insight into the relationships between land cover and honey chemistry as described in Chapter 3 i.e. how specific land-uses, for example GUAs, can be more beneficial in terms of honey quality.

Managed bumble bees are most commonly used in enclosed production systems (glasshouses and poly-tunnels) [7]. Bumble bee honey extracted during this research was obtained from nests used for the pollination of different crops (blackberry, blueberry, cherry, peppers, raspberry, and strawberry). Differences in the physiochemical properties within bumble bee honey according to crop type were observed (Appendix C, Fig. C-2a - C-2d). Given these differences, bumble bee honey could be used to assess differences in pesticide contamination depending on what crop the bees foraged. Due to their high risk to honey bees, from December 2018, two of the neonicotinoids detected in Irish honey (reported in Chapter 4) will no longer be used outdoors within the EU. However, they will be used on crops in permanent greenhouses once the crop stays within the greenhouse during its entire life cycle (EU Regulations 2018/783, 2018/784, 2018/785) (2). Bumble bee honey harvested from managed bees used in controlled environments (e.g. permanent greenhouses) may be a useful matrix to monitor the exposure levels of these neonicotinoids to bees in the future. Enclosed systems are ideal environments to study the accumulation of neonicotinoids in honey and the pathway of neonicotinoids (i.e. from plant to bee to honey) in a controlled experiment. These systems could also provide information on how the different neonicotinoids, or other pesticides, accumulate in honey depending on the crop the bees foraged on.

5.4.2 Confirming the floral origin of honey

It is common that honeys from different botanical origins have distinctive organoleptic properties (colour, aroma and taste). The differences in honeys' aroma are due primarily to differences in the volatile compound composition (3). Therefore understanding the structure and content of volatile compounds in honey might result in a better understanding of honey's flavour, botanical origin and its potential human and bee health properties. This could provide further understanding of honey physiochemistry and could have practical applications in the food, regulatory and medical industries. The volatile fraction of honey has previously been used to determine the botanical origin of ivy honey (4). Makowicz *et al.* (2018) proposed that headspace solid phase micro-extraction (HS-SPME) coupled with GC-MS can provide a simple visual determination of honey's authenticity (4). The volatile fraction of heather honey has never before been reported and HS-SPME may provide further insight into the potential medicinal properties of heather honey.

The question of whether the fluorescence characteristics of Irish honey can be used as an indicator of honey's floral origin remains unanswered. Characteristic excitation–emission spectras have previously been used to differentiate between honey types (5). Identifying characteristic fluorescence profiles in honeys from different floral origins may give an indication of the floral origins of honey. Measuring honey fluorescence has potential as a rapid and non-destructive method to assess honey authentication and adulteration in the food regulatory industry.

5.4.3 The relationship between land use and honey chemistry

Modifications in land-use can affect the botanical origin and quantity of nectar collected by honey bees and thus the honey produced. Floral resource abundance throughout the year and floral diversity are important for the honey bee's diet and are thus important for bee health (6), as well as honey production. Some of the most important components in honey from a human health point of view are phenols (7). The variation in agricultural management practices may explain, in large part, the variation in phenol composition found here. Given that less intensive/semi-natural grazed grassland can contain higher floral diversity compared to intensively managed grassland (8), it is not surprising that the honey from such areas has a higher diversity of phenols.

Understanding the relationship between honey composition, honey quality, and land-use could enable characterisation of habitat composition and configuration which results in high quality honey that could promote both bee and human health. This understanding could also provide information to beekeepers to support their decisions as to where to place hives to optimise honey quality and could indicate the value of different habitat types for other pollinators, including wild bees, which may be useful for bee conservation policies. Honey composition profiles also have the potential to be linked to the floral source and therefore provide some insight into bee foraging patterns and preferences.

This study has identified for the first time elements of landscapes i.e. specific land-uses that can be beneficial (in terms of honey quality) for honey production, for example green urban areas (GUAs). Given the range of habitats associated with GUA within the CORINE data set, a more in-depth analysis of areas classified as GUA could provide additional insight into the potential correlations between landscape and phenol content. Given that some phenols have been shown to promote many beneficial processes in bees, these results

support the hypothesis that differences in both the abundance and diversity of pollinators in anthropogenic habitats may be driven by a direct impact of how land-use influences floral resources (9). Recording the plant species present around each of the hive sites would provide further insight into exactly what species are available for the bees. By comparing the phenol composition of the nectar of these species with the phenol composition of the honey, the exact plant species on which the bees are foraging could be identified. This information would add to the current knowledge of bee foraging preference but could also identify plant species that are potentially more beneficial for bee health. By combining the data of floral resource availability and phenol composition profiles, the preferred plant species of the honey bee could be identified. However, ground-truthing to an appropriate area (up to 5 km radius) around each hive site would be very time-consuming, and was beyond the scope of this research.

5.4.4 Essential and toxic elemental composition of Irish honey in relation to anthropogenic activity

Honey has been shown to be a sensitive and accurate barometer of heavy metal contamination (10). Honey toxic element analyses, specifically identifying and quantifying heavy metals associated with potential health risks have been carried out primarily using atomic absorption spectrometry (10) or inductively coupled plasma atomic emission spectrometry (11). Examples of studies include detection of four heavy metals in commercially bought honeys in Greece (11), three heavy metals in honeys from Italy (12) and three heavy metals in Polish honey (10). Honey heavy metal content can be used to assess food contamination and bee health as increased metal concentration can have negative effects on honey bees (13). The economic value of insect pollination to Irish rape seed oil alone has been estimated as €3.9 million per annum (14). It may therefore be beneficial for Irish crop production to determine if there are increased levels of metals in honey that could affect pollination services, and therefore crop production. Additionally, using honey to identify potential environmental detriments has not previously been carried out in Ireland. Lead is one of the most widespread metal pollutants in regions in Ireland and has no beneficial role in human metabolism. It can cause progressive toxicity and health disorders such as fatigue, sleeplessness and hearing and weight loss in humans (15). Little research has been carried out on the effects of heavy metals on bees; however, recent studies have shown that metals had an effect on the overall hive health status by lowering honey production, increasing dead pupae in capped cells, and lowering the relative pupae

growth index (13,16). It will therefore be advantageous for beekeepers to know what locations in Ireland are related to higher honey metal concentrations and will be beneficial to the growing Irish honey market. Metals can be harmful to humans and bees and identifying them in honey can be useful for detecting environmental pollution. An essential element profile for single origin Irish honeys (for example heather honey) can be beneficial in proving authenticity and enabling traceability, and can therefore add value to established products and contribute to growth in the Irish food industry. By investigating the essential and toxic elemental composition of Irish honey and comparing it with land cover, further insight can be gained into the relationship between anthropogenic activity and honey chemistry. This research will also provide an understanding of the effect of urbanisation on the metal and essential element content of honey and will provide a profile for Irish honey in terms of its essential elements.

5.4.5 Other contaminants in honey

Although the results presented in this thesis show that urban environments have a positive relationship with honey quality in terms of phenolics, there may be negative impacts beyond neonicotinoid content, on honey that were not assessed here. Given that air quality improves as areas become more rural (17), honey originating from urban areas may contain more contaminants. Urban environments have been linked to a reduction in the honey bee's ability to recognise floral odours by altering floral volatiles (18,19) and various studies have detected environmental pollutants in bees, honey and pollen (20). Nectar chemical composition has been shown to alter the foraging behaviour of *Bombus koreanus* and *Apis mellifera* (21) and diesel fumes can affect floral scent (19). To our knowledge there are no data available on the environmental pollutants in nectar.

Because honey can be contaminated not only by neonicotinoids but by a multitude of agrochemicals such as other systemic chemicals (22) and pyrethroid insecticides (20), a multi-residue analysis of these honey samples is likely to give more information on the pesticide contamination of honey. It is likely that honey bees are subject to year-long routes of pesticide exposure and therefore it also recommended that analysis of the temporal changes in pesticide composition of honey be carried out to determine at what times of the year honey bees are more frequently exposed.

Neonicotinoids are likely to be bioavailable to honey bees and potentially to other pollinators throughout the year. Given that the moratorium on two of these pesticides was introduced in 2013 it is suggested that honeys from the same 30 hive sites are collected in the future and analysed using the methods detailed herein to better understand the longevity of these pesticides in the landscape.

5.5 Implications for policy

As well as producing honey, a wide variety of crops are pollinated by honey bees, (23) and visitation by bees has been shown to increase crop yield (24). Thus honey bees do not just contribute to our economies via honey production, but insect pollination of food crops for human consumption. The pollination services of bees has an estimated economic value of €14.2 billion per year in the EU alone (25). Despite this, bees are currently under threat in Europe (26) and in need of conservation. The protection of the earth's biodiversity is important not only for its economic value but also to protect the non-market and intrinsic value of nature. Regrettably, the value of biodiversity to economies and societies is often not reflected in either public policy or private enterprise decisions. However, the aim of the EU's Biodiversity Strategy adopted in 2011 is to protect and restore biodiversity and the services it provides. In Ireland we have adopted the All Ireland Pollinator Plan (AIPP), which has been endorsed by the National Biodiversity Plan (27) which aims to build the foundations towards creating a landscape where pollinators can flourish, and explicitly supports beekeepers (28). My research provides evidence on where beekeepers can place hives to optimise honey quality (in terms of phenols in honeys). This may help beekeepers achieve sustainable populations of managed honey bees (one of the aims of the AIPP) by placing hives in these locations. However, it is important that the sustainable populations of managed bees do not negatively impact wild bee populations or other pollinators by increasing competition for forage resources. My research has detected pesticides in honey and provides evidence that honey bees are exposed to pesticides in honey at concentrations that have been shown to cause sub-lethal effects. There is a growing body of evidence that agrochemicals have many sub-lethal effects on pollinators (20,29–33) and thus the pollination services they provide. For a PPP to get onto the market, the concentration at which it is applied must only kill half the members of a tested population after a specified test duration (the LD_{50}); other negative effects are not tested. Given their widespread

occurrence and the concentrations found in Irish and other honeys that have been associated with sub-lethal effects it is recommended that the criteria for approving PPP at EU level be re-assessed and a more sensitive method be developed that includes the testing of sub-lethal effects as well as the lethal effects.

5.6 Concluding remarks

The research discussed in this thesis shows that Irish honey is a high quality product that society should value more. By showing that the phenolic content in Irish heather honey is similar to that of manuka honey, there is more potential to increase the commercial value of Irish heather honey. The high phenolic content of manuka honey is just one characteristic that indicates the human health benefits of this honey brand. Other characteristics such as the anti-bacterial and anti-microbial properties are widely recognised. Although this research did not examine the anti-bacterial or anti-microbial properties of Irish heather honey there is no reason to believe that they would be dissimilar to those of manuka honey. Because of this research, interest in beekeeping and honey production may increase in Ireland. More hives may now be located where there is heather, and so more use may be made of Irish bogs and heaths while preserving them and these complex, dynamic ecosystems which are not found elsewhere. This research may also be useful to strengthen the case for the conservation of our peatlands. However, an increase in the number of honey bee populations in these landscapes may have negative knock-on effects to wild bee populations. The quantity and quality of wildlife habitats are highly threatened by human activities and bees are just one group of organisms that are affected. There are currently 2,000 species of bees native to Europe and 99 of these native to Ireland. The honey bee is just one of these bee species, however, because of the numerous commercially extractable products it provides; the honey bee surpasses all other bees in terms of its popularity with the general public. With regard to food security, the pollination services provided by bees (not just the honey bee) are essential and a significant decrease in bee populations has the potential to negatively influence crop yields. In recent years, declining bee population trends have been observed throughout Europe (26), however, the population trend for 79% of Europe's bees (including the honey bee) is unknown. Recent studies have reported a considerable decline in bee colony health and a decline in the number of managed honey bee colonies (34–36). These declines have been associated with

habitat loss, a reduction in floral resources and the use of neonicotinoids and other agrochemicals. Continued evidence-based research, building on the work detailed here, in the development of new land policies and with regard to changes in land management, is urgently required to support efforts to reverse or even reduce losses in bee diversity and a potential resultant decrease in crop pollination. This research has illustrated that honey provides an ideal matrix to evaluate the extent to which pollinators interact with their foraging landscape.

We need to try to find a balance between enabling beekeepers to produce a good valuable product and, protecting biodiversity and pollination services. Both can be achieved with the right policies in place. Habitats rich in botanical diversity are not only good for bees but also good for people's health and well-being and for healthy functioning ecosystems and ultimately the whole planet.

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Appendices

Appendix A

Table A-1 Compounds characteristic to unifloral honeys.

Floral honey type	Chemical Marker	Geographical Origin	Author
Asphodel	Methyl syringate	Sardinia	Tuberoso, 2009 (1)
Chestnut	4-Hydroxybenzoic acid Phenylacetic acid Ferulic acid	Europe	Dimitrova *et al.* 2007 (2)
Citrus	Hesperetin	Europe	Ferres *et al.* 1991 (3); Tomás-Barberán *et al.* 2001 (4)
Heather (*Calluna vulgaris*)	Phenylacetic acid	Europe	Guyot *et al.* 1998 (5)
Lime	3-Hydroxybenzoic acid	Germany & Netherlands	Dimitrova *et al.* 2007 (2)
Rapeseed	cis-trans-Abscisic acid trans-trans-Abscisic acid Kaempferol 8-Methoxykaempferol Quercetin	Europe	Dimitrova *et al.* 2007 (2)
Rosemary	Kaempferol	Europe	Gil *et al.* 1995 (6); Tomás-Barberán *et al.* 2001 (4)
Strawberry tree	Homogentisic acid	Sardinia	Cabras *et al.* 1999 (7); Tuberoso 2010 (8)
Sunflower	Quercetin	Europe	Gil *et al.* 1995 (6); Tomás-Barberán *et al.* 2001 (4)

Table A-2 Total flavonoids of honeys from various geographical locations expressed as milligrams of quercetin equivalents (QE) per 100 g honey.

QE concentration (mg QE / 100g)	Country of origin	Floral origin	Author
5.8 ± 2.2	Spain	Blackberry	Escuredo *et al.*2013 (9)
7.7 ± 2.5	Spain	Chestnut	Escuredo *et al.*2013 (9)
4.2 ± 1.3	Spain	Eucalyptus	Escuredo *et al.*2013 (9)
9.58 ± 0.03	Tunisia	Eucalyptus	Boussaid *et al.* 2014 (10)
6.5 ± 2.0	Spain	Heather	Escuredo *et al.*2013 (9)
11.03± 0.07	Tunisia	Horehound	Boussaid *et al.* 2014 (10)
22.45 ± 0.1	Tunisia	Mint	Boussaid *et al.* 2014 (10)
5.5 ± 1.8	Spain	Multi-floral	Escuredo *et al.*2013 (9)
2.57 ± 2.09	Africa	Multi-floral	Meda *et al.* 2005 (11)
6.11	Brazil	Multi-floral	Sant'ana *et al.* 2013 (12)
3.52 ± 1.19	Argentina	Multi-floral	Ciappini and Stoppani 2014 (13)
11.12 ± 0.03	Tunisia	Orange	Boussaid *et al.* 2014 (10)
16.24 ± 0.08	Tunisia	Rosemary	Boussaid *et al.* 2014 (10)
14.77 ± 0.04	Tunisia	Thyme	Boussaid *et al.* 2014 (10)

Table A-3 Total phenolic content of honeys from various geographical locations expressed as milligrams of gallic acid equivalents (GAE) per 100 g honey.

GAE concentration (mg GAE/ 100 g)	Country of origin	Floral origin	Author
1.56 ± 0.105	Malaysia	Coconut	Aljadi and Kamaruddin 2002 (14)
2.14 ± 0.129	Malaysia	Gelam	Aljadi and Kamaruddin 2002 (14)
62.756 ± 4.403	Germany	Black Forest (Acacia)	Alzahrani *et al.*2012 (15)
11.142 ± 0.354	Saudi Arabia	Lavender	Alzahrani *et al.*2012 (15)
89.909 ± 1.175	New Zealand	Manuka (Medihoney®)	Alzahrani *et al.*2012 (15)
50.0309 ± 0.829	Algeria	Wild carrot	Alzahrani *et al.*2012 (15)
5.52 ± 0.28	Unknown	Acacia	Beretta *et al.* 2005 (16)
48.22 ± 0.24	Mexico	Buckwheat	Beretta *et al.* 2005 (16)
21.12 ± 0.55	Unknown	Chestnut	Beretta *et al.* 2005 (16)
6.71 ± 0.56	Unknown	Clover	Beretta *et al.* 2005 (16)
10.21 ± 1	Unknown	Dandelion	Beretta *et al.* 2005 (16)
2.556 ± 0.75	Unknown	Honeydew	Beretta *et al.* 2005 (16)
17.04 ± 0.17	Unknown	Multi-floral	Beretta *et al.* 2005 (16)
78.96 ± 1.38	Unknown	Strawberry tree	Beretta *et al.* 2005 (16)
20.16 ± 1.68	Poland	Buckwheat	Kaškonienė *et al.* 2009 (17)
20.12 ± 0.55	Poland	Heather	Kaškonienė *et al.* 2009 (17)
15.31 ± 0.55	Poland	Lime	Kaškonienė *et al.* 2009 (17)
7.17 ± 0.13	Poland	Rape	Kaškonienė *et al.* 2009 (17)
2.62 ± 0.056	Malaysia	Gelam	Khalil *et al.* 2011 (18)
5.263 ± 0.121	New Zealand	Manuka Honey Active 5+ Comvita	Khalil *et al.* 2011 (18)
0.24 ± 0.15	Turkey	Rhododendron	Silici *et al.*2010 (19)
108.21	Turkey	Chestnut	Sagdic *et al.* 2013 (20)
141.83 ± 1.68	Turkey	Rhododendron	Silici *et al.*2010 (19)

Appendix B

B1 Detailed information on phenol extraction and analysis

All chemicals and reagents were analytical or HPLC grade. Water was deionised using a Mili-Q water purification system to a resistance of 18 MΩ cm @ 298 K. Methanol, phosphoric acid and amber vials and screw caps were purchased from Sigma Aldrich Ireland. Phenolic acids (benzoic, chlorogenic, ellagic, ferulic, gallic, homogentisic, 2, 4, dihydroxybenzoic, p-coumaric, o-coumaric, salicylic and trans-cinnamic) and flavonoids (chrysin, galangin, luteolin, myricetin, pinocembrin, quercetin, and rutin), commonly found in honey samples were used as reference compounds. All reference compounds were obtained from Sigma Aldrich Ireland. VWR Syringe filters with nylon membrane, (pore size 0.45 µm and 25mm diameter), LiChrosorb® C18 reversed phase column (LiChroCART® 125 x 4 mm, particle size 5µm), manu-CART® NT cartridge holder and LiChrospher® 100 RP-18 5µm guard cartridge were all purchased from VWR International Ltd.

Solid phase extraction (SPE) was used to extract phenolic compounds from the honey samples. Initially, five grams of honey was dissolved in 50 mL of acidified water (pH 2.0, achieved with trifluoroacetic acid) using a stir plate. The phenols were adsorbed onto preconditioned C18 cartridges (Supelco, Supelclean LC-18, 0.5g, Sigma Aldrich). Preconditioning was achieved by sequentially passing 3 mL each of methanol and acidified water (pH 2.0) at a drop wise flow rate. Subsequently 10 mL of honey solution was passed through the cartridge at a drop wise flow rate to ensure efficient absorption of phenols. Finally elution was achieved by passing 1.5 mL of 90% (v/v) methanol/water solution. These three steps were repeated 3 times and the elutes were combined. The resulting solution (4.5 mL) of each eluted sample was stored in an amber vial in the fridge at 4℃.

The HPLC system consisted of a Shimadzu LC-20AD XR solvent delivery module coupled with SPD 20A UV/VIS detector, DGU-20A 5R degassing unit and SIL-20AC XR auto sampler. The system was operated using Lab Solutions Lite 5.82 chromatography software. UV chromatograms were recoded using this software and were then imported into Microsoft Office Excel. A LiChrosorb® C18 reversed phase column (LiChroCART® 125 x 4 mm, particle size 5µm) and guard column was used.

A binary mobile phase was employed consisting of methanol with 0.1% phosphoric acid (A) and ultra-pure water with 0.1% phosphoric acid (B) with elution from the column with the following gradient: 0 min to 3 min A increased from 30% to 35%; 3−18 min A increased to 55%; 18-25 min A increased to 62%; 25-30 min A increased to 85%; 30-35 min A decreased to 35% and was kept constant until 40 min. A 40 µL injection volume, a flow rate of 0.5 mL min-1 and a 40 min runtime were the selected parameters with UV detection at 265 nm. The identification of eluted components was achieved by comparing the retention time of reference standards with the honey samples. Further confirmation was achieved by visually inspecting the chromatograms. Quantification was achieved using calibration curve data. Calibrations were carried out within the range of 0.1 to 100 mg/L. Limits of detection (LOD) and limits of quantification were determined using the calibration data.

Three replicates of each honey sample were analysed except for three honey samples where two replicates were analysed. Any peaks with a RSD greater than 5% were disregarded.

B2 Detailed information of the estimation of total phenolic content

Honeys were analysed using a modified Folin-Ciocalteu method (11,21) to determine total phenolic content (TPC). Each honey sample was dissolved in deionised water (10% w/v) and filtered through Whatman No. 1 filter paper (pore size 11µm). This solution (0.5 mL) was mixed with 2.5 mL of 0.2 N Folin-Ciocalteu reagent for 5 min, and 2 mL of 75 g/L sodium carbonate was then added. After incubation for one hour at room temperature the absorbance of the reaction mixture was measured at 760 nm against a methanol blank (Varian Cary® 50 UV-Vis Spectrophotometer). The mean of three replications was recorded and results were expressed as mg of gallic acid equivalents (GAE) per 100 g of honey.

B3 Supplementary tables and figures for Chapter 3

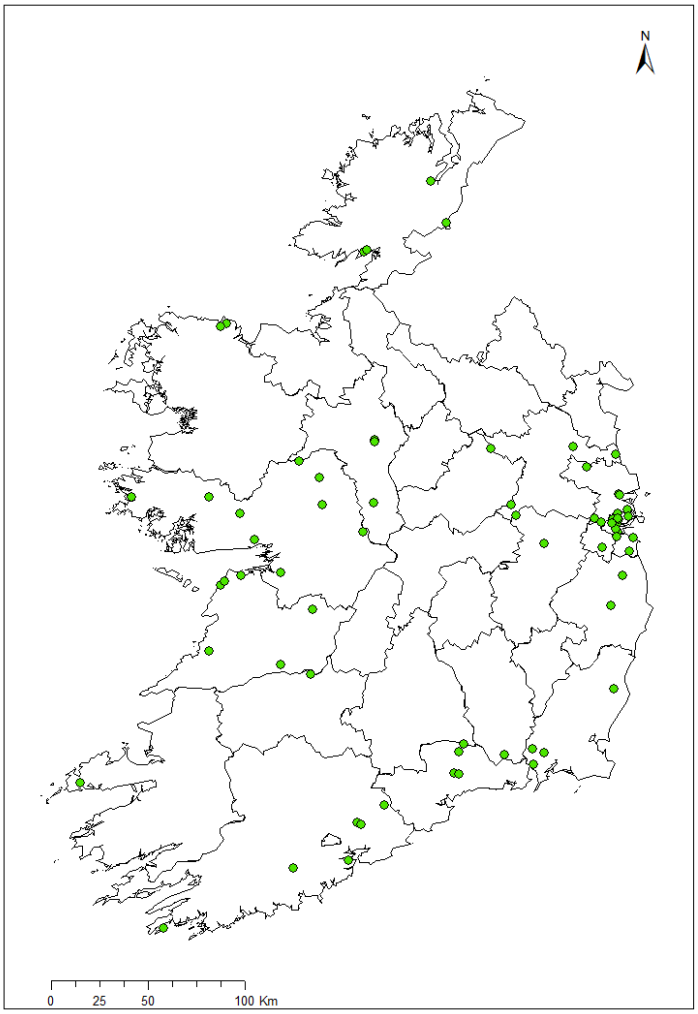

Figure B3-1 Honey sampling sites across the island of Ireland (n=76).

Table B3-1 list of land covers used in analysis

Code	Name
111	Continuous urban fabric
112	Discontinuous urban fabric
122	Road and rail networks and associated land
141	Green urban areas
142	Sport and leisure facilities
211	Non irrigated arable land
231	Pastures
242	Complex cultivation patterns
243	Land principally occupied by agriculture
311	Broad leaved forest
312	Coniferous forest
313	Mixed forest
314	Natural grassland
322	Moors and heathland
324	Sclerophyllous vegetation
331	Transitional woodland scrub
333	Sparsely vegetated areas
411	Inland marches
412	Peat bogs
421	Salt marches
423	Intertidal flats
511	Water courses
512	Water bodies
521	Coastal lagoons
522	Estuaries
523	Sea and ocean

Table B3-2 Summary statistics associated with of each phenol detected and/or quantified in honey samples. The maximum concentration is given in mg/L together with mean, the standard deviation, the standard error, the limits of detection (LOD) and quantification (LOQ) (all units in mg/L) and the percentage of samples in which LOQ were quantified.

Phenol	Number detected	Number quantified	Maximum conc.	Mean	Stdev	SE	LOD	LOQ	>LOQ (%)
Benzoic acid	49	27	72.42	4.71	12.12	2.33	1.39	4.22	26
Chlorogenic acid	15	5	61.55	1.25	6.97	3.12	3.42	10.38	5
Chrysin	8	3	1.12	0.02	0.12	0.07	0.08	0.24	3
Ellagic acid	44	11	18.95	0.83	2.85	0.86	1.10	3.33	11
Ferulic acid	5	3	3.87	0.11	0.62	0.36	0.90	2.74	3
Galangin	3	2	1.79	0.03	0.22	0.16	0.41	1.25	2
HBA	85	43	13.19	1.53	2.45	0.37	0.59	1.80	42
Homogentisic acid	2	2	17.89	0.22	1.82	1.29	0.79	2.39	2
Luteolin	31	11	19.47	0.50	2.14	0.65	0.53	1.60	11
Myricetin	68	36	35.92	2.37	5.12	0.92	1.19	3.62	30
O-Coumaric acid	22	12	39.68	0.74	4.20	1.40	0.53	1.60	9
p-Coumaric acid	100	95	24.81	4.00	3.42	0.35	0.37	1.13	92
Pinocembrin	53	5	15.74	0.65	2.91	1.30	3.71	11.25	5
Quercetin	41	11	7.86	0.32	1.14	0.34	0.34	1.04	11
Rutin	92	80	96.64	7.97	15.04	1.68	0.36	1.10	78
Salicylic acid	17	4	32.24	0.71	4.36	2.18	2.56	7.76	4
trans-Cinnamic acid	27	6	3.89	0.13	0.55	0.22	0.39	1.17	6

Table B3-3 Validation parameters for the quantification of phenols in Irish honey: calibration range (linearity), limits of detection (LOD) and quantification (LOQ) and precision (%RSD).

Phenol	Linearity (mg/L)	LOD (mg/L)	LOQ (mg/L)	Precision (%RSD)
Benzoic acid	0.5-100	1.39	4.22	2.31
Chlorogenic acid	0.1-100	3.42	10.38	3.17
Chrysin	0.1-15	0.08	0.24	0.73
Ellagic acid	0.1-20	1.10	3.33	3.37
Ferulic acid	0.5-15	0.90	2.74	1.86
Galangin	0.5-15	0.41	1.25	5.54
HBA	1-15	0.59	1.80	3.78
Homogentisic acid	5-20	0.79	2.39	0.73
Luteolin	1-15	0.53	1.60	0.54
Myricetin	1-20	1.19	3.62	0.61
O-Coumaric acid	0.5-100	0.53	1.60	0.78
p-Coumaric acid	0.5-20	0.37	1.13	1.73
Pinocembrin	1-15	3.71	11.25	1.59
Quercetin	0.1-20	0.34	1.04	0.51
Rutin	0.5-100	0.36	1.10	0.68
Salicylic acid	5-20	2.56	7.76	1.24
trans-Cinnamic acid	0.1-20	0.39	1.17	0.65

Table B3-4 Highest concentration of each phenol from a single honey sample and the harvest season, degree of urbanisation and dominant land covers (>30% total area) in which that concentration was found.

Phenol	Concentration (mg/L)	Harvest season	Degree of urbanisation	Dominant land covers
Benzoic acid	72.42	Later	Rural area	412
Chlorogenic acid	61.55	Later	Urban centre	112
Chrysin	1.12	Late	Urban centre	112 & 231
Ellagic acid	18.95	Early	Rural area	231 & 322
Ferulic acid	3.87	Late	Urban centre	112
Galangin	1.79	Late	Rural area	231
HBA	13.19	Late	Urban centre	112
Homogentisic acid	17.89	Late	Urban centre	112 & 231
Luteolin	19.47	Later	Rural area	231
Myricetin	35.92	Later	Rural area	312 & 412
O-Coumaric acid	39.68	Later	Rural area	312 & 412
p-Coumaric acid	24.81	Later	Rural area	312 & 412
Pinocembrin	15.74	Late	Urban centre	112
Quercetin	7.86	Mid	Urban centre	112
Rutin	96.64	Early	Rural area	231
Salicylic acid	32.24	Late	Rural area	231
trans-Cinnamic acid	3.89	Late	Rural area	231

Table B3-5 Kruskal-Wallis analysis of variance for each phenol according to honey harvest season (early, mid, late and later), chi-squared values, and p values (p) for each phenol are listed. Degrees of freedom (df) = 3. P values <0.05 are in bold.

Phenol	Kruskal-Wallis chi-squared	p
Benzoic acid	14.55	**0.002**
Chlorogenic acid	3.72	0.293
Ellagic acid	8.38	**0.039**
Ferulic acid	1.05	0.790
HBA	8.30	**0.040**
Luteolin	14.24	**0.003**
Myricetin	3.25	0.355
o-Coumaric acid	11.37	**0.010**
p-Coumaric acid	4.24	0.237
Pinocembrin	0.65	0.885
Quercetin	13.03	**0.005**
Rutin	6.90	0.075
trans-Cinnamic acid	6.61	0.085
TPC	10.81	**0.013**

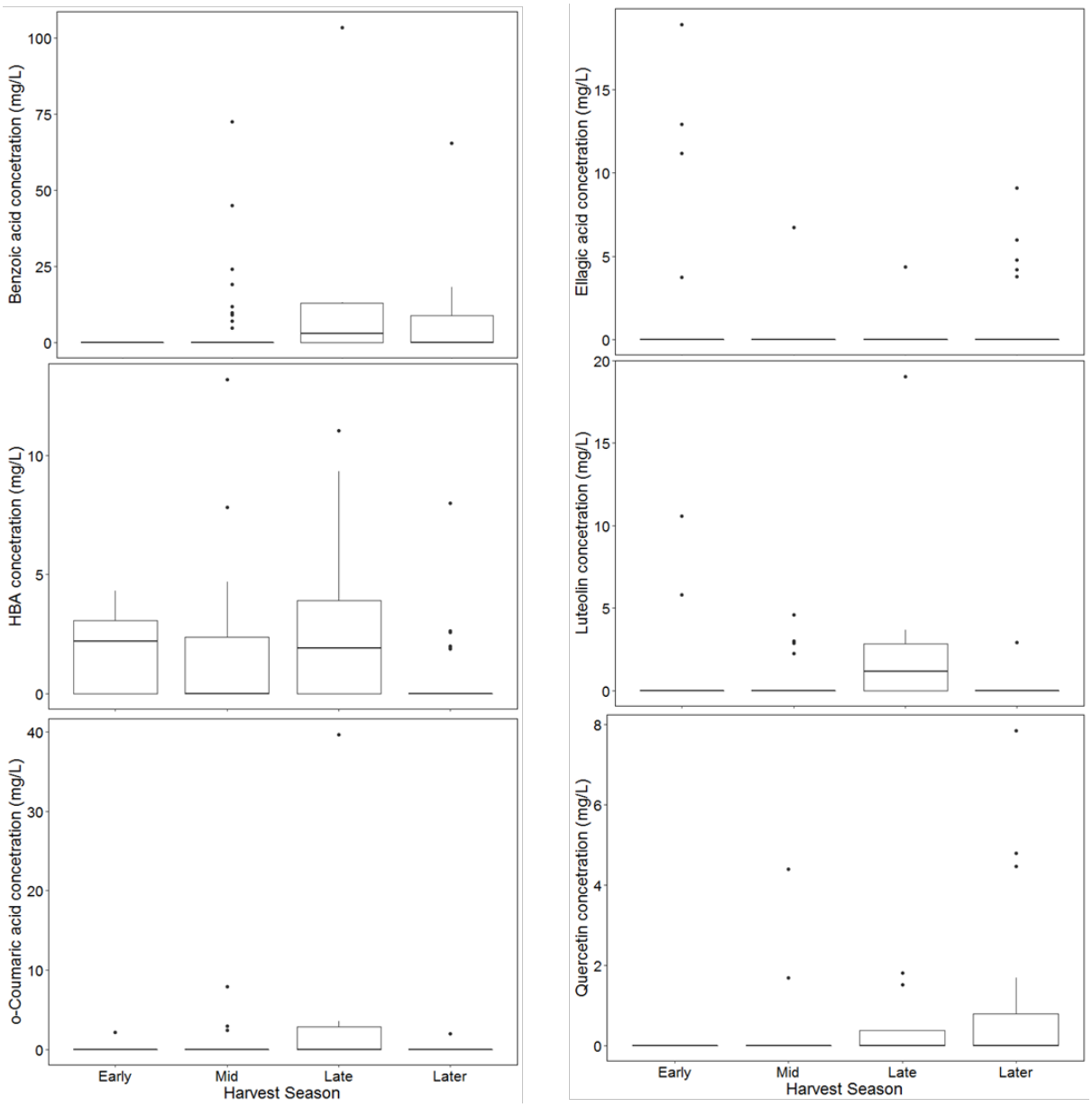

Figure B3-2 Concentrations of six phenols in honey samples that showed significant differences in median across the four harvest seasons, early (May, n=18), mid (June-July, n=26), late (August-September, n= 50) and later (October, n=8). Box plots represent the median (horizontal line in the box), 1st and 3rd quartiles (bottom and top box lines), the upper and lower whiskers and the outliers (dots).

Table B3-6 Statistically significant results from Wilcoxon's signed rank test of harvest season and phenol concentration. The results are show for four phenols (BE = benzoic acid, HBA = 2, 4 hydroxybenzoic acid, Qu= quercetin and Ru = rutin) and TPC (total phenolic content). Medians (M) and the range per harvest season are given along with the test statistic (V) (* = $p<0.05$, ** = $p<0.005$).

Phenols	Tested seasons	Early M	Mid M	Late M	Later M	Early range	Mid range	Late range	Later range	V
BA	Mid & Late	0	0	0	2.77	0	0 - 72.4	0-65.5	0-103.6	86*
HBA	Early & Mid	2.2	0	0	1.9	0-4.3	0-8	0-13	0-11	72*
HBA	Early & Late	2.2	0	0	1.9	0-4.3	0-8	0-13	0-11	67*
Qu	Mid & Late	0	0	0	0	0	0-7.9	0-4.4	0-1.8	0*
Ru	Early & Late	9.12	2.70	3.14	3.62	0-96.1	0-74.8	0-26.6	0-28.8	13**
TPC	Early & Late	13.6	20.3	25	21.3	6.4-33.5	9.9-35.6	2.6-63.3	3.5-33	142*

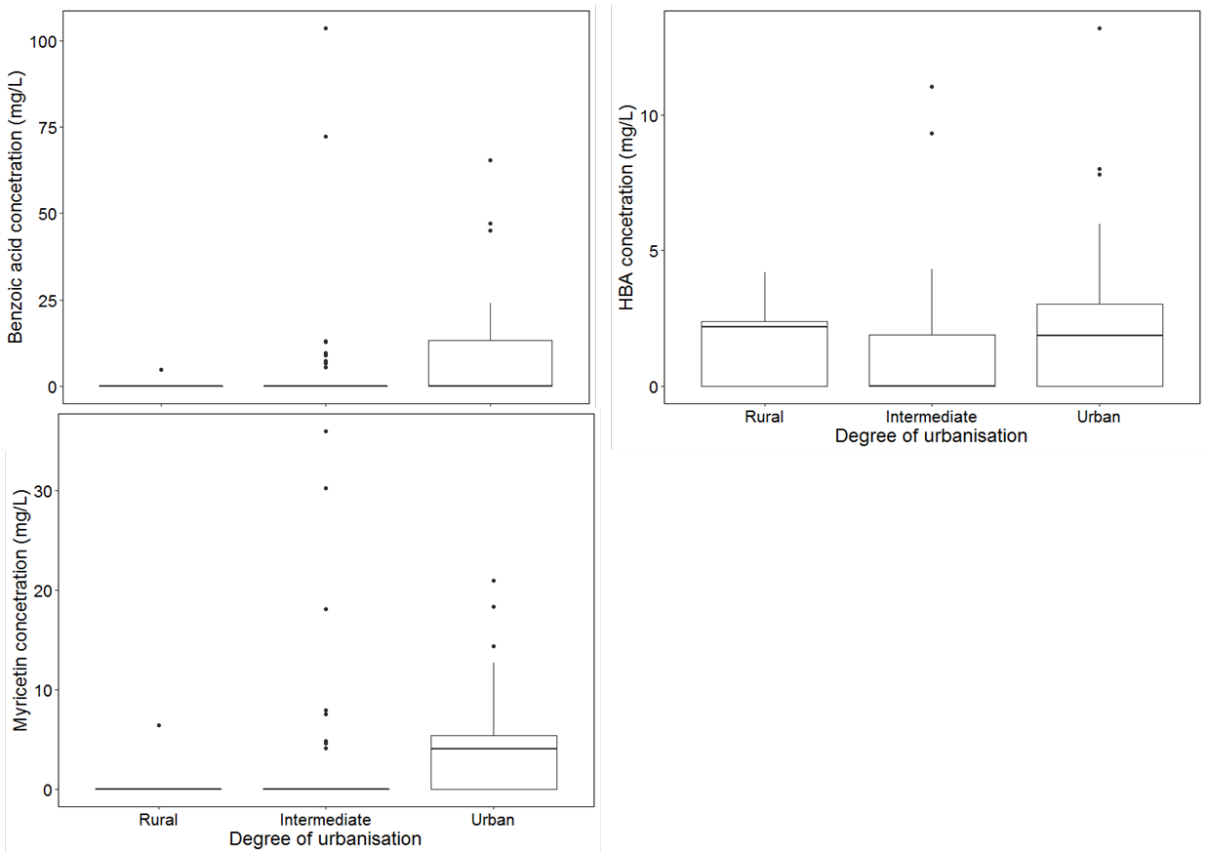

Figure B3-3 Concentrations of three phenols in honey samples that showed significant differences in median across the three levels of urbanisation, rural (n=56), intermediate (n=9) and Urban (n=38). Box plots represent the median (horizontal line in the box), 1st and 3rd quartiles (bottom and top box lines), the upper and lower whiskers and the outliers (dots).

Table B3-7 Kruskal-Wallis analysis of variance for each phenol according to degree of urbanisation (rural area, intermediate, urban centre), chi-squared values, and p values (p) for each phenol are listed. Degrees of freedom (df) = 2. P values <0.05 are in bold.

Phenol	Kruskal-Wallis chi-squared	p
Benzoic acid	9.16	**0.010**
Chlorogenic acid	9.14	**0.010**
Ellagic acid	0.01	0.996
Ferulic acid	5.38	0.068
HBA	6.72	**0.035**
Luteolin	3.90	0.142
Myricetin	20.03	**0.000**
o-Coumaric acid	0.56	0.757
p-Coumaric acid	2.98	0.225
Pinocembrin	1.05	0.593
Quercetin	4.49	0.106
Rutin	3.97	0.138
trans-Cinnamic acid	0.50	0.778
TPC	13.41	**0.001**

Table B3-8 Statistically significant results from Wilcoxon's signed rank test of degree of urbanisation and phenol concentration. The results are show for four phenols (BA = benzoic acid, HBA = 2, 4 hydroxybenzoic acid and My= myricetin) and TPC (total phenolic content). Medians (M) and the range per harvest season are given along with the test statistic (V) (* = p<0.05, ** = p<0.005, *** = p<0.001).

Phenol	Tests	Rural M	Intermediate M	Urban M	Rural range	Intermediate range	Urban range	V
BA	Rural V Urban	0.00	0.00	0.00	0-72.4	0 - 4.7	0-65.5	169**
HBA	Rural V Urban	0.00	2.22	1.85	0-11.3	0-4.3	0-13.2	236*
HBA	Intermediate V Urban	0.00	2.22	1.85	0-11.3	0-4.3	0-13.2	21*
My	Rural V Urban	0.00	0.00	3.81	0-39.5	0-6.4	0-18.4	248**
TPC	Rural V Urban	18.54	20.60	26.40	2.6-54.6	9.2-58	10.4-63.3	601***
TPC	Intermediate V Rural	18.54	20.60	26.40	2.6-54.6	9.2-58	10.4-63.3	41*

Appendix C

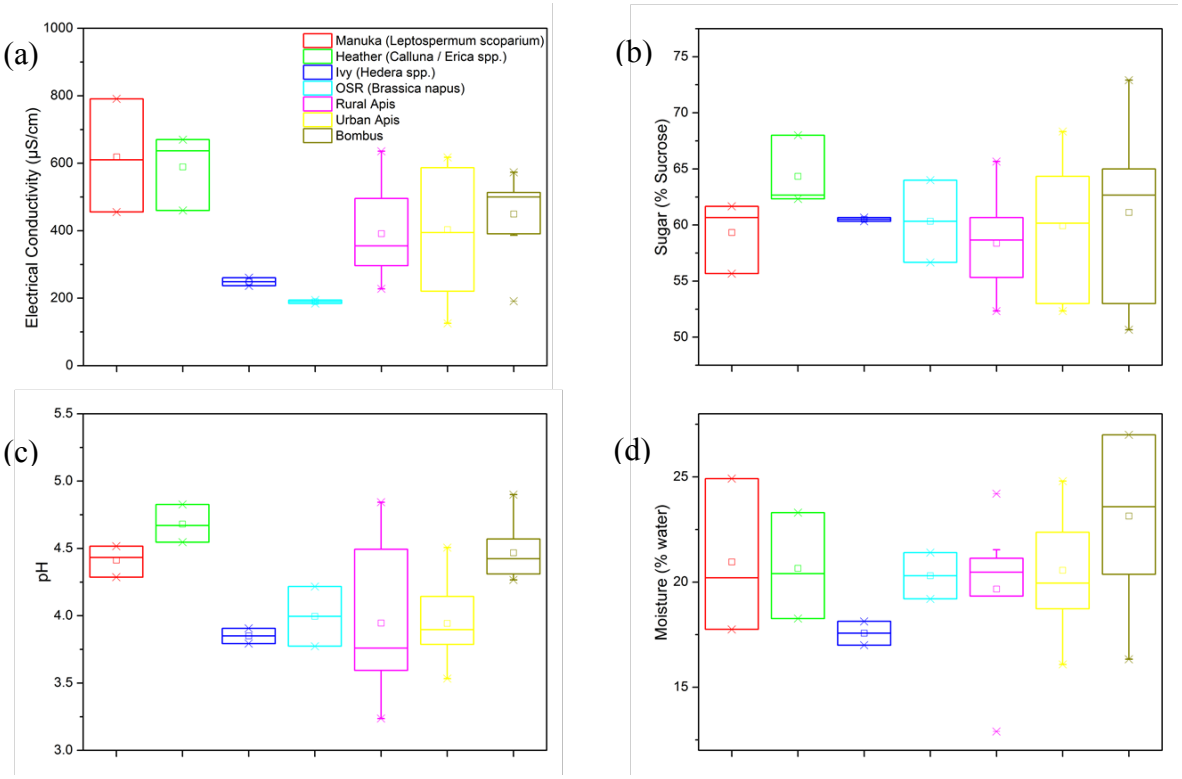

Figure C-1 Electrical Conductivity (a), sugar, (b), pH (c) and moisture (d) for Irish honey types (heather (n=3), ivy (n=2), OSR (oil seed rape) (n=2), multi-floral rural (n=10) and multi-floral urban (n=10)) compared to Manuka honey (n=3) and bumble bee honey (*Bombus*) (n=16). Box plots represent the median (horizontal line in the box), standard deviation (bottom and top box lines) and the mean (small square in the box) and the range (x).

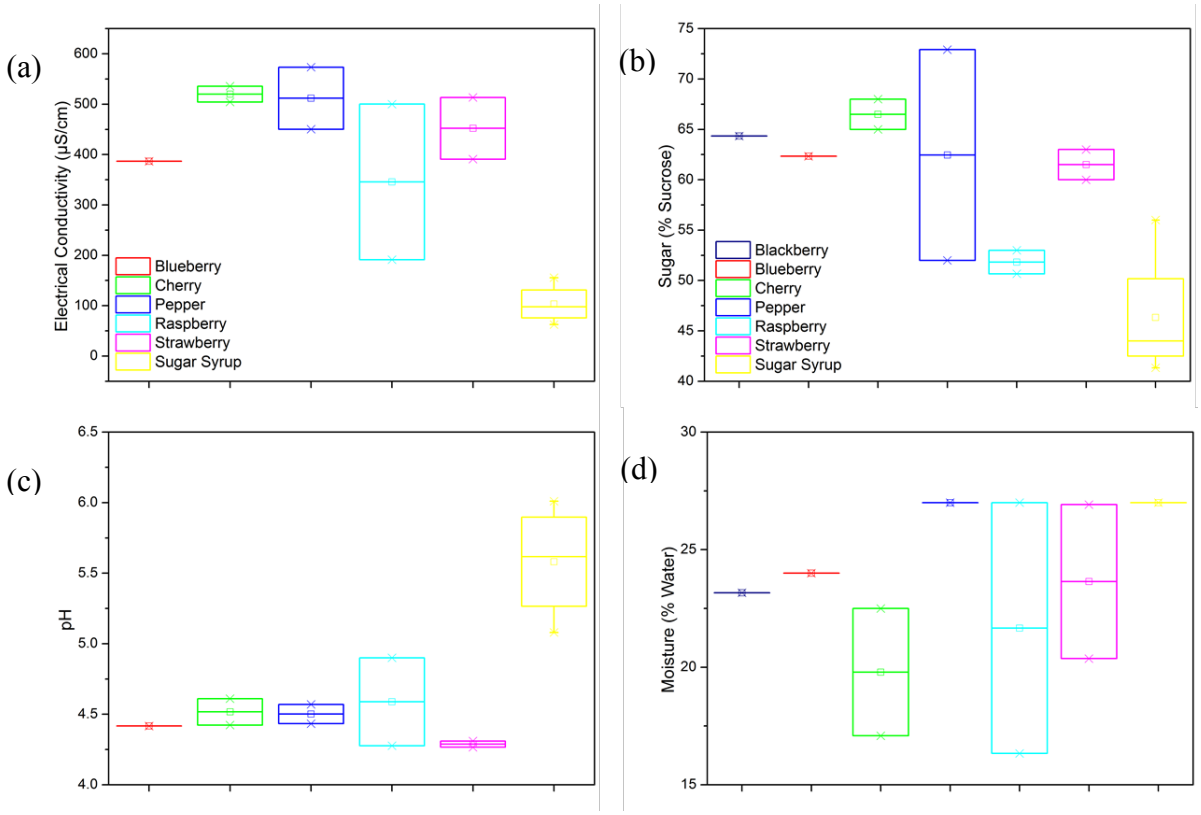

Fig C-2 Electrical Conductivity (a), sugar, (b), pH (c) and moisture (d) for bumble bee honey according to crop type: blackberry (n=3), blueberry (navy, n=2), cherry (green, n=3), peppers (dark blue, n=3), raspberry (light blue, n=2), and strawberry (pink, n=3) compared to sugar (yellow, n=3). Box plots represent the median (horizontal line in the box), standard deviation (bottom and top box lines) and the mean (small square in the box) and the range (x).

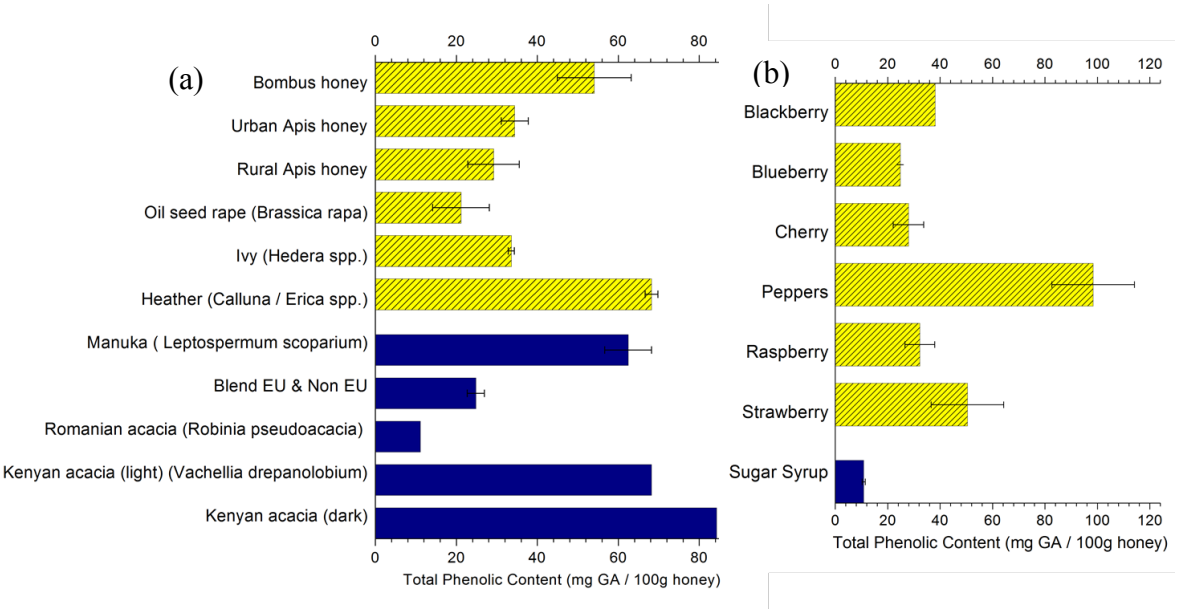

Figure C-3 Mean total phenolic content and standard error of (a) different Irish honey types Bombus honey (bumble bee), (multi-floral urban and rural, oilseed rape (*Brassica rapa*), ivy (*Hedera spp.*) and heather *Calluna / Erica spp.*) in yellow, compared with international honeys (Manuka (*Leptospermum scoparium*) blended, Romanian acacia (*Robinia pseudoacacia*), and Kenyan dark (*Vachellia drepanolobium*) and light) in blue and (b) different types of bumble bee honey according to crop type in yellow compared with sugar syrup in blue.

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