

# Investigating Antibacterial Plant-Derived Compounds from Natural Honey



A thesis submitted for the degree of  
Philosophiae Doctor (*Ph.D*)  
*by*

***Jennifer Hawkins***

School of Pharmacy and Pharmaceutical  
Sciences

Cardiff University

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## DECLARATION

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# ABBREVIATIONS

ACN	Acetonitrile
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BCE	Before the Common Era
BOLD	Barcode of Life Database
BSA	Bovine serum albumin
CA-MRSA	Community associated methicillin resistant <i>S. aureus</i>
CBOL	Consortium for the Barcoding of Life
CCS	Conventional corn syrup
CFU	Colony forming units
CLSI	Clinical and Laboratory Standards Institute
DAD	Diode array detection
DDT	Dichlorodiphenyltrichloroethane
DHA	Dihydroxyacetone
diH <sub>2</sub> O	Deionised water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EU	European Union
EC	European Commission
ESI	Electrospray ionisation
GC	Gas chromatography
h	Hour
H <sub>2</sub> O	Water
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
HCL	Hydrogen chloride
HFCS	High fructose corn syrup
HMF	Hydroxymethylfurfural
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectrometry
HTS	High throughput sequencing
ICBB	International Commission of Bee Botany
IHC	International Honey Commission
IL	Interleukin
INT	Iodonitrotetrazolium chloride
IS	Invert syrup
ISO	Iso-sensitest agar
kDa	Kilodaltons
Kg	Kilogram
L	Litre
LLE	Liquid-liquid extraction
LB	Lysogeny broth
M	Molar
MAE	Microwave assisted extraction
MGO	Methylglyoxal
MH	Mueller-Hinton
MIC	Minimum inhibitory concentration

MBC	Minimum bactericidal concentration
min	Minute
mg	Milligram
mL	Millilitre
MRJP	Major royal jelly proteins
MRP	Millard reaction product
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
MS	Mass spectrometry
<i>m/z</i>	Mass-to-charge ratios
n	Number of replicates
NA	Nutrient agar
NaOH	Sodium hydroxide
NB	Nutrient broth
NCIMB	National Collection of Industrial, Food and Marine Bacteria
NCTC	National Collection of Type Cultures
NGS	Next generation sequencing
nm	Nanometre
NMR	Nuclear magnetic resonance
O <sub>2</sub>	Oxygen
°C	Degrees Celsius
OD	Optical density
ONS	Office of National Statistics
<i>p</i>	Value of significance
PCR	Polymerase chain reaction
PHE	Public Health England
PLE	Pressurized liquid extraction
ROS	Reactive oxygen species
PAGE	Polyacrylamide gel electrophoresis
PWG	Plant Working Group
Q-Q	Quantile-quantile
<i>r</i>	Effect size
r r-value	Correlation coefficient
<i>r</i> <sup>2</sup>	Coefficient of determination
Rf	Retention factor
RS	Revamil® source
SBS	Sequence by synthesis
SDE	Steam distillation extraction
SDS	Sodium dodecyl sulfate
sdw	Sterile deionised water
SE	Standard error of the mean
sec	Second
SFE	Supercritical fluid extraction
SPE	Solid phase extraction
spp.	Species
SPS	Sodium polyacrylate
TCA	Trichloroacetic
TLC	Thin layer chromatography
TNF	Tumour necrosis factor
TSA	Tryptone soya agar

TSB	Tryptone soya broth
TVC	Total viable count
U	Mann-Whitney U test value
UMF	Unique manuka factor
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
V	Volts
v/v	Volume per volume
VOC	Volatile organic compounds
w/v	Weight per volume
μ	Micro
%	Percentage

## PUBLICATIONS AND COMMUNICATIONS

Hawkins J., de Vere N., Griffith A., Ford C. R., Allainguillaume J., Adams-Groom B., Hegarty M. and Baillie L. – Using DNA Metabarcoding to Identify the Floral Composition of Honey: A New Tool for Investigating Honey Bee Foraging Preferences- (**Appendix A**) **Due to be published in the 26<sup>th</sup> August 2015 in PLOS Journals**

Hawkins, J. and de Vere, N. (2014) - DNA barcoding honey: pollinator focused plant surveying and drug discovery. The 58th Ecological Genetics Group conference, Copthorne Hotel, Newcastle. **Oral Presentation.**

Hawkins, J., Baillie, L., White, A. and de Vere, N. (2014) - Wild honey as a drug discovery tool. Welsh School of Pharmacy Postgraduate Research Day, Cardiff. **1<sup>st</sup> Prize Oral presentation.**

Hawkins J. (2013) Wild honey as a drug discovery tool. The Central Association of Bee-keepers booklet. Pages 1-16

Hawkins, J., Baillie, L., White, A. and de Vere, N. (2013) - Wild honey as a drug discovery tool. Welsh School of Pharmacy Postgraduate Research Day, Cardiff. **Poster presentation.**

Hawkins, J., Baillie, L., White, A. and de Vere, N. (2012) - Wild honey as a drug discovery tool. Mechanism-Based Natural Product Development conference, Whistler Mountain, Canada. **1<sup>st</sup> Prize Poster Presentation.**

Hawkins, J., Baillie, L., White, A. and de Vere, N. (2012) - Wild honey as a drug discovery tool. Annual meeting of the Microbiology and Infection Translational Research Group, Liberty stadium, Swansea. **Poster Presentation.**

Hawkins J. (2011) Honey and MRSA. Beecraft magazine. **93**(11): 7-8

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## SUMMARY

Honey possesses therapeutic properties which are the result of a range of factors including high sugar content, low pH, hydrogen peroxide and bee-derived peptides. Honey also contains antimicrobial phytochemicals which represent a rich source of leads for the development of drugs for the treatment of microbial infections.

Honey samples donated by UK beekeepers (217) and Manuka samples (3) were screened for the presence of novel antibacterial compounds by determining activity against methicillin resistant *Staphylococcus aureus* (MRSA) using optimised agar well diffusion and broth microdilution assays. The majority (92%) of the honeys showed inhibitory activity. Identification of unknown factors was performed by neutralising antibacterial honey components previously described in the literature. Of the samples screened four samples were found to contain potentially novel antibacterial compounds.

The pollen present in honey represents a record of the plants which contributed to the making of the honey and may be the source of specific antibacterial factors. For this reason pollen was extracted from honey samples which demonstrated high levels of antimicrobial activity. Microscopic and DNA metabarcoding (454 and Illumina) analysis was performed. Plant species identified with DNA metabarcoding provided superior discrimination and greater repeatability. Key species identified in the antibacterial samples included woodruff (*Galium odoratum*), bluebell (*Hyacinthoides non-scripta*) and dandelion (*Taraxacum officinale*).

Extracts from active honeys and characterised plants demonstrated antibacterial activity against MRSA, *E. coli* and *P. aeruginosa* using broth based methods and thin layer chromatography (TLC) bioautographic overlay methods. Activity-guided characterisation using a TLC/mass spectrometry (MS) interface and high performance liquid chromatography (HPLC) was performed. Compounds identified using these approaches included known pinobanksin derivatives and unknown compounds suggesting that the plants may be the original source of active compounds. The demonstration of antibacterial activity may provide new lead compounds that could serve as selective agents against MRSA and other antibiotic resistant bacteria.

# Chapter 1

## **GENERAL INTRODUCTION**

## 1.1 Natural Products as Therapeutics

A natural product is a chemical compound or secondary metabolite derived from terrestrial or marine organisms (Beutler, 2009). Natural products are the source of many small molecules characterised with biologically useful activity (David *et al.*, 2014), natural product compounds have many modern therapeutic applications (Table 1.1). They represent a rich reservoir of potential drugs for the treatment of many diseases and illnesses.

Table 1. 1: Representative therapeutics of natural product origin

Natural Source	Therapeutic Compound	Medical Applications
<i>Salix</i> (Willow plants)	Aspirin	Analgesic and anti-inflammatory drug
<i>Opium</i> (Poppy plant)	Morphine	Analgesic drug
<i>Digitalis</i> (Fox glove plant)	Glycoside digoxin	Drugs for the treatment of various heart conditions
Cinchona bark	Quinine	Pain relief, fever-reducing drug - The first effective treatment for malaria
<i>Bugula neritina</i> (Californian bryozoan)	Bryostatin 1	Protein Kinase C-activating cancer cell toxin
<i>Pseudopterogorgia elisabethae</i> (Marine Coral)	Pseudopterosin	Anti-inflammatory and analgesic activity
Cyanobacteria within <i>Dolabella auricularia</i> (Sea slug)	Dolastatin	Anticancer molecules
<i>Conus magus</i> (Cone Snail)	Prialt toxin	Powerful analgesic drug
<i>Podophyllum</i> species roots and rhizomes	Podophyllotoxin	Antitumor agents
<i>Penicillium notatum</i>	Penicillins	Antibiotics
<i>Streptomyces venezuelae</i>	Chloramphenicol	Antibiotics
<i>Amycolatopsis orientalis</i>	Vancomycin	Antibiotics
<i>Streptomyces cattleya</i>	Thienamycin	Antibiotics
<i>Streptomyces griseus</i>	Streptomycin	Antibiotics - The first antibiotic treatment for tuberculosis

(Singh and Barrett, 2006, Newman and Cragg, 2007, Simmons *et al.*, 2008).

### 1.1.1 The History of Drug Discovery

Throughout the ages humans have relied on nature as a source of many traditional remedies and therapeutics. With the earliest Egyptian records, dating from 2400BCE, it is clear oils and plant material were utilised for their medicinal properties (David *et al.*, 2014). The Greeks and the Romans also utilised nature as a source of drug discovery (Beutler, 2009), a tradition that has been upheld through to modern medicine today as plants are the source of many nutraceuticals and pharmaceuticals (Sumner *et al.*, 2015). At the beginning of the 19<sup>th</sup> century plants were thoroughly studied to determine their therapeutic potential and during the 1970s the ocean was

also targeted as a source for natural products (David *et al.*, 2014). 50% of the currently marketed drugs approved from 1981 to 2010 are of natural product origin (Newman and Cragg, 2012, Schmitt *et al.*, 2011). New drugs were predominantly discovered through sheer luck, inherited knowledge or trial and error up until rational drug design was developed.

Drug design starts with a hypothesis that a biological molecule may have the potential to be used as a therapeutic. Bioactive compounds have been traditionally characterised following the fractionation and purification of extracts (Sumner *et al.*, 2015). In the mid-1990s large drug companies utilised fragment based molecular modelling and computational chemistry technology to discover and produce synthetic drugs (Erlanson, 2012). The production and screening of synthetic compounds has become more accessible due to the introduction of high throughput screening methods (HTS) and modern advances in synthetic chemistry and has led to a focus on laboratory driven drug development (Cragg and Newman, 2013).

Combinatorial chemistry is a high throughput technique which has been utilised for the discovery of novel therapeutics. Points of diversity are assessed in an initial starting compound or pharmacophore. Different constructs can be created based on starting material and mathematical models (Beutler, 2009). Huge libraries can be produced and the molecular constructions can be analysed for activity. However disadvantages include limited yield, poor solubility and low purity of the created compounds (Beutler, 2009). The success rate of drug discovery has subsequently been lower than originally expected (Newman and Cragg, 2007).

Natural product structures are not limited by the chemist's imagination and are attractive for drug discovery due to the evolution of novel bioactive secondary metabolites (Beutler, 2009). However, the use of HTS and natural products as leads for drug discovery has diminished in the past two decades (Harvey *et al.*, 2015). This trend has arisen due to the complexity of identifying, extracting and isolating new novel compounds from natural sources (Beutler, 2009). The decline or levelling out of the discovery of lead compounds by pharmaceutical companies has been evident between 1981 and 2010 (Newman and Cragg, 2012). However, natural products as a source of novel drugs are re-emerging and pharmaceutical companies are realising that these sources need to be re-explored and combined with diversity-orientated synthetic methodologies (Newman and Cragg, 2012, David *et al.*, 2014).

Due to the significant advances in our understanding of natural-product biosynthesis, with considerable developments in approaches for natural-product isolation and synthesis new paradigms and new enterprises have recently evolved (Beutler, 2009, Singh and Barrett, 2006). Transcriptomics, proteomics and metabolomics studies have recently uncovered new knowledge on biosynthesis of bioactive molecules (Sumner *et al.*, 2015, Harvey *et al.*, 2015). The production

of artemisinic acid has been induced in the tobacco plant *Nicotiana benthamiana* for the treatment of malaria (Van Herpen *et al.*, 2010). The enhanced sensitivity of HTS technologies including high-performance liquid chromatography (HPLC), mass spectrometry (MS) and nuclear magnetic resonance (NMR) has advanced the ability to elucidate chemical structures from natural products (Eldridge *et al.*, 2002, Harvey *et al.*, 2015).

With the emergence of high throughput drug screening technologies related to genetic information, new lines of research are emerging to rapidly and effectively identify novel lead compounds (Singh and Barrett, 2006, Cragg and Newman, 2013). A total of 25 natural product and natural product derivatives drugs were approved for marketing from January 2008 to December 2013, 10 of these are considered to be semisynthetic natural products and 10 were natural-product derivatives (Butler *et al.*, 2014). Surprisingly, less than 10% of the earth's biodiversity has been examined for biological activity, many more useful natural therapeutics may yet to be discovered (Harvey, 2000). By combining high throughput technology with natural product screening, nature will continue to play a vital role in the drug discovery process.

### **1.1.2 Plant-derived Natural Products**

Plants in particular, have played a leading role in providing drugs or templates for secondary metabolites (Beutler, 2009). Traditional Ayurvedic and Chinese medicines have exploited the medicinal benefits of plant derived components for thousands of years. The 'Ebers Papyrus' (1500BCE) describes the use of over 700 drugs of plant origin.

William Withering discovered the medicinal value of foxglove (*Digitalis purpurea*) in 1785 when he identified a glycoside derivative which is now used to treat patients with cardiac failure (Krikler, 1985). The cardiac glycosides are used to increase cardiac contractility and as an antiarrhythmic agent to control the heart rate (Krikler, 1985). These cardenolide glycosides have been identified in several other plants including milk weed (*Asclepias*), and are now commonly used as therapeutics (Seiber *et al.*, 1978).

Similarly, Friedrich Sertürner discovered morphine in 1804, a poppy plant (*Papaver somniferum*) derivative (Lockermann, 1951). Morphine, like many other plant derived products, is now used globally as a potent opiate analgesic drug. In 1985 the World Health Organisation (WHO) estimated approximately 65% of the world population rely on plants for medication (Cragg and Newman, 2013). Only 5% to 15% of the approximately 250,000 species of higher plants have ever been investigated for bioactive compounds, suggesting there is still scope for new areas of research and drug discovery within untapped plant research (Cragg, 2002).

Plants which have been used in traditional Chinese medicines have attracted renewed interest in modern medicine, specifically in cancer prevention and therapy as well as the treatment of bacterial infections (Cai *et al.*, 2004). Paclitaxel (Taxol®) (Figure 1.1) (Gaspar *et al.*, 2008) and its precursors (the baccatins) are the most recently discovered plant-derived chemotherapy drugs routinely used (Kingston, 2012). Phenolic compounds including phenolic acids, flavonoids and tannins that possess anti-inflammatory, antitumour, antibacterial and antiviral activities have been described (Liu *et al.*, 2013). These compounds have an important role in medicine and drug discovery and have many therapeutic uses (Table 1.1) (Seiber *et al.*, 1978, Beart *et al.*, 1985).

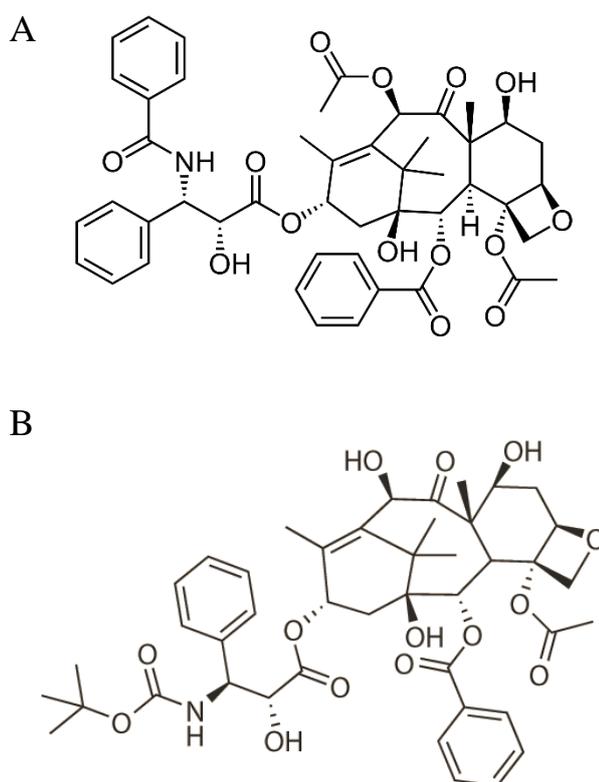


Figure 1. 1: Plant-derived anticancer agent Paclitaxel (A) and its analogues Docetaxel (B)

### 1.1.3 Antimicrobial Natural Products

Microorganisms are common sources of novel drugs and lead compounds, which are extensively used in modern medicine (Davidson, 1995, Distler *et al.*, 1987, Akagawa *et al.*, 1975). The modern era of antimicrobial therapy began in 1929, with Fleming's accidental discovery of the bactericidal substance, penicillin (Fleming, 1929). It was observed that the growth of a fungus, from the *Penicillium* genus, had a bactericidal effect on neighbouring *Staphylococci* species (Figure 1.2). An observation which eventually resulted in the production of many antibiotic derivatives of penicillin (Bruggink *et al.*, 1998).

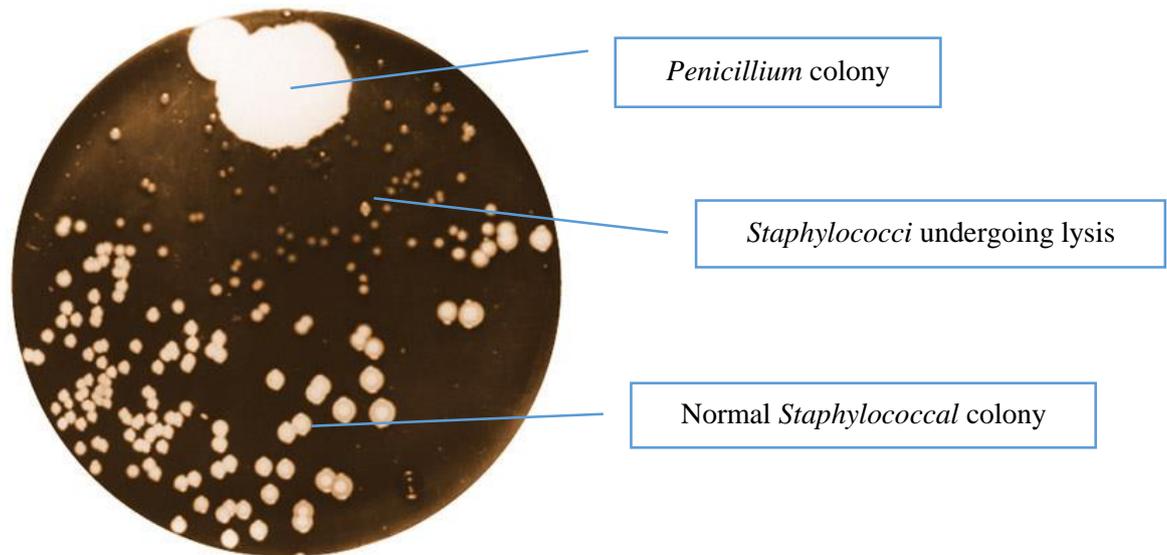


Figure 1. 2: Alexander Fleming's original culture plate

The zone of inhibited staphylococcal growth can be seen around a *Penicillium* colony (Fleming, 1929).

The discovery of penicillin prompted increased interest in identifying novel classes of antibiotics from natural products and up till 1962 nearly all new antibiotics came from this source (Singh and Barrett, 2006). *Streptomyces* is the largest antibiotic-producing genus of bacteria, producing various antimicrobials including streptomycin (Distler *et al.*, 1987) and chloramphenicol (Akagawa *et al.*, 1975). Antifungals including nystatin, have also been isolated from *Streptomyces noursei* (Brown *et al.*, 1953). These are a few of many natural products derived from microorganisms. There is also a diverse array of unexplored potential for microbial diversity; environmental samples, extremeophiles, endophytes, marine microbes and microbial symbionts are yet to be explored (Cragg and Newman, 2013).

Evolutionarily preserved antimicrobial peptides (host defence peptides) are a diverse family of cystein-rich cationic molecules which act against a range of different microorganisms. Defensins are key elements of the innate immune response and are produced upon infection or injury to protect the host (Dossey, 2010). Naturally occurring peptides from various biological sources are utilised in modern medical therapeutics (Matsunaga *et al.*, 1985, Hopkins *et al.*, 1994, Klaudiny *et al.*, 2005).

Defensins kill bacteria by increasing the permeability of their cytoplasmic membrane resulting in a reduction of cellular cytoplasmic content (Nakajima *et al.*, 2003). Peptides have a broad antimicrobial spectrum and disrupt microbial membranes via peptide–lipid interactions by defensin oligomers. Cationic peptides interact with the negative charge of the outer membrane, disruption occurs and peptides can enter the cell. Peptides can also aggregate into the membrane

forming barrel-like structures which span the membrane causing disruption of cell death (Sahl *et al.*, 2005). The inner membrane is also depolarized, cytoplasmic ATP is reduced and respiration is inhibited resulting in bacterial cell death (Cociancich *et al.*, 1993). Three antimicrobial peptides from the marine sponge *Discodermia kiiensis*, discodermins models were among the first peptide antibiotics to be discovered and were shown to have antibacterial activity against a range of bacteria including *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus subtilis*, and *Mycobacterium smegmatis* (Matsunaga *et al.*, 1985).

Antimicrobial insect defensins are a large family of peptides commonly found in the hemolymph or fat cells of several insect orders, including honey bees (Ilyasov *et al.*, 2012). Honey bees produce antimicrobial defence peptides when responding to an infection (Klaudiny *et al.*, 2005). Four immune system peptides have been isolated from honey bees; apidaecin, abaecin hymenoptaecin and defensins (Casteels *et al.*, 1989, Casteels *et al.*, 1990, Casteels *et al.*, 1993). These honey bee defensins are known to leak into naturally produced bee products. Antimicrobial defensin molecules have been isolated from royal jelly (Klaudiny *et al.*, 2005, Fontana *et al.*, 2004) and more recently in Revamil® (RS) honey (Kwakman *et al.*, 2010).

#### **1.1.4 The Emergence of Antibiotic Resistant Bacteria**

The unearthing of penicillin initiated the ‘Golden Age’ (1940–1962) of antibiotic discovery. Many novel natural products were discovered leading to overwhelming excitement and excessive overestimations about their role in medicine (Singh and Barrett, 2006). Inappropriate and extensive use of antimicrobials in medicine, veterinary, food animal production and agriculture sectors encouraged the microorganism to mutate or acquire resistance genes, resulting in the emergence of bacterial strains with resistance to novel therapeutics (Levy and Marshall, 2004).

The mass-production and use of penicillin began in 1943 and within 4 years resistant strains of *Staphylococcus aureus* began to emerge (Figure 1.3), a trend commonly seen with many antibiotics. Methicillin resistant *Staphylococcus aureus* (MRSA), which is resistant to practically all  $\beta$ -lactam antibiotics acquires resistance due to the integration of staphylococcal cassette chromosome *mec* (SCC*mec*) element. The SCC*mec* element encompasses the *mecA* gene complex and the *ccr* gene complex which encode resistance and genetic element motility and integration (Deurenberg and Stobberingh, 2008).

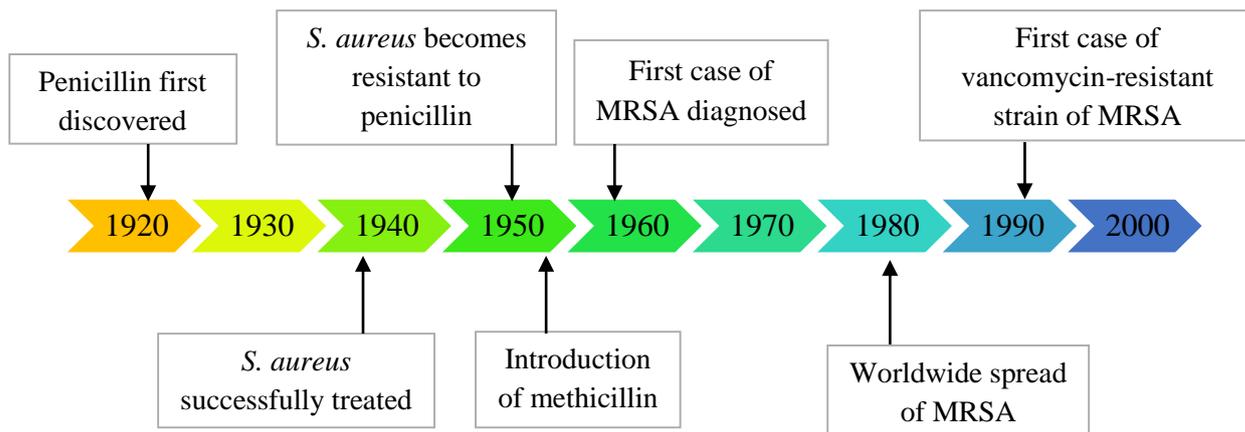


Figure 1. 3: The evolution of antibiotic resistance of Methicillin resistant *S. aureus* (MRSA)

A report on antimicrobial resistance produced in 2014 predicts that 300 million people may die prematurely because of antimicrobial drug resistance over the next 35 years (O’Neill, 2014). The WHO reported that in the EU (and in Norway and Iceland), an estimated 25,000 people die every year because of infections related to antibiotic resistance, most of them contracted in the health care environment (WHO, 2014). These occurrences result in considerable increases in health and social costs, estimated to be € 0.9 billion annually across Europe.

The WHO global report from 2014 on surveillance of antimicrobial resistance recognises the problems surrounding the global increase in bacterial resistance and acknowledges that MRSA is a significant threat to hospitalised and community patients (WHO, 2014). MRSA is isolated in about 5% of all infections associated with healthcare. The WHO report (2014) highlighted that all-cause mortality, intensive care unit (ICU) mortality and bacterium-associated mortality all increase significantly with MRSA infection.

The resistance of *E. coli*, *N. gonorrhoeae* and *K. pneumoniae* to multiple drugs is on the rise (WHO, 2014). To combat this problem the WHO aim to strengthen national co-ordination and communication, to improve surveillance, to promote strategies which reduce the misuse of antimicrobials and to promote research into novel therapeutics and technologies. These strategies aim to reduce the morbidity, mortality and related expenses associated with antibiotic resistance of hospital acquired infections. Resistance management is now part of the process of identifying novel drugs as it is accepted that the emergence of resistant microorganisms is inevitable (Singh and Barrett, 2006).

## 1.2 Honey – A ‘Rediscovered’ Therapy

### 1.2.1 Production of Honey

The honey bee (*Apis mellifera*) is of great importance for humans as a pollinator of both commercial and domestic crops and provider of honey, a high-value nutritional commodity (Potts *et al.*, 2010, Ratnieks and Carreck, 2010). Honey bee loss due to the interacting drivers of pests and diseases, exposure to agrochemicals, apicultural mismanagement and lack of genetic diversity have led to widespread concern about the future potential of honey bees to provide these services (Ratnieks and Carreck, 2010, Potts *et al.*, 2010). The quality and composition of honey produced is affected by many factors including flower composition, geographical position of the hive, bee health and annual changes in local flora and flowering phenology (Linsens and Jorde, 1997, Galimberti *et al.*, 2014). Various physical types of honey are also commercially available (comb, chunk, crystallized or granulated, creamed) with many different levels of processing (pressed, centrifuged, drained, heat processed) (Anklam, 1998).

Within a honey bee hive there are three castes – queen (alpha), worker (beta) and drone (gamma) bees (Havenhand, 2010), a collective effort allows for the production of honey. Honey is produced by honey bees using nectar from flowering plants, nectar is a sugar-rich liquid that is produced in glands called nectaries. Nectar is collected by worker bees, travelling up to 9 km in one trip (Havenhand, 2010). Sucrose in nectar is hydrolysed to produce glucose and fructose (Kubota *et al.*, 2004). Upon return to the hive the nectar is swallowed and regurgitated by thousands of worker bees within the honey comb. The regurgitation process and wing fanning causes evaporation and the water content is reduced, the honey is ripened over time.

Honey bees keep the honey as food stores for the winter period when no nectar or pollen is available. Any excess honey can be extracted for human consumption (Havenhand, 2010). Kubota *et al.*, (2004) described how glucosidase III is produced in the hypopharyngeal gland of European honey bees. This enzyme is secreted into the nectar and is responsible for the production of hydrogen peroxide (Bucekova *et al.*, 2014).

Pollen grains are collected by honey bees as they visit flowering plants to feed honey bee larvae (Galimberti *et al.*, 2014). Dense pollen pellets are produced from these grains using a nectar-saliva mixture. Honey bees collect the exudate from sap-sucking insects as an alternative to nectar. Honeydew collection is often recorded from sap feeding insects feeding on conifers and other anemophilous species (Oddo *et al.*, 2004). Tree resin is also actively collected from a range of species and combined with wax to make propolis that is deposited within the hive as it has antimicrobial properties (Wilson *et al.*, 2013).

### 1.2.2 History of Honey

Honey bees (*Apis mellifera*) have been exploited by human beings throughout history (Havenhand, 2010). There is strong evidence of honey being used throughout human civilisation with the ancient Egyptians (5500 BC), Chinese, Greeks and Romans all using honey for different applications. Natural honey has historically been used in medicine where it was employed for the treatment of wounds and diseases of the gut (Zumla and Lulat, 1989). Bees were kept for honey and hive products. Honey, beeswax, propolis, pollen, and royal jelly were all utilised. The earliest documented evidence of honey being used as a therapeutic is found in the ancient Egyptian text 'Ebers Papyrus' (Zumla and Lulat, 1989). Hieroglyphic symbols translated from the text describes a mixture of grease, honey and lint/fibre which has wound healing potential (Zumla and Lulat, 1989).

Honey is an organic, natural sugar alternative and due to its prolonged shelf-life was an important food source for ancient civilisations (Allsop and Miller, 1996). It also was highly valued by both Christian and Islamic religions as a precious gift and as a symbol of good health (Havenhand, 2010). It was even traded as currency by the Egyptians and Romans (Havenhand, 2010).

During the medieval period (AD 450-1485) lower classes kept bees for their personal use whilst abbeys and monasteries were centres of beekeeping and honey production. Honey was used to sweeten food, candles were made from beeswax and alcoholic mead was produced by honey fermentation (Allsop and Miller, 1996). During the period 1485-1700 it is believed that almost every small holding kept their own bees and utilised the products they produced. Accounts of food trade from this period describe honey being sold by the gallon or barrel at relatively low prices, two traits which would only be possible if honey production was common practice (Allsop and Miller, 1996).

In the early 1700s sugar cane was being imported into the UK and the wealthiest households replaced honey with sugar. Within 80 years sugar became widely available and honey was no longer considered the most accessible sweetening agent. Since the 18<sup>th</sup> century the number of beekeepers and the production of honey in the UK have dramatically declined. There are an estimated 285,000 honey bee hives in the UK a 75% drop in the last 100 years (Breeze *et al.*, 2011). Honey is used in modern medicine but the majority of honey for human consumption is imported to meet the supermarket demand.

### 1.3 Chemical Composition of Honey

Honey contains an array of minor constituents including carbohydrates, volatiles and phenolic compounds including flavonoids and non-flavonoid phenolic compounds (Table 1.2) (Baroni *et al.*, 2006). These compounds originate from plants foraged upon by the bees and from the bees themselves. Phenolic compounds are affected by the storage and processing of the honey, microbial or environmental contamination, geographical distribution and botanical source of nectar and pollen (Manyi-Loh *et al.*, 2011).

Table 1. 2: Average composition of honey

Component	Average value in%
Water	17
D-Fructose	38
D-Glucose	32
Sucrose	1.3
Maltose	7.3
Oligosaccharides	1.5
Protein	0.3
Minerals	0.2
Vitamins & amino acids	1.0
Other	1.4

(Anklam, 1996)

#### 1.3.1 Carbohydrates

Sugars (saccharides) comprise the major portion of honey; approximately 85-95 % (w/v) of the total honey. Honey consists mostly of the monosaccharides fructose and glucose (Table 1.2). Twenty five other oligosaccharides (disaccharides, trisaccharides, tetrasaccharides) have also been described (Anklam, 1998). Invert syrup (IS), conventional corn syrup (CCS) and high fructose corn syrup (HFCS) is also used in honey adulteration (Anklam, 1998). Honey is a variable and complex mixture of sugars and other components.

#### 1.3.2 Proteins and Amino Acids

Honey normally contains between 0.1-0.5% protein (Won *et al.*, 2009). Eighteen amino acids are found in honey; proline represents 50-85% of the total amino acid profile. Arginine, tryptophan, and cystine are characteristic amino acids in some honey types (Anklam, 1998). Enzymes make up a small fraction of these proteins. Enzymes found in honey which originate from both nectar and the bees are common (Weston, 2000). Predominant enzymes are diastase (amylase), which breaks down starch into smaller units; invertase (glucosidase) which converts glucose to fructose and glucose oxidase which catalyses the reaction of glucose to gluconolactone, resulting in the

production of gluconic acid and hydrogen peroxide (Bucekova *et al.*, 2014). Catalase occurs naturally in some pollen grains, catalase neutralises hydrogen peroxide (Assia and Ali, 2015, Weston, 2000).

### 1.3.3 Vitamins and Minerals

Trace amounts of B vitamins (riboflavin, niacin, folic acid, pantothenic acid and vitamin B6) and C vitamins (ascorbic acid) are found in honey. Many different minerals (calcium, iron, zinc, potassium, chromium, phosphorous, magnesium and manganese) are found in unprocessed honey.

### 1.3.4 Volatile Compounds

More than 600 volatile organic compounds (VOCs) have been identified in honey. Volatiles are organic chemicals that have a high vapour pressure at standard room temperature. Seven major groups have been previously characterised in honey; aldehydes, ketones, acids, alcohols, esters, hydrocarbons and cyclic compounds (Manyi-Loh *et al.*, 2011). The dominant volatiles can be seen in figure 1.4 (Kaškonienė and Venskutonis, 2010).

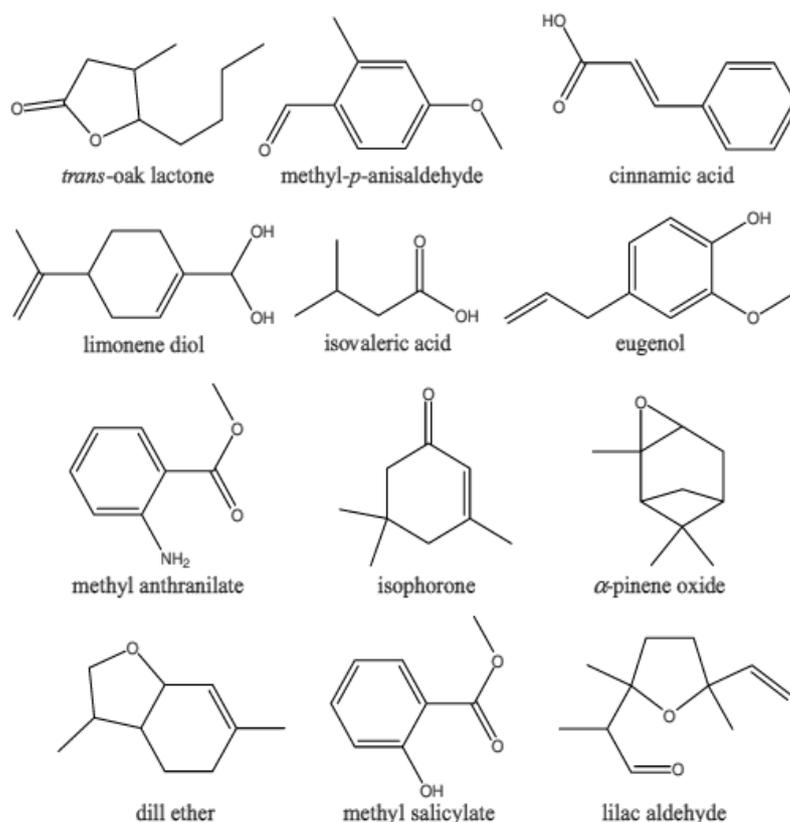


Figure 1. 4: Structure of selected volatile components found in honey

Honey contains numerous VOCs in low concentration however, VOCs affect the sensory characteristic of honey; flavour, aroma, colour and texture are all effected by the type of plants and flowers bees visit (Manyi-Loh *et al.*, 2011). Some VOCs originate from the plants or nectar source whereas others are created during the processing or storage of honey (Jerkovic *et al.*, 2006, Jerković *et al.*, 2011, Castro-Vázquez *et al.*, 2008). The Maillard reaction occurs when honey is heat treated; a non-enzymatic browning reaction occurs between sugars and amino acids resulting in the production or transformation of VOCs (Castro-Vázquez *et al.*, 2008). Microbial and environmental contamination can also contribute to the number of VOCs (Manyi-Loh *et al.*, 2011).

### **1.3.5 Phenolic Compounds**

The major phenolic compounds identified in honey are flavonoids: quercetin, pinocembrin, pinobanksin, chrysin, galangin, kaempferol and luteolin (Dong *et al.*, 2013, Kaškonienė and Venskutonis, 2010, Pyrzynska and Biesaga, 2009). Aromatic acids contain an aromatic ring and an organic acid function (C6-C1 skeleton). Phenolic compounds are an example of aromatic acids as they containing a phenolic ring and an organic carboxylic acid function. Phenolic acids can be found in many plant species (Cai *et al.*, 2004, Pinho *et al.*, 2014, Lin and Harnly, 2007). Flavonoids are plant specialized metabolites which fulfil many functions and are important for plant pigmentation, UV filtration and symbiotic nitrogen fixation (Dixon and Pasinetti, 2010). Flavonoids are widely distributed in plants and their basic molecular structure is 2-phenyl-1,4-benzopyrone. Plant derived phenolic acids include benzoic, ferulic, gallic, chlorogenic, caffeic, p-coumaric, ellagic and syringic acids. Phenolic compounds have antibacterial, anti-inflammatory and antioxidant activities. The composition of phytochemicals has an effect on the bioactivity of honey (Kaškonienė and Venskutonis, 2010).

### **1.3.6 Other Compounds**

Organic acids including acetic, formic, citric, lactic, malic and gluconic acids are found in honey (Cherchi *et al.*, 1994). Gluconic acid is produced in the breakdown of glucose by glucose oxidase. Hydroxymethylfurfural (HMF) is also found in honey, a natural product of the breakdown of simple sugars (Assia and Ali, 2015). Microelements also include metals such as copper, cadmium manganese and iron (Erbilir and Erdoğrul, 2005).

Low levels of hydrogen peroxide is commonly found in natural honey. Hydrogen peroxide is produced due to the action of the enzyme glucose oxidase which catalyses the conversion of glucose into gluconic acid and hydrogen peroxide (Bang *et al.*, 2003) (Figure 1.5). Glucose oxidase is produced in the hypopharyngeal gland of the bee during the ripening process of nectar

(Bucekova *et al.*, 2014). The ripening process inactivates glucose oxidase; this activity is only restored on dilution of honey (Kwakman and Zaat, 2012, Bang *et al.*, 2003). It has been suggested that the lower pH of concentrated honey is outside the range needed for activation of the glucose oxidase (White *et al.*, 1963). Upon dilution, when honey is eaten or applied to wounds, hydrogen peroxide can be produced increasing the antibacterial potency of the honey (Bogdanov, 1997).

The concentration of hydrogen peroxide varies between honeys and is effected by different factors including excess heat or light, the levels of catalase found in nectar or chemical scavenging activity (Brudzynski *et al.*, 2011, White *et al.*, 1963, Kwakman *et al.*, 2011b, Weston, 2000). Honey contains varying amounts of catalase, peroxidases, methylglyoxal (MGO) and antioxidants which can affect the levels of glucosidase activity (Weston, 2000, Pyrzynska and Biesaga, 2009, Majtan *et al.*, 2012).

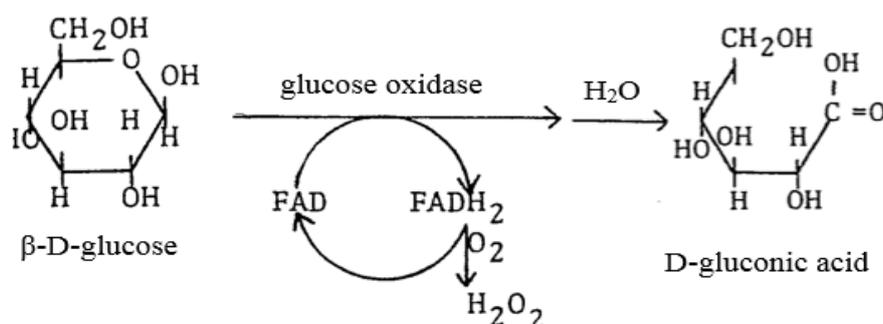


Figure 1. 5: Glucose oxidase chemical reaction in honey

(White *et al.*, 1963)

### 1.3.7 Pollen, Propolis and Royal jelly

Honey bees collect pollen and nectar from flowering plants, supplying the hive with protein for nourishment. Pollen is commonly found in honey. Wind pollinated pollen from trees and plants also frequently feature within honey (Bruni *et al.*, 2015). Pollen contains carbohydrates, amino acids, DNA, nucleic acids, proteins, lipids, vitamins, minerals, phenolic compounds and flavonoids (Morais *et al.*, 2011).

Propolis is produced from the exudates of plants; bees seal the hive with the resinous substance creating a protective barrier against intruders (Viuda-Martos *et al.*, 2008). Propolis is comprised of resin (50%), wax (30%), essential oils (10%), pollen (5%), and other organic compounds (5%) (Viuda-Martos *et al.*, 2008). More than 300 compounds including phenolic compounds, esters, flavonoids, terpenes and anthraquinones have been found in propolis (Kalogeropoulos *et al.*, 2009, Bertrams *et al.*, 2013, Gardana *et al.*, 2007)

Royal jelly is a proteinous liquid secreted by glands in the hypopharynx of worker bees; it is produced exclusively for the adult queen bees, it is a vital nutritional source (Viuda-Martos *et al.*, 2008). More than 50% of the dry mass of royal jelly is proteins, major royal jelly proteins (MRJPs) have been researched and analysed (Won *et al.*, 2009). Royal jelly is used as a dietary supplement for the treatment of many conditions including asthma, high cholesterol and seasonal allergies.

#### 1.4 Characterisation of Honey

Honey bees collect nectar and pollen from flowering plants for the production of honey, thus the resulting sample is a reflection of the plants that the bees have visited. The characteristics of a honey can vary greatly due to variations in the flora on which the bees forage (Downey *et al.*, 2005). The colour and taste is dependent on the presence of plant derived pigments and phytochemicals, the darker the honey the higher the bioflavonoid and mineral content (Havenhand, 2010, Isla *et al.*, 2011).

Standard methods for the characterisation of honey have been devised by the International Honey Commission (IHC) in order to improve analytical methods for honey analysis and to underpin quality criteria (Bogdanov *et al.*, 1999). The characterisation of the floral source of a honey for sale is a requirement (Oddo and Bogdanov, 2004). The IHC have set out a selection of methods for use in routine honey characterisation (Figure 1.6). These controls have been designed to include all the characteristics which are influential in the determination of honey quality (Bogdanov *et al.*, 1999).

Moisture content	<ul style="list-style-type: none"> <li>•In general - not more than 21 %</li> <li>•Industrial honey or baker's honey- not more than 25 %</li> </ul>
Hydroxymethylfurfural (HMF) content	<ul style="list-style-type: none"> <li>•Not more than 40 mg/kg (ppm)</li> </ul>
Diastase activity (Schade scale)	<ul style="list-style-type: none"> <li>•Not less than 8</li> </ul>
Invert sugar	<ul style="list-style-type: none"> <li>•Blossom honey - not less than 65%</li> <li>•Honeydew honey and blends - not less than 60%</li> </ul>
Free acidity	<ul style="list-style-type: none"> <li>•Not more than 40%</li> </ul>
Sucrose content	<ul style="list-style-type: none"> <li>•In general- not more than 5%</li> <li>•Honeydew honey and blends - not more than 5%</li> </ul>
Mineral (ash) content	<ul style="list-style-type: none"> <li>•In general - not more than 0.6 %</li> <li>•Honeydew honey and blends - not more than 1.2 %</li> </ul>
Water insoluble solids content	<ul style="list-style-type: none"> <li>•In general - not more than 0.1 %</li> <li>•Pressed honey - more than 0.5 %</li> </ul>

(Bogdanov *et al.*, 1999, Anklam, 1996)

Figure 1.6: International Honey Commission (IHC) compositional criteria described for standardised analysis

## **1.5 Analytical Methods to Determine the Geographical and Botanical Origin of Honey**

The composition of honey is strongly associated with its botanical and geographical origin and understanding the floral composition of honey has a wide variety of applications. It can indicate what plant species are being used as food sources for honey bees in the habitat surrounding hives, providing a method for pollinator-focused plant surveying (Batáry *et al.*, 2010). It can be used to verify the stated floral sources of commercial honey and verify the honey's origin (Kaškonienė and Venskutonis, 2010). Monofloral honeys differ from multifloral honeys by the dominance of nectar and pollen collected from a single type of plant species; Louveaux *et al.* (1978) classifies honey as monofloral if it contains >45% pollen from one species.

Monofloral honeys are prone to fraudulent adulterations and incorrect labelling due to their higher commercial value (Oddo and Bogdanov, 2004). Food safety and quality is also of concern as traces of several poisonous plants have been detected in honey including *Atropa belladonna* (Bruni *et al.*, 2015) and *Rhododendron* spp. (Koca and Koca, 2007). Hepatotoxic pyrrolizidine alkaloids (PAs) have been detected in honeys after bees have foraged on plants within the Boraginaceae (Edgar *et al.*, 2002). EU guideline 2001/110/EC states that except in the case of filtered or baker's honey, the origin and composition of honey must be indicated, but samples from more than one Member State or third country can be labelled 'blend of EC and non-EC honeys'. The botanical profile can therefore be used to scrutinise the composition of honey, ensuring products are safe for the consumer (Olivieri *et al.*, 2012). It is also possible to use the botanical profile to characterise the source of plant derived antimicrobials which contribute to the therapeutic applications of honey.

A number of methods have been used to investigate the floral composition of honey. The traditional approach is melissopalynology; the morphological examination of pollen under a light microscope (Louveaux *et al.*, 1978). Chemical methods include the analysis of free amino acids, organic acids, phenolics and aromatics. A complex mixture of phenolic acids and flavonoids contribute to honey's chemical composition (Molan, 1998). It is difficult to indicate only one, individual marker to identify the origin and quality of honey so 'fingerprints' are generally used (Jasicka-Misiak *et al.*, 2012). The use of DNA-based methods for botanical classification of honey has attracted interest in recent years.

### **1.5.1 Melissopalynology**

Pollen analysis has revealed that honey contains a fingerprint of the plants visited by the bees (Anklam, 1998, Sodré *et al.*, 2007). Melissopalynology (pollen identification) has been the

standard method for determining the floral origin of honey for many years (Vorwohl, 1967). Plants which contribute to the honey produced by worker bees can be identified via microscope analysis (Vorwohl, 1967), but this technique is time consuming and requires a specialist with a well trained eye and a high level of experience (Bruni *et al.*, 2015, Downey *et al.*, 2005). Some pollen grains can only be identified to genus level due to the limited specificity of microscope analysis (Valentini *et al.*, 2010). Electronic, size-based particle counters and haemocytometers can be used to obtain a rapid estimate of pollen grain numbers and provide estimates on number and size of grains (Shubharani *et al.*, 2012).

### 1.5.2 Biochemical Profile

It has been proposed that ratios between the concentration of amino acids and proteins can be used to characterise the origin of plants in honey (Anklam, 1998, Bogdanov *et al.*, 2004). Pirini *et al.*, (1992) described the presence of amino acids such as arginine, tryptophan, and cystine as being characteristic for some honey types (Pirini *et al.*, 1992). Immunoblot assays of honey proteins, originating from pollen can be performed for authentication (Baroni *et al.*, 2002). More recently, protein fingerprinting and barcoding using advanced mass spectra techniques have been used for the authentication and determination of geographic origin (Wang *et al.*, 2009). Protein and amino acid fingerprints provide an alternative method to microscopy for the analysis of plants in honey, but are often best combined with other characterisation techniques.

Aroma compounds including volatile and semi-volatile organic compounds have been analysed based on their relationship with the floral origin of honey (Bogdanov *et al.*, 2004). Volatiles contribute to the flavour and aroma of honey but analysis and characterisation is difficult (Anklam, 1998). It has been reported that some honey samples could be characterised by one compound. Methyl anthranilate for citrus honey (Ferrerres *et al.*, 1994), isophorone for strawberry-tree honey (De La Fuente *et al.*, 2007) and Ericaceae family (Guyot *et al.*, 1999), however a combination of markers is often required for comprehensive characterisation (Anklam, 1998, Kaškonienė and Venskutonis, 2010).

The composition of phenolic compounds and flavonoids depends on the floral source and are subsequently used as floral markers. Quercetin has been proposed as a marker for sunflower honey (Tomás-Barberán *et al.*, 2001), hesperitin for citrus honey (Ferrerres *et al.*, 1993) and naringenin for lavender honey (Andrade *et al.*, 1997). A recent review by Kaškonienė and Venskutonis (2010) lists the characteristic compounds of some unifloral honeys. Other organic compounds (aliphatic) and microelements have been described as possible floral markers (Kaškonienė and Venskutonis, 2010). Trace elements and minerals including sodium, potassium, calcium, magnesium, copper, iron, manganese, phosphorus, chlorine, silicon, ash, lead and

cadmium have also been investigated (Anklam, 1998). Enzymes, carbohydrates (sugars) and stable isotopes have also been examined for botanical and geographical characterisation. The analysis of different compounds should be combined for accurate characterisation.

### 1.5.3 DNA Barcoding

DNA based identification has the potential to reduce processing time, increase the level of species discrimination and does not require the high level of taxonomic expertise required for melissopalynology (Schnell *et al.*, 2010, Valentini *et al.*, 2010, Jain *et al.*, 2013, Bruni *et al.*, 2015). There are many applications which utilise this novel DNA approach. By identifying plant species through DNA analysis and next generation sequencing it is possible to accurately characterise pollen in honey.

It is possible to extract sufficient pollen DNA from honey to identify plant species. Previously molecular genetics including real-time PCR has been used (Laube *et al.*, 2010), but this requires an *a priori* knowledge of the plants visited by the bees which may contribute to the honey sample. Specific primers and probes were designed for the identification of each plant (Laube *et al.*, 2010). Honey samples containing a mixture of plant DNA can be more accurately assessed using an approach based on DNA barcoding and next generation DNA sequencing (Valentini *et al.*, 2010). This novel approach involves identifying a standardized DNA region for characterisation of plants within honey (Valentini *et al.*, 2010). Initially marker regions of DNA such as *ITS* or *trnL* were isolated and used to characterise plants (Valentini *et al.*, 2010, Cheng *et al.*, 2007), but the accuracy and discrimination was low.

In an effort to generate a universal database, researchers from across the world have been involved in a project to identify a short region of unique, discriminative DNA. The Plant Working Group (PWG) of the Consortium for the Barcoding of Life (CBOL) compared recoverability, sequence quality and levels of species discrimination of plastid DNA regions (CBOL Plant Working Group *et al.*, 2009). This motion led the way to a global DNA barcoding initiative to standardise the marker gene regions and protocols for plant DNA classification. The group settled on two gene regions in the chloroplast, *matK* and *rbcL* to serve as universal barcodes, with the recognition that additional markers may be required for some plant species (CBOL Plant Working Group *et al.*, 2009, Kress *et al.*, 2010). The identification of unknown plant DNA in honey is made considerably easier when open access international databases such as Barcode of Life Database (BOLD) and also GenBank® can be utilised. These unique DNA sequences are added to the BOLD and GenBank® standard databases and used as a tool for biological species identification, this process is known as DNA barcoding.

Accurate species identification using DNA barcoding requires comprehensive databases of known reference samples. The Barcode Wales project created a reference database of DNA barcodes for the Welsh native flora (de Vere *et al.*, 2012). Using the standardised markers *rbcL* and *matK* a DNA barcode database was created for the 1143 native flowering plants and conifers of Wales, this database represents the most comprehensive sampling of any national flora to date. Discrimination levels of 69.4 to 74.9% of all species and 98.6 to 99.8% of genera were achieved when using both markers. While this database represents an important tool it should be remembered that it is based on native plants and does not contain alien or cultivated plants that bees may forage upon. Using these valuable resources and next generation DNA sequencing technologies it is possible to create a detailed fingerprint of the plants which may be present in the honey. The antibacterial activity of honey is in part due to the plants which contribute to its making (Morais *et al.*, 2011), thus by accurately identifying the pollen it may be possible to discover the plant which contains the antibacterial compound.

## **1.6 The Use of Honey in Modern Medicine**

### **1.6.1 Antibacterial Activity**

The renewed interest in honey as a source of antibacterial compounds is due primarily to the rise in prevalence of antibiotic-resistant bacteria (Kwakman *et al.*, 2010). Many bacteria including; *Helicobacter pylori*, *B. subtilis* and *E. coli* have been shown to be susceptible to raw honey (Kwakman *et al.*, 2010, Jenkins *et al.*, 2011, Mavric *et al.*, 2008, Boorn *et al.*, 2010, Manyi-Loh *et al.*, 2010). High levels of antibacterial activity has been described against a broad range of bacterial species which cause wound infections; including *P. aeruginosa* from burn wounds (Cooper *et al.*, 2002a) and *S. aureus* and vancomycin-sensitive *enterococci* from infected wounds (Cooper *et al.*, 2002b, Cooper *et al.*, 1999). The anti-inflammatory and antibacterial effects also make honey suitable for the treatment of other conditions including coughs and sore throats (Paul *et al.*, 2007, Cohen *et al.*, 2012). Honey is often used as a supplementary component to many over the counter remedies. Similarly bacterial gastroenteritis is an acute inflammation of the GI tract, honey has shown to reduce the recovery period and reduce the symptoms of acute infantile diarrhoea (Elnady *et al.*, 2011).

To date a number of compounds isolated from honey have been shown to possess antibacterial activity (Figure 1.7) (Kwakman and Zaat, 2012). Two distinct mechanisms of activity have been described, peroxide based activity and non-peroxide based activity (Kwakman *et al.*, 2011b). These antibacterial factors also vary greatly from honey to honey and are often effected by storage, processing and floral origin of the sample (Atrott *et al.*, 2012). The exact mechanisms by which honey is able to inhibit bacterial growth remains unclear but a synergistic effect has been

proposed (Kwakman *et al.*, 2010). Traces of pollen, royal jelly and propolis which have leaked into the honey may also contribute to the antimicrobial effect (Weston, 2000).

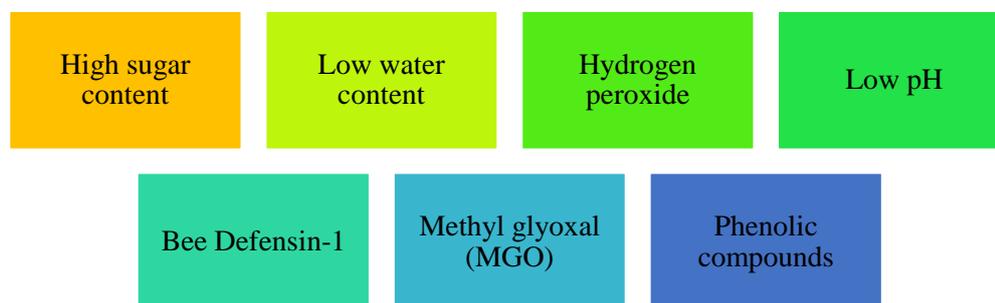


Figure 1. 7: Antibacterial components of honey

### 1.6.1.1 Sugar

At its very basic level, honey consists of a mixture of simple carbohydrates which create a highly osmotic environment. The combination of low levels of water (~18%) and high levels of sugar (~80%) are enough in themselves to prevent the spoilage of honey by microorganisms (Kwakman *et al.*, 2010). Disruption of the bacterial cell wall occurs due to the osmotic effect (Zumla and Lulat, 1989). The osmotic effect has been shown to be an important parameter for killing *H. pylori* (Osato *et al.*, 1999), however honey has other antibacterial factors beyond the osmotic effect (Cooper *et al.*, 1999, Kwakman and Zaat, 2012). An artificial honey solution is used to distinguish between the osmotic effects of sugars and antibacterial activity in a study by Cooper *et al.*, (2002b).

### 1.6.1.2 Hydrogen Peroxide

In the 1960s, hydrogen peroxide ( $H_2O_2$ ) was identified as a major antibacterial compound in honey. Hydrogen peroxide is commonly used in cleaning products such as bleach but it is also produced naturally during glucose oxidation of honey (Brudzynski *et al.*, 2011). Hydrogen peroxide is also a contributing factor to a honeys acidity and sterility.

Hydrogen peroxide and honey phenolics with pro-oxidant activities are involved in oxidative damage resulting in bacterial growth inhibition and DNA degradation (Brudzynski *et al.*, 2011, Brudzynski *et al.*, 2012). Brudzynski *et al.*, (2012) concluded that hydrogen peroxide is involved in oxidative damage, which causes bacterial DNA degradation and growth inhibition. Further studies revealed the bacteriostatic effect was directly related to the generation, and therefore concentration of hydroxyl radicals generated from the hydrogen peroxide (Brudzynski and Lannigan, 2012). It is believed that the hydrogen peroxide effects are modulated by other honey components (Brudzynski *et al.*, 2011).

### 1.6.1.3 Low pH

The low pH (3.5-5.5) also contributes to the bactericidal activity of honey (Bogdanov, 1997). Honey has a low pH, mainly due to the high levels of hydrogen peroxide and gluconic acid. Honey contains a highly variable number of different acids, including amino, organic, aliphatic, phenolic and aromatic acids. The acidity of honey increases upon storage and heat treatment of honey.

### 1.6.1.4 Bee Derived Antibacterial Peptides

Bee derived defensins are cysteine-rich cationic peptides produced in the salivary glands and fat body cells and are involved in social and individual immunity (Klaudiny *et al.*, 2005). Two defensins have been characterised, royalisin (from royal jelly) and defensin (from the haemolymph) (Table 1.3), which are both encoded by *defensin1*. *Defensin-2* which shows 55% similarity to *defensin1*, has also been identified (Ilyasov *et al.*, 2013). Defensin-1 (5.5KDa) has been shown to possess potent antibacterial activity against Gram-positive micro-organisms including *S. aureus* and *B. subtilis* (Bucekova *et al.*, 2014, Kwakman *et al.*, 2010) and *Paenibacillus* larvae. This is the causative agent of American Foulbrood (AFB) which is a major pathogen of bees (Katarína *et al.*, 2002).

Table 1. 3: Amino acid sequences of honey bee defensin1 variants

Defensin	VTCDLLSFKGQVNSACAANCLSLGKAGGHCEKVGVCICRKTSFKDLWDKRF
Royalisin	VTCDLLSFKGQVNSACAANCLSLGKAGGHCEKVGVCICRKTSFKDLWDKRF

Revamil® source (RS) honey work has identified that these peptides are effective antibiotics with a broad spectrum of activities, and add to the antimicrobial properties of honey (Kwakman *et al.*, 2011a). RS Honey is produced in greenhouses under controlled conditions, by the Dutch group Bfactory and they do not disclose any details on the origin of this honey. The honey is not registered as an antimicrobial but as a wound healing stimulant where it is claimed to stimulate tissue regeneration and reduce inflammation. The *in vitro* bactericidal activity of Revamil® honey against *B. subtilis*, *S. aureus*, *S. epidermidis*, *E. coli* and *P. aeruginosa* was assessed and a bactericidal effect was seen within 24 h by 10-40% (v/v) honey (Kwakman *et al.*, 2010). The peptide (defensin-1) and the other factors contributing to this bactericidal effect were also characterised (Kwakman *et al.*, 2010). Other proteinaceous antibacterial compounds have previously been reported in six of twenty six honeys, but identification of these proteins was not performed (Mundo *et al.*, 2004).

### 1.6.1.5 Plant Derived Antibacterial Phytochemicals

Plant derived phytochemicals play an important role in the antibacterial activity of honey; methylglyoxal (MGO) from Manuka honey is an example of honey which attributes its activity to plant derived chemicals. Non-peroxide activity has been described in investigations of bactericidal factors within honey (Manyi-Loh *et al.*, 2012, Aljadi and Yusoff, 2002, Pinho *et al.*, 2014), particular attention has been paid to Manuka honey (Adams *et al.*, 2009).

Plant derived phenolic compounds isolated from honey have been investigated and identified by different research groups, but the contribution to the overall activity remains unclear (Isla *et al.*, 2011, Manyi-Loh *et al.*, 2012, Aljadi and Yusoff, 2002, Kwakman and Zaat, 2012, Liu *et al.*, 2013) It has been suggested that the contribution of plant derived components to the antibacterial activity of honey is too low to detect (Kwakman *et al.*, 2010, Molan, 1992), but when extracted phenolics and flavonoids are regarded as a very promising source of natural medicinal therapeutics.

Solid phase extraction (SPE) and HPLC analysis was used to extract phenolic compounds and antimicrobial agents from *Rubus* honey (Escuredo *et al.*, 2012). The phenolics caffeic, *p*-coumaric and ellagic acids and the flavonoids chrysin, galangin, pinocembrin, kaempferol and tectochrysin were isolated (Escuredo *et al.*, 2012). The phenolic fraction samples showed antimicrobial activity against various organisms including *Salmonella typhimurium*, *Proteus mirabilis*, and *P. aeruginosa*. The most susceptible species were *P. mirabilis* and *Bacillus cereus* (Escuredo *et al.*, 2012). The antioxidant and antimicrobial activities of phenolics extracted from *Rhododendron* honeys from the Black Sea region of Turkey have also been studied (Silici *et al.*, 2010). High levels of antimicrobial activity was described against *P. aeruginosa* and *P. mirabilis* (Silici *et al.*, 2010). The combination of different phenolics, instead of individual compounds may contribute to the activity of honey, but further investigations are required in order to assess these interactions (Manyi-Loh *et al.*, 2012). The minor constituents in honey have high levels of antimicrobial activity due to a combination of these factors, often working in unison. These plant derived compounds have high potential to be used as therapeutics in human health.

It has been shown that the flavonoids, phenolic and organic acids in honey may act in various processes including hydrogen donating, oxygen quenching, radical scavenging and metal ion chelation resulting in bacterial growth inhibition (Manyi-Loh *et al.*, 2012). The antibacterial activity of phenolic compounds should not be dismissed; phytochemicals have an influence on the antimicrobial activity of honey (Molan, 2011). Peroxide and non-peroxide factors may also be working in synergy and inhibiting bacterial growth (Manyi-Loh *et al.*, 2011).

In order to analyse these compounds, the sugars which are the major components in honey must be removed. Various analytical techniques can be used to identify these components (Cuevas-Glory *et al.*, 2007, Pontes *et al.*, 2007). Thin Layer Chromatography (TLC) and Gas

Chromatography-Mass Spectrometry (GC-MS) have been used to extract the phenolic compounds which have demonstrated antibacterial activity against *H. pylori* (Manyi-Loh *et al.*, 2012). *H. pylori*, which causes chronic active gastritis and peptic ulcers, showed susceptibility to various fractions of South African honey (Manyi-Loh *et al.*, 2012, Manyi-Loh *et al.*, 2013). The activity was attributed to the combination or separate action of volatile compounds including acetic acid (Manyi-Loh *et al.*, 2012).

Other VOCs have been identified in honey; ( $\pm$ )-3-Hydroxy-4-phenyl-2-butanone and (+)-8-hydroxylinalool show high levels of antimicrobial activity against bacteria including *S. aureus*, *E. coli*, *K. pneumonia* and human pathogen fungi *Candida albicans* (Melliou and Chinou, 2011). Despite only being present in low concentrations the VOCs may contribute to the overall antimicrobial activity and have the potential to be used as natural therapeutics to treat a range of pathogenic microbial organisms.

### 1.6.2 Manuka Honey

Manuka honey has been approved for marketing as a therapeutic honey and is a valuable consumer product on the market. These honeys have shown to have *in vivo* activity and are used for the treatment of ulcers, wounds and many other skin infections (Cooper *et al.*, 2002a, Visavadia *et al.*, 2008). Manuka honey has been approved for the treatment of various different infections, including burns, thrush and gastrointestinal problems (Somal *et al.*, 1994). It is produced when bees forage on the Manuka bush *Leptospermum scoparium* (Figure 1.8) which is indigenous to New Zealand and Australia (Kwakman *et al.*, 2011b). Dihydroxyacetone (DHA) is a degradation product of carbohydrates (Atrott *et al.*, 2012); the nectar of this plant contains dihydroxyacetone which is the precursor of a potent antibacterial compound MGO (Adams *et al.*, 2009). MGO is a reactive metabolite that can exert toxic effects (Kalapos, 1999), it is responsible for **log** phase extension during microbial growth and morphological changes to bacterial cells; which effect cell division and DNA formation (Lu *et al.*, 2013). MGO is known to inhibit DNA replication in bacterial cells (Fraval and McBrien, 1980).



Figure 1. 8: Manuka bush from which bees gather nectar to produce antibacterial mono-floral Manuka honey

Upon storage the high level of collected DHA is converted to MGO. The level of MGO in Manuka fluctuates based on many factors including the age and variety of *L. scoparium* from which the nectar was collected (Stephens *et al.*, 2010, Rogers *et al.*, 2014, Adams *et al.*, 2009). As a consequence Manuka honey contains high levels of MGO at concentrations ranging from 38 to 761 mg/kg, which is up to 100-fold higher than the levels found in the majority of other honeys (Mavric *et al.*, 2008). The high levels of MGO results in non-peroxide antibacterial activity which is unique to Manuka honey (Mavric *et al.*, 2008). Recent reports suggest MGO is also responsible for suppressing the generation of hydrogen peroxide in Manuka honey (Majtan *et al.*, 2014).

Manuka honey has broad-spectrum antibacterial activity, with bactericidal effects against many strains of bacteria including MRSA (Jenkins *et al.*, 2011) and *P. aeruginosa* (Cooper *et al.*, 2002a). The high osmolarity caused by Manuka honey induces cell lysis in *P. aeruginosa* cells, it inhibits cell-to-cell adhesion and induces abnormal cell phenotypes by reducing structural integrity (Cooper *et al.*, 2002a). Manuka honey has multifactorial antibacterial activity, Jenkins *et al.*, (2011) and Kwakman *et al.*, (2011b) have both concluded that the inhibition of cell division was the result of antibacterial components, other than MGO and sugar in Manuka honey. Previous studies suggest it is possible that other acidic and phenolic compounds are in part responsible for the bactericidal activity of Manuka honey (Bogdanov, 1997, Weston, 2000).

The antibacterial activity of Manuka honey is an important commercial property. MGO is a specific example of a plant derived chemical which has antimicrobial activity (Kwakman *et al.*, 2011b, Stephens *et al.*, 2010). Mundo *et al.*, (2004) reported that after neutralisation of hydrogen peroxide and removal of proteinaceous compound some honeys retain activity against MGO resistant *B. stearothermophilus* suggesting the presence of additional antibacterial factors, which have not previously been described (Mundo *et al.*, 2004). Similarly, research has proven that neutralisation of MGO abolishes the activity of Manuka honey against *S. aureus* and *B. subtilis*, but not against *E. coli* and *P. aeruginosa* (Kwakman *et al.*, 2011b). The cell division cycle in MRSA (NCTC 13142) is interrupted when MGO and sugars have been accounted for, again suggesting the presence of other contributing factors (Jenkins *et al.*, 2011). This suggests MGO is not totally responsible for Manuka non-peroxide antimicrobial activity, but plays an important antibacterial role.

#### **1.3.1.6 Other antimicrobials in honey from pollen, propolis and royal jelly**

The phenolic composition, antioxidant properties and antibacterial activity of pollen collected by honey bees has been studied to identify natural components which may be beneficial for human health (Morais *et al.*, 2011). Phytochemicals are considered to be beneficial to human health by

reducing oxidative stress and inhibiting macromolecular oxidation (Pulido *et al.*, 2000). Some phytochemicals are also reported to have anti-carcinogenic properties (Loa *et al.*, 2009). The antibacterial properties of pollen extracts have been assessed against *B. cereus*, *S. aureus*, *S. typhi* and *E. coli* (Morais *et al.*, 2011). The findings described good levels of antimicrobial activity suggesting pollen-derived phytochemicals could be a good source of new antimicrobial pharmaceuticals (Morais *et al.*, 2011). Propolis extracts have potent antibacterial, antiviral, antifungal and anti-inflammatory activities (Kalogeropoulos *et al.*, 2009, Kasote *et al.*, 2015, Kujumgiev *et al.*, 1999, Borrelli *et al.*, 2002). Propolis was part of ancient medicine but is now used in a variety of modern biological and pharmaceutical applications.

Royal jelly contains antimicrobial proteins known as Jelleins; including major royal jelly protein 1 (MRJP1). These proteins are produced by the bees and secreted specifically into royal jelly (Fontana *et al.*, 2004). Jelleine 1 (PFKLSLHL-NH(2)), Jelleine 2 (TPFKLSLHL-NH(2)) and Jelleine 3 (EPFKLSLHL-NH(2)) showed activity against eleven different bacteria including *S. aureus*, *B. subtilis*, *E. coli* and *K. pneumoniae* (Fontana *et al.*, 2004). Royal jelly is sold commercially as a skin care and natural beauty product as it contains B-complex vitamins, including vitamin B<sub>5</sub> and vitamin B<sub>6</sub>. Phenolic compounds including flavonoids found in pollen, propolis and royal jelly are often also present in honey, and may subsequently contribute to the antimicrobial activity (Kaškonienė and Venskutonis, 2010). There is scope to discover novel therapeutics by further characterising the minor constituents in honey.

### **1.6.3 Wound Healing Capabilities**

The healing properties of honey has long been documented (Majno, 1975), it has been extensively studied since it was rediscovered as a therapy more than a century ago (Molan, 1992, Allen *et al.*, 1991). When honey is applied to a wound a combination of factors are involved in the healing process (Table 1.3). The physical nature of honey makes it desirable for wound healing and wound dressings. Honey has high viscosity and adheres easily to dressings, this creates a protective barrier between the wound and other air borne microorganisms (Molan, 2002).

Applying honey to wounds directly promotes healing by reducing inflammation, debriding necrotic tissue and reducing oedema (Tonks *et al.*, 2001, Molan, 2011). There is a lack of high-quality evidence from randomised controlled trials (RCTs) for the use of honey in wound treatments, often due to the low number of patients treated (Molan, 2011). Despite this positive findings from 17 RCT's involving a total of 1,965 patients provided evidence supporting the use of honey in wound care (Molan, 2011). Honey dressing have successfully been used for the treatment of many conditions, including neuropathic plantar foot ulcers (Mohamed *et al.*, 2013).

Table 1. 4: Therapeutic benefit of honey in wound care

<b>Mechanism of Activity</b>	<b>Suggested Rationale</b>
Preventing the entry of other foreign pathogens	Viscosity of honey provides a protective barrier
Assists and accelerates healing	Stimulates macrophages, lymphocytes and phagocytes
Dressings do not adhere or become embedded into wounds	A protective interface forms between wound bed and dressing due to the viscosity of honey
Reducing infection	Antibacterial properties - found to be effective against a range of bacteria
Creates a moist wound healing surface	Osmotic pressure draws fluid from underlying tissues
Anti-inflammatory	Number of inflammatory cells reduced in honey-treated wounds

(Subrahmanyam, 1998, Gupta et al., 2011, Al-Waili and Saloom, 1999, Molan, 2011)

The rationale for the healing mechanisms of honey is well established (Molan, 2011). The application of honey results in rapid debridement, increased antioxidant activity and a reduction in inflammation which all assist wound healing. The osmotic effect of honey draws a replenishing supply of proteases to the interface of the wound bed and the necrotic tissue (Molan, 2002). The process also draws out lymph fluid from the wound removing dirt and washing the bed from below (Molan, 2011). Honey is known for its debriding action; it is believed honey activated plasminogen produced in macrophages result in the digestion of fibrin (Molan, 2009a). The production of eschar and scabs are reduced due to the reduction of fibrin which attaches slough to the wound surface (Molan, 2011). Slough is made up of a clustering of dead cells which prevents the wound healing.

Numerous studies have reported honey reducing oedema and stimulating exudate production, soothing the wound and reducing scarring (Efem, 1988, Subrahmanyam, 1993). Honey has been demonstrated to reduce oedema and decrease proinflammatory mediators (Hussein *et al.*, 2012). Honey also directly increases the rate of healing; research has shown that it stimulates proliferation of the monocytic cells including B-lymphocytes and T-lymphocytes. These cells induce the expression of cytokines such as tumour necrosis factor (TNF- $\alpha$ ) and interleukin (IL)-1 and IL-6 which help to fight the infecting bacteria and activate tissue repair (Tonks *et al.*, 2001).

Honey scavenges reactive oxygen species (ROS) produced during the inflammation process. The reduction in ROS limits the amount of tissue damage by activating macrophages during the healing process which reduces hypertrophic scarring. This decrease in production of ROS is reportedly due to the antioxidant components in honey (Molan, 2011, Ma *et al.*, 2003), the healing of burns has also been accredited to the antioxidants found within honey (Subrahmanyam *et al.*, 2003). The agents responsible remain to be isolated, hydrogen peroxide may be responsible. The

acidity of honey also helps provide oxygen to regenerating tissue. The overall effect of applying honey to wound dressings is to enhance the rate of wound healing, reduce infection of opportunistic microorganisms, reduce inflammation and increase debridement.

The phenolic content of honey has been associated with its antioxidant potential and represents compounds which can remove oxygen radicals and thus reduced their toxicity (Dong *et al.*, 2013). Flavonoids, phenolic and organic acids which have been identified in natural honey are known to scavenge for free superoxide and other reactive oxygen metabolites, reducing their potential to cause oxidative damage (Manyi-Loh *et al.*, 2010). They are commonly used to treat or reduce the incidence of many conditions including strokes and heart disease (Piljac-Žegarac *et al.*, 2009). It is believed that Maillard reaction products (MRPs) act as antioxidants, HMF is one of the major antioxidant products of the Maillard reaction (Manyi-Loh *et al.*, 2011). Due to the numerous health benefits of dietary antioxidants, the botanical profile of honey should be considered when determining the potential of honey as an antioxidant-containing food supplement.

## **1.7 Microorganisms in Healthcare Settings**

### **1.7.1 Methicillin resistant *Staphylococcus aureus* (MRSA)**

*Staphylococcus aureus* is a facultative anaerobic, Gram-positive coccal bacterium which can cause a variety of self-limiting to life-threatening diseases in humans (Deurenberg and Stobberingh, 2008). It is an opportunistic pathogen found on the skin of 25–30% of the population and is the most common cause of wound infections (Perencevich and Diekema, 2010). *Staphylococcus aureus* is a bacterium that is relatively simple to handle and manipulate and is commonly used for the screening of antimicrobial compounds in honey (Cooper *et al.*, 1999, Maeda *et al.*, 2008).

Recent reports suggest that there has been a decrease in MRSA infection rates among patients in US hospitals between 2005 and 2008 (Kallen *et al.*, 2010) and in the UK between during the period 2002–2008 (Pearson *et al.*, 2009). The Office of National Statistics also reported a reduction of MRSA related deaths by 79% in males and 76% in females between 2008 and 2012 (ONS., 2013). Despite these reductions MRSA is still a prevalent hospital acquired infection; in 2012 across England and Wales a total 557 death certificates mentioned *S. aureus* (ONS., 2013). Annual counts and rates of MRSA bacteraemia by NHS acute trusts were determined by Public Health England (PHE), 13,667 cases of MRSA were recorded between April 2007 and March 2015 (PHE, 2014).

MRSA is predominantly contained within the health care setting but can also be found in the community in immuno-compromised patients. The prevalence of community-acquired MRSA (CA-MRSA) has steadily increased since the 1980s with many skin and soft tissue infections being reported, in particular in elderly patients (Skov *et al.*, 2012). The increased prevalence of MRSA infection within the community, and the number of MRSA cases that still occur annually in hospitals is a cause for concern. Furthermore with the pervasiveness of antibiotic resistance *S. aureus* is still an area which requires the investment of research into novel therapeutics. The evolution of resistance to antibiotics is a constant threat and novel drugs will always be required. In summary MRSA is an ideal model organism, it is relatively easy to culture and has been shown to be sensitive to the antimicrobial properties of honey.

### **1.7.2 *Escherichia coli* (*E. coli*) and *Pseudomonas aeruginosa* (*P. aeruginosa*)**

*E. coli* is a common Gram-negative, facultative, anaerobic, rod shaped bacterium. It is found in the environment, foods, and intestines of people and animals. *E. coli* can cause a range of infections including urinary tract infection and intestinal infection. *P. aeruginosa* is a Gram-negative, aerobic, coccobacillus bacterium often found in soil and ground water. *P. aeruginosa* is an opportunistic pathogen which commonly causes infections due to its ability to colonise critical body organs, respiratory equipment and catheters (Baltch and Smith, 1994). These bacteria can both be cultured easily and cost effectively in a laboratory setting. *E. coli* and *P. aeruginosa* have been used for testing the antibacterial activity of honey (Kwakman *et al.*, 2011b, Brudzynski and Lannigan, 2012).

### **1.7.3 *Bacillus subtilis* (*B. subtilis*)**

*B. subtilis* is a Gram-positive, catalase-positive bacterium commonly found in soil and also as a commensal in the gut of humans. It is not pathogenic to humans or animals but vegetative *B. subtilis* is used to assess the antibacterial properties of honey due to its susceptibility for non-peroxide bactericidal activity, it is used for activity-guided identification of novel components (Kwakman *et al.*, 2010, Kwakman *et al.*, 2011b). *B. subtilis* forms stress-resistant endospores, enabling survival in extreme environment.

## **1.8 PhD Aims and Objectives**

### **1.8.1 Aims**

The main aim of this project is to identify, isolate and characterise plant-derived antibacterial compounds in honey which may have the potential for therapeutic application and the treatment of clinically relevant pathogens.

### **1.8.2 Objectives**

- To obtain a range of honey samples from across Wales and the UK which represent as diverse a range as possible of the native flora
- To develop laboratory based methods capable of identifying honey with non-peroxide based antibacterial activity
  - To develop isolation and characterisation techniques with which to extract and identify novel antibacterial compounds from honey
- To develop next generation sequencing (NGS) techniques to characterise DNA from pollen
  - To determine the plants which contribute to the making of active honey samples
  - To compare NGS to traditional microscopic methods for pollen characterisation
- To characterise the compounds using activity-guided separation chromatography and analytical chemistry techniques
  - Identify compounds from honey and plant extracts which have the potential to be used for the treatment of clinically associated pathogens

## Chapter 2

### **GENERAL MATERIALS AND METHODS**

## **2.1 Materials**

### **2.1.1 Honey Sample Collection**

In total 217 unprocessed raw honeys were provided by bee keepers from across Wales and the rest of the UK for the study. Details of the source of each sample are shown in table 2.1.

Bee keepers were asked to keep any post-harvest processing such as filtration and heating to a minimum to reduce any potential damage to pollen and antibacterial compounds. Three samples of Manuka honey with unique Manuka factor values of 5<sup>+</sup>, 10<sup>+</sup> and 15<sup>+</sup> (a measure of antibacterial activity linked to a phenol standard) were purchased from Holland and Barrett (Cardiff, UK). All honey samples ( $n=220$ ) were stored in the dark at room temperature prior to sampling.

### **2.1.2 Chemicals and Reagents**

Chemicals and reagents were from Sigma Aldrich Ltd., UK, solvents were from Thermo Fisher Scientific Ltd., UK, or VWR Ltd., USA and reagents for DNA analysis were purchased from Bioline Ltd., UK or Qiagen Ltd., Germany unless otherwise stated in the text.

Table 2. 1: The honey ID and source of each honey sample

Description of the details held for each individual honey, UK sample was assigned if no details were held. Manuka samples highlighted in red.

Honey ID	Description	Honey ID	Description
H1	SA33 5NE - Mel Pur Cymraeg	H58	West Sussex
H2	SA33 5NE - Mel Pur Cymraeg	H59	LU13TQ - Luton Zoo Garden
H3	SA33 5NE - Mel Pur Cymraeg	H60	Kent honey
H4	SA33 5NE - Mel Pur Cymraeg	H61	Kent honey
H5	SA43 2PQ - Beelief botanics	H62	UK honey
H6	SA43 2PQ - Beelief botanics	H63	Surry honey
H7	Pembrokeshire - West valley honey farm	H64	UK honey
H8	SA67 8NR - Amroth Woodreef	H65	Kent honey
H9	UK sample	H66	Hereford honey
H10	UK sample	H67	Hereford honey
H11	Barry honey	H68	Lancashire honey
H12	CF32 9QB - Rectary close, Sarn	H69	UK honey
H13	SA34 OJD - Brynderi honey farm	H70	Wiltshire honey
H14	SA34 OJD - Brynderi honey farm	H71	Wiltshire honey
H15	Rhos, Llandysul honey	H72	Brimecombe apiaries - Devon
H16	UK sample	H73	Brimecombe apiaries - Devon
H17	SA44 6NN - New Quay honey farm	H74	Brimecombe apiaries - Devon
H18	SA44 6NN - New Quay honey farm	H75	Brimecombe apiaries - Devon
H19	Aberteifi honey	H76	Boullin Surrey garden honey
H20	SY23 1AA- Aberystwyth – Tropical forest products – heather honey	H77	UK honey
H21	LL55 1YD - Caernarfon honey	H78	UK honey
H22	LL16 5LH - Pentre reidiog, Llansannan,	H79	WV8 1PG – Wolverhampton honey
H23	LL16 5LH - Pentre reidiog, Llansannan,	H80	Shop bought Shropshire honey
H24	LL36 9EW – Aberdovey, Gwynedd	H81	UK honey
H25	LL36 9EW – Aberdovey, Gwynedd	H82	TN19 7JT –Etchingham
H26	SA44 5NA - Ceredigion	H83	TN19 7JT – Westdown Lane, Etchingham
H27	SA67 8NR – Amroth Woodreef	H84	TN19 7JT – Westdown Lane, Etchingham
H28	SA67 8NR - Amroth Woodreef	H85	TN19 7JT – Westdown Lane, Etchingham
H29	SA67 8NR - Amroth Woodreef	H86	UK honey
H30	Shop bought heather honey, Brecon	H87	UK honey
H31	SA32 8HN – National Botanic Garden of Wales	H88	PO18 8RW – Sussex
H32	SA19 7XL - Llandeilo, Carmarthenshire	H89	UK honey
H33	SA44 5NL - Penty parc, Ceredigion	H90	UK honey
H34	Bridgend bee keepers association (BKA)	H91	RM15 4DR – Purfleet Road, Ockendon
H35	Bridgend BKA honey	H92	DE22 5JW – Montpelier Quarndon, Derby
H36	CF32 0EE – Bridgend	H93	UK honey
H37	Bridgend BKA honey	H94	BN20 8DY – Pashley Road, Eastbourne
H38	Bridgend BKA honey	H95	BT30 9HJ - Kilmore Road, Downpatrick
H39	Bridgend BKA honey	H96	DE55 6DN – Wessington, Alfreton
H40	Bridgend BKA honey	H97	EX14 9RY – Ramsden Lane, Honiton
H41	Bridgend BKA sample	H98	HG2 9NS - Harrogate, North Yorkshire
H42	Bridgend BKA sample	H99	RH12 4BT – Horsham, West Sussex
H43	Bridgend BKA sample	H100	RH12 4BT - Horsham, West Sussex
H44	CF44 9JZ – Penderyn, Aberdare	H101	UK honey
H45	Bridgend BKA sample	H102	UK honey
H46	CF44 9JZ - Caerhowell, Penderyn	H103	RH5 6PG - Holmbury St Mary, Surrey
H47	LL61 6PY - Gwynedd	H104	RH5 6PG - Holmbury St Mary, Surrey
H48	LL61 6PY - Gwynedd	H105	UK honey
H49	LL61 6PY - Gwynedd	H106	UK honey
H50	Welsh honey - Wigfa apiaries, Rhos	H107	PO32 6NG - East Cowes, Isle of Wight
H51	Cornish honey	H108	UK honey
H52	Rowse Manuka – 5+ activity rating	H109	UK honey
H53	Manuka – 15+ activity rating	H110	Llangymir, Carmarthenshire
H54	Manuka - +10 activity rating	H111	UK honey
H55	SA44 5NA - Llandysul	H112	UK honey
H56	Cambridge honey	H113	UK honey
H57	UK honey	H114	SA32 8RD – Dryslwyn, Carmarthen
		H115	UK honey
		H116	UK honey
		H117	UK honey
		H118	UK honey

H119	KT14 6RL - West Byfleet, Surrey
H120	PR3 2QY - Chipping, Preston, Lancashire
H121	SY8 3LJ - Knowbury, Ludlow, Shropshire
H122	WS5 3BH - Walsall, West Midlands
H123	WS5 3BH - Walsall, West Midlands
H124	WS5 3BH - Walsall, West Midlands
H125	UK honey
H126	UK honey
H127	UK honey
H128	UK honey
H129	UK honey
H130	UK honey
H131	SW18 5QR – London
H132	KT10 – Claygate, Surrey
H133	ME9 8JT - Borden, Sittingbourne, Kent
H134	CR2 0SB - South Croydon, London
H135	KT21 1QF - Ashted
H136	NP7 0DG – Abergavenny
H137	NP15 2ET - Usk, Monmouthshire
H138	Essex honey
H139	Llanpumsaint honey
H140	UK honey
H141	UK honey
H142	GU23 6QG - Surrey
H143	GU23 6QG - Surrey
H144	UK honey
H145	UK honey
H146	CM5 9RT – Norton hall cottages, Essex
H147	GY3 5ND – Guernsey
H148	UK honey
H149	TQ2 7HA - Torquay, Torbay
H150	UK honey
H151	LL77 8JD - Talwrn, Isle of Anglesey
H152	SA44 - Llandysul, Ceredigion
H153	SY23 3DG - Commin coch, Aberystwyth
H154	SY24 5AX – Bow street, Ceredigion
H155	Pont-rhyd-y-groes, Ceredigion
H156	Salisbury, Dorset, Field honey farms
H157	Salisbury, Dorset, Field honey farms
H158	LL48 6SH - Penrhyndeudraeth, Gwynedd
H159	UK honey
H160	Aberystwyth honey
H161	Aberystwyth honey
H162	UK honey
H163	UK honey
H164	LL39 1YR - Arthog, Gwynedd
H165	LL51 9AX - Garndolbenmaen, Gwynedd
H166	UK honey
H167	UK honey
H168	UK honey
H169	LL130YT –Wrexham
H170	HG1 2PY - Harrogate, North Yorkshire
H171	HG1 2PY - 3 Rutland road, Harrogate, North Yorkshire,
H172	Wollaton, Nottinghamshire
H173	Wollaton, Nottinghamshire
H174	UK honey
H175	UK honey
H176	UK honey
H177	Mike Barrie
H178	UK honey
H179	TN32 5QX - Roberts bridge, East Sussex
H180	LL36 9EW – Aberdovey, Gwynedd
H181	SA62 4PS - Wiston, Haverfordwest, Pembrokeshire
H182	UK honey

H183	UK honey
H184	RG28 7DZ - Whitchurch, Hampshire
H185	RG28 7DZ - Whitchurch, Hampshire
H186	SL4 4PZ - Windsor and Maidenhead
H187	UK honey
H188	UK honey
H189	UK honey
H190	UK honey
H191	UK honey
H192	UK honey
H193	SY20 8ND - Machynlleth, Ceredigion
H194	SO19 9LJ - The Grove, South Hampton
H195	SO19 9LJ - The Grove, South Hampton
H196	SY23 5NJ - Nant heuloog, Ceredigion
H197	LA11 6JX - Grange-over-Sands, Cumbria
H198	LA11 6JX - Grange-over-Sands, Cumbria
H199	BA3 4RH - Radstock, Bath
H200	TN9 2BT - Tonbridge, Kent
H201	Bournmouth honey – buffalo boat
H202	SY15 6BB - Montgomery, Powys
H203	SY15 6BB - Montgomery, Powys
H204	SY21 8BB - Welshpool, Powys
H205	SY21 8BB - Berriew, Powys
H206	SY15 6LD - Llwynmadoc, Abermule
H207	SY16 3JL - Newtown, Powys
H208	SY22 5LZ - Llanfyllin, Powys
H209	SY16 4IZ - Kerry, Powys
H210	SY15 6HU - Church Stoke, Montgomery
H211	SY16 3LB - Newtown, Powys
H212	SY17 5JP - Carno, Caersws, Powys
H213	SY163AX – Newtown, Powys
H214	SY17 5AT - Llandinam, Powys
H215	SY16 3PE - Tregynon, Newtown, Powys
H216	SY18 6NX - Llanidloes, Powys
H217	SY16 3AX - Newtown, Powys
H218	SY16 3PU - Newtown, Powys
H219	SY18 6NQ - Llanidloes, Powys
H220	SY156HU - Church Stoke, Montgomery

### 2.1.3 Biological Culture Media

All agar and broths were from Fisher Scientific Ltd., UK, unless otherwise stated (Table 2.2). Supplemented agar and broth were prepared following manufacturers' instructions and all media was sterilised, by autoclaving (Prestige Medical, UK) for 15 min at 121 °C prior to use. The following media was used;

Table 2. 2: Culture media used to support for bacterial growth

#### Culture media

Iso-sensitest agar (ISO)  
Lysogeny broth (LB)  
Mueller Hinton agar (MHA)  
Nutrient agar (NA)  
Nutrient broth (NB)  
Tryptone soya agar (TSA)  
Tryptone soya broth (TSB)

De-ionised water (diH<sub>2</sub>O) was obtained from an ELGA Purelab Option BP15 dispenser (ELGA labwater, UK). Solutions were sterilised by autoclaving at 121 °C for 15 min.

### 2.1.4 Bacterial Cultures

Methicillin-resistant *Staphylococcus aureus* (11939) (MRSA) was purchased from the National Collection of Type Cultures (NCTC, UK).

*Bacillus subtilis* (6633) and a non-sporogenic mutant (39090) were purchased from the American Type Culture Collection (ATCC, USA).

*Escherichia coli* (12210) was purchased from the National Collections of Industrial, Marine and Food Bacteria (NCIMB, UK).

*Pseudomonas aeruginosa* (10548) was purchased from the National Collections of Industrial, Marine and Food Bacteria (NCIMB, UK).

All cultures were stored in 10% glycerol on Microbank™ cryoprotective beads (Pro-Lab Diagnostics Ltd., UK) at -80 °C until further use.

## **2.2 Methods**

### **2.2.1 Freezer Storage**

Freezer stock cultures for each strain were prepared using PROTECT® beads which were stored at -80 °C (Technical service Consultants Ltd, UK). Bacteria were cultured following manufacturers' rehydration instructions. A 1ml sample of 24 h pure culture (section 2.3.3) was aseptically pipetted and added to cryopreservative fluid. The vial was vigorously shaken and the liquid removed, the beads were stored at -80 °C until required for experimentation.

### **2.2.2 Preparation of Fresh Bacterial Culture Slope**

McCarty bottles were filled with 30 ml of molten tryptone soya agar (TSA) and allowed to set at a 45 degree angle. An inoculated loop from an overnight culture of bacteria was used to coat each slope, the overnight cultures (section 2.3.3) were prepared directly from the freezer beads. The slopes were grown at 37 °C for 24 h and were then stored at 4 °C in a fridge. All culture slopes were stored for no longer than 2 weeks.

### **2.2.3 Preparation of an Overnight Culture**

Falcon tubes (50mL) containing 10ml of tryptone soya broth (TSB) were inoculated with a bacterial slope colony using an inoculation loop. Inoculated broths were incubated overnight at 37 °C in a shaking incubator (Thermo scientific MAXQ 4450) at 1 g. After 24 h the broths were centrifuged at 5000 g for 15 mins at 18 (MSE, Mistral 1000. London, UK). The supernatant was discarded and the pellet resuspended in broth or buffer to an optical density (OD<sub>600</sub>) indicating the desired bacterial cell count.

### **2.2.4 Miles Misera Viable Bacterial Cell Counting Method**

The Miles and Misera method was adopted in order to determine the number of colony forming units within a suspension of bacteria (Miles *et al.*, 1938). A bacterial suspension was prepared by centrifuging (MSE, Mistral 1000. London, UK) a 10 ml overnight culture for 10 mins at 5000 g at room temperature. The supernatant was discarded and the pellet was re-suspended in 10 ml of sterile phosphate buffered saline (PBS) (Sigma) and vortexed until fully suspended. The optical density was measured at 600 nm using a spectrophotometer (Ultraspec 3100 *pro*). The bacterial suspension was serially diluted by adding 1x of suspension to 9x of diluent (PBS), this was repeated until dilutions were made to at least 10<sup>-8</sup>. A 1 x 10 µl drop of each dilution is dropped

onto the surface of the TSA agar plates in triplicate. The drops were allowed to dry and plates were incubated overnight at 37 °C (Memmert INE 600, Germany). The individual colonies were counted and colony forming units (CFU) were counted using the following equation;

$$CFU \text{ per ml} = \text{Average number of colonies for a dilution} \times 100 \times \text{dilution factor.}$$

Standard curves can be produced using the Miles Misera method. To construct an optical density against total viable count standard curve, mean count and corresponding density of each dilution was plotted on a graph. Two fold serial dilutions of the bacterial suspension were produced using PBS to produce dilutions ranging from neat to 1/264. The optical density of each dilution was measured at 600 nm using a spectrophotometer (Ultraspec 3100 *pro*).

The number of viable organisms in each dilution was determined in triplicate using the drop count, serial dilution method described above. This experiment was repeated three times and an average of the nine drop counts was calculated. To construct the OD-TVC curve the mean count and corresponding optical density of each dilution was plotted on a graph. The mean value of the data produced was fitted on a line plot graph. A linear equation was then used in order to establish the optical density required to reach a preferred bacterial concentration in CFU/ml (Figure 2.1- 2.3).

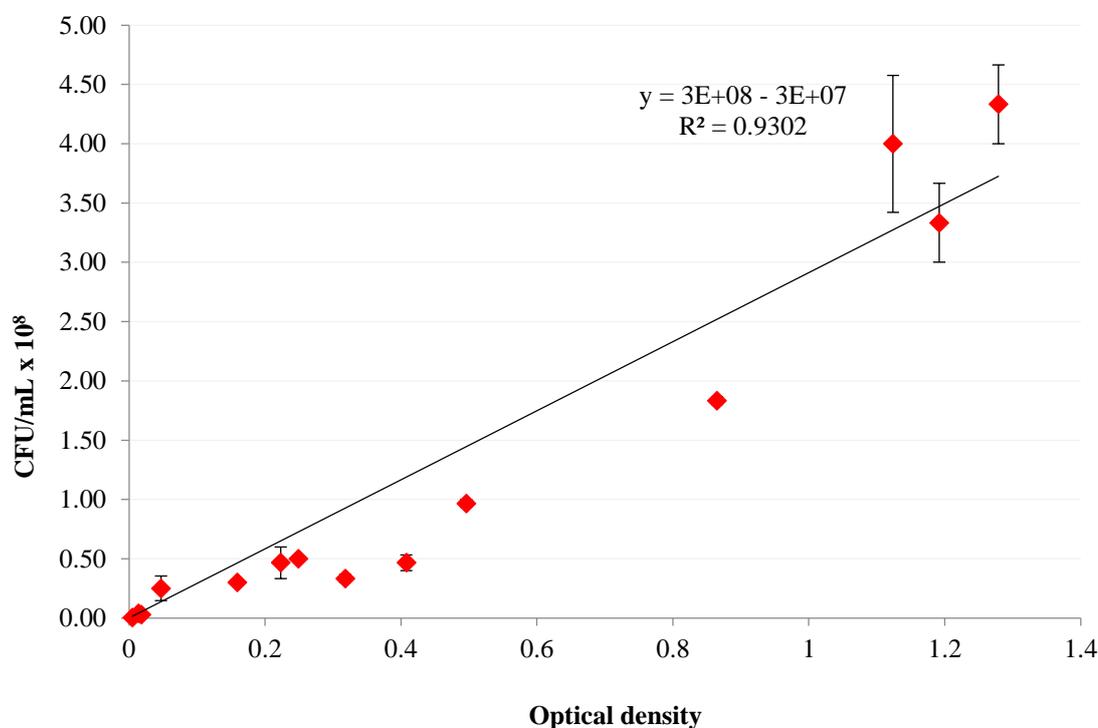


Figure 2. 1: MRSA (NCTC 11939) standard curve

The optical density measured at 600 nm ( $OD_{600}$ ) against the number of colony forming units (CFU) of MRSA NCTC 11939 (Error bars = SE; n = 3).

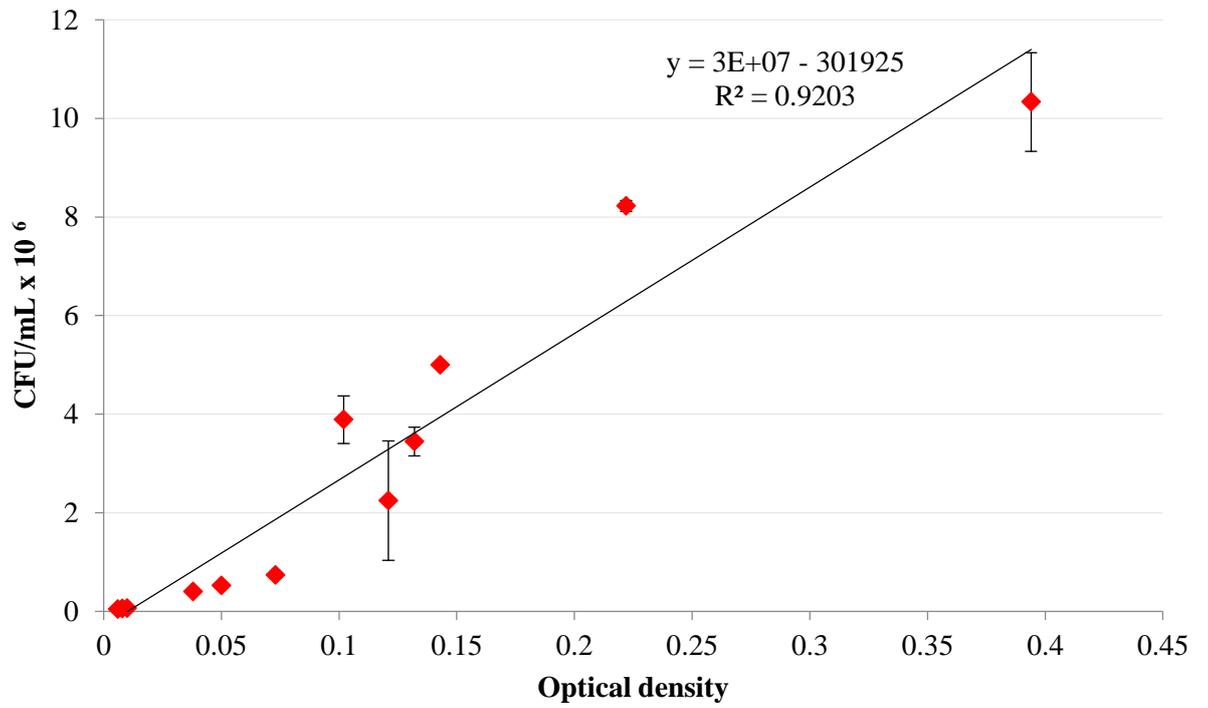


Figure 2. 2: *B. subtilis* (ATCC 6633) standard curve

The optical density measured at 600 nm ( $OD_{600}$ ) against the number of colony forming units (CFU) of *B. subtilis* (6633) (Error bars = SE;  $n = 3$ ).

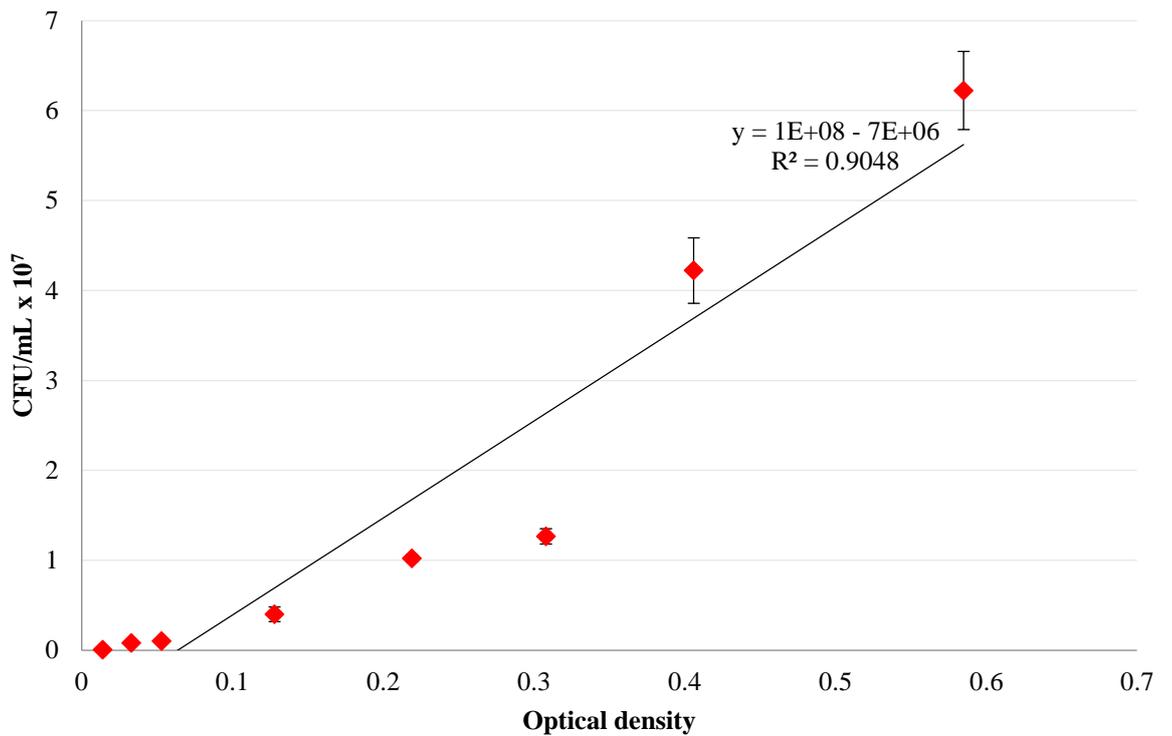


Figure 2. 3: *B. subtilis* (ATCC 39090) standard curve

The optical density measured at 600 nm ( $OD_{600}$ ) against the number of colony forming units (CFU) of *B. subtilis* (39090) (Error bars = SE;  $n = 3$ ).

The McFarland standard of 1 ( $\sim 10^8$  CFU/mL) was used for the *E. coli* and *P. aeruginosa* screening assays. To ensure that suspensions of cultures with turbidity equal to a McFarland standard of 1 approximately contained  $1 \times 10^8$  CFU/mL, viable counts were performed on these strains using the Miles and Misra method (Miles *et al.*, 1938) as described above in section 2.3.4.

### 2.2.5 Streak Plate Method for Isolating Pure Bacterial Colonies

A 10 $\mu$ l loop full of broth culture was aseptically removed from an overnight culture, a section of an agar plate was streaked (Figure 2.4). The plate was rotated anticlockwise and a quarter of the plate streaked again. This process was repeated, dragging a small amount of culture each time (Figure 2.4). A streak in each quarter thins the number of bacteria, forming single colonies on the final streak.

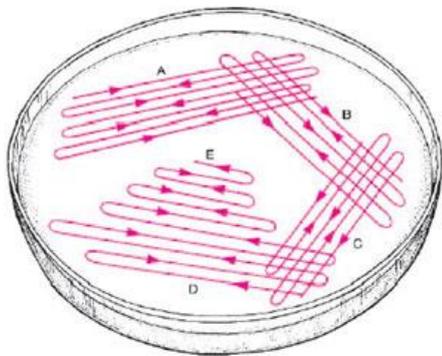


Figure 2. 4: Streak plate method

Highlighting the direction of streaking A- E.

### 2.2.6 Gram Staining

To examine bacteria at microscopic level and confirm basic structure a Gram stain was performed. A 10  $\mu$ l loop was used to remove a colony of bacteria and emulsify it in a drop of sterile distilled water (sdw) on a clean microscope slide. It was allowed to thoroughly air dry and then heat fixed. The slides were then treated as follows;

- Flood slide with crystal violet. Leave on for one minute
- Wash off the stain with sdw
- Cover with the slides with Gram's iodine. Leave on for one minute.
- Wash off the iodine with sdw
- Decolorize with 95% ethanol
- Rinse with sdw to remove excess chemicals
- Counterstain with safranin for 10 seconds
- Rinse with sdw, drain off excess water and dry by blotting gently with paper towel
- Examine the slide under a light microscope using oil immersion (100x) objective

## 2.3 Statistical Analysis

The results presented in this study are the mean of three repeats from within three separate experiments (n=9), unless otherwise stated. All statistics were performed using IBM® SPSS® Statistics version 20.

### 2.3.1 Normal Distribution

Three assumptions must be met to ensure data is normally distributed, if all three assumptions are met parametric tests can be performed (Table 2.3)(Cohen, 1988).

- 1) The observations of the data were independent from each other.
- 2) The results follow normal distribution and pass normality tests.
- 3) The data sets to be compared show homogeneity of variance.

The D'Agostino and Pearson normality tests were given priority but these test were also reviewed in conjunction with the results of the Kolmogorov-Smirnov and Shapiro-Wilk tests for normality. Kurtosis and skewedness were also analysed and their deviance from normal was assessed. Levine's test of the equality of variances was conducted to determine if the data sets showed homogeneity of variance. If any of the assumptions were violated, an appropriate transformation of the data will be attempted (Log10, square root, etc.) to normalise the data or the equivalent nonparametric test was used (Table 2.3).

Table 2. 3: Choice of statistical test

Parametric Test	Data	Non-Parametric Test	Data
One sample T-test	<i>Passes normality assumptions Interval data</i>	Wilcoxon Sign Rank Test	<i>Normality assumptions not met Ordinal /Skewed data</i>
Two sample T-test		Mann-Whitney U Test	
Paired T-test		Wilcoxon Sign Rank Test	
One-Way ANOVA		Kruskal Wallis Test	
Two-Way ANOVA		Friedman Test	

### 2.3.2 Mann-Whitney U Test

If the assumptions were not met to perform the independent samples t-test, then the non-parametric Mann-Whitney U test was used to compare the difference between the medians of two groups.

If a significant difference was found ( $p < 0.05$ ) then interpretation of effect size of ( $r$ ) between two statistically different groups was calculated, this was achieved using the following equation:

$$r = \frac{z}{\sqrt{n}}$$

Where  $r$  = effect size,  $z$  =  $z$  value generated from test and  $n$  = total sample size. Effect size was interpreted (Cohen, 1988): Small 0.10 - 0.29, Moderate 0.30 - 0.49, Large  $\Rightarrow$  0.50

### **2.3.3 One-way Analysis of Variance (ANOVA)**

The one-way ANOVA test was employed to determine if there was significant difference in the means between two or more groups of data. This parametric test was performed if the assumptions described in section 2.3.1 were met.

If a significant difference between groups was discovered a Tukey's honestly significant difference test was performed to determine which groups were significantly different from each other.

### **2.3.4 Kruskal Wallis Test**

If the assumptions in section 2.3.1 were not met The Kruskal Wallis test was used to determine if the medians of each group were significantly different. The Kruskal Wallis test is the non-parametric equivalent to the one-way ANOVA. Effect size was also calculated as described in section 2.3.2 if a significant difference was observed.

If a significant difference was found, the Bonferroni-Dunn's multiple comparisons test was used to determine which groups were significantly different.

### **2.3.5 Pearson's Correlation**

Correlation analysis was used to determine the strength and direction of a linear relationship between two variables. Pearson's correlation, or parametric correlation, was used if the following assumptions were met (Cohen, 1988):

- 1) The relationship between two or more data sets is linear.
- 2) Observations in the data sets were independent.
- 3) Each data set in the analysis followed normal distribution.
- 4) The data sets showed homoscedasticity.

The strength of the relationship between variables was determined by the guidance set out by Cohen (1988). The correlation coefficient ( $r$ ) obtained between two variables was calculated. The coefficient of determination ( $r^2$ ) was calculated by squaring the correlation coefficient ( $r$ ) obtained between two variables.

### **2.3.6 Spearman's Correlation**

If normal distribution was observed but the other assumptions described in section 2.3.5 were met, then Spearman's correlation was employed to determine the strength and direction of the linear relationship between variables. Results were expressed as Spearman's rho (Cohen, 1988). Bonferroni correction for multiple testing was performed to determine the effect of sample size on the results.

## Chapter 3

### **THE ANTIBACTERIAL ACTIVITY OF HONEY**

### **3.1 Introduction**

#### **3.1.1 The Antibacterial Activity of Honey**

Honey has a long medical history, it has been used as a traditional remedy for the treatment of microbial infections since ancient times (Zumla and Lulat, 1989). Honey based treatments have shown renewed favour due to the rapid evolution of antibiotic resistant bacteria (Molan, 2011). Laboratory studies and clinical trials have shown natural unheated honey to be an effective broad-spectrum antimicrobial agent with activity against numerous aerobes and anaerobes, Gram positive and Gram negative bacteria (Mundo *et al.*, 2004, Molan, 1992, Osho and Bello, 2010, Adetuyi *et al.*, 2009, Cooper *et al.*, 1999, Cooper *et al.*, 2002a, Cooper *et al.*, 2000, Mohammed *et al.*, 2014). Research has also been conducted on Manuka (*L. scoparium*) honey; Visavadia *et al.*, (2008) describes the use of Manuka embedded wound dressings for the treatment and management of chronic wound infections (Visavadia *et al.*, 2008).

The chemical composition of honey is dependent in part on the flowers visited by the bees. Plant pigments, phytochemicals, pollen and nectar all influence the colour and content of the honey (Havenhand, 2010, Dong *et al.*, 2013, Yao *et al.*, 2004). The low water content, high acidity and high sugar content of honey creates an unfavourable environment for microorganisms to survive (Kwakman *et al.*, 2010, Kwakman and Zaat, 2012). These characteristics need to be taken into account when performing assays to determine their influence on the antimicrobial activity of a sample (Kwakman *et al.*, 2010).

Antibacterial activity is further enhanced by the presence of a number of additional factors such as hydrogen peroxide, antioxidants, phenolic acids, flavonoids, methylglyoxal, bee derived antibacterial peptides, proteins and amino acids (chapter 1, section 1.5) (Kwakman *et al.*, 2010, Israili, 2014, Kwakman *et al.*, 2011a). Hydrogen peroxide is thought to be the main antimicrobial factor in the majority of honey samples (Brudzynski and Lannigan, 2012). The contribution of antimicrobial phytochemicals to the overall activity of honey remains unclear as these compounds are yet to be fully characterised (Kwakman *et al.*, 2010, Molan, 2002, Kwakman and Zaat, 2012). The presence and concentration of these compounds varies significantly between samples and further studies are required to isolate and identify these chemicals.

#### **3.1.2 Methods for Determining the Activity of Honey**

In one form or another two standard techniques, agar diffusion and broth/agar microdilution assays, have been used extensively to determine the antibacterial activity of honey (Allen *et al.*, 1991, Boorn *et al.*, 2010, Cooper and Wheat, 2008). The agar diffusion assay involves applying honey in a well in the centre of a nutrient plate inoculated with a microbial culture. Honey diffuses

into the agar and any inhibitory activity is represented by a clear zone of inhibition. The size of the zone is proportional to the concentration of antibacterial compounds present in the honey. A series of different concentrations is commonly assessed via a broth dilution assay in which honey is resuspended with an inoculated bacterial culture and growth is assessed. Bactericidal concentrations can be identified by sub-culturing the samples onto fresh medium. Disc diffusion, overlay assays and time-kill methodologies have also been explored to assess the overall activity of unprocessed honey samples (Boorn *et al.*, 2010, Valgas *et al.*, 2007, Taormina *et al.*, 2001).

With agar diffusion assays a phenol standard is also often included as a reference by which to compare the relative antimicrobial activity of different honey samples (Allen *et al.*, 1991). This standard is directly comparable to the Unique Manuka Factor (UMF<sup>®</sup>) which is used to quantify the antibacterial activity of New Zealand Manuka honey (Stephens *et al.*, 2010). The UMF<sup>®</sup> represents the concentration of a phenol solution which yields a defined zone of growth inhibition when tested in an agar diffusion assay against *S. aureus* (Allen *et al.*, 1991).

The antibacterial activity of honey is commonly assessed by determining the extent to which the growth of an indicator bacterium such as *S. aureus*, is inhibited (Irish *et al.*, 2011). The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) can be established using dilution techniques. The MIC is the lowest concentration of the tested sample with the ability to inhibit growth and the MBC is the lowest concentration to kill the bacteria. (Estevinho *et al.*, 2008). For example, Osho *et al.*, (2010) tested two multifloral honey samples from Nigeria against hospital pathogens and concluded that the MIC for *E. coli*, *S. aureus* and *B. subtilis* was 25% w/v (Osho and Bello, 2010). These were more sensitive than *P. aeruginosa* and *K. pneumoniae* which were assigned an MIC of 50% w/v (Osho and Bello, 2010). The inhibitory effect is evidently affected by the test pathogen, experimental conditions, the floral source and the concentration of honey used (Taormina *et al.*, 2001, Osho and Bello, 2010).

The properties of honey from a particular hive will be affected by a range of factors which include the diversity of the plants on which the bees have fed, bee health, environmental conditions or contamination and the production of antimicrobial compounds by bacteria present in honey (Lee *et al.*, 2008). Agar diffusion and microdilution methods represent a simple, inexpensive way of screening large numbers of honey samples for antibacterial activity against a range of bacterial species (Irish *et al.*, 2011). These techniques can be used to identify honey samples which possess high levels of antibacterial activity (Carina *et al.*, 2014). The peroxide effect is well described and is the main antimicrobial factor in many of these studies (Carina *et al.*, 2014, Cooper and Wheat, 2008).

The antibacterial screening and analysis of phytochemicals of honey and essential plants has been of great interest in the discovery of novel drugs for the treatments of hospital associated infections (Nwankwo *et al.*, 2014). Successive neutralisation of known antibacterial compounds is an approach which has been successfully used to identify novel antibacterial elements in honey (Kwakman *et al.*, 2011b, Kwakman *et al.*, 2010). Using such an approach Kwakman *et al.*, (2010) were able to identify the presence of bee Defensin-1 by neutralising the activity of hydrogen peroxide and methylglyoxal (MGO) (Kwakman *et al.*, 2010). By adopting this successive neutralisation approach it should be possible to detect the presence of novel plant derived phytochemicals with antibacterial activity.

### **3.2 Chapter Aims and Objectives**

The aim of this chapter is to eliminate all known antimicrobials contained in honey and identify samples which contain antibacterial activity due to other compounds which may well be novel antimicrobial phytochemicals.

- Collect a representative number of honey sample from across Wales and the UK which capture the floral diversity of the country
- Characterise the water content and pH of all honey samples
- Develop laboratory based methods capable of determining the antibacterial activity of honey
- Optimise the sensitivity of honey antimicrobial screening assays described previously in literature
- Develop methods to allow the identification of novel antimicrobial factors

### **3.3 Materials and Methods**

#### **3.3.1 Honey Samples**

In total 220 honeys were collected and analysed, details of the source of each sample are shown in chapter 2, table 2.1.

#### **3.3.2 Physiochemical Honey Properties**

The pH and water content of the 220 honeys was assessed, as these factors contribute to the antibacterial activity of honey.

##### **3.3.2.1 pH Analysis**

All honeys are acidic with a pH-value ranging from 3.5 to 5.5, due to the presence of organic acids (Bogdanov *et al.*, 1999). The pH of each of the honey samples included in this study was determined using a Hanna 2210 pH meter by dissolving 10 g of honey in 75 mL of distilled water at room temperature (Bogdanov, 1997). The pH of each honey was determined in triplicate and the mean value determined.

##### **3.3.2.2 Water Content**

The moisture in each honey sample was determined using the refractometric method recommended by the International Honey Commission. Water content was calculated using an Abbè refractometer which was calibrated with distilled water which has a refractive index (nD) at 20 °C of 1.3330. To assess the water content 1 mL of water/honey was placed on the visualisation platform and a reading was taken.

The water content was determined from the refractive index value of each honey sample using the standard conversion table (Appendix B). Refractive index increases with solid content, the conversion table was constructed from a plot of the logarithm of the refractive index minus unity plotted against the water content as determined by vacuum drying (Bogdanov *et al.*, 1999). The water content was determined three times, and mean calculated.

#### **3.3.3 Antimicrobial Assay Optimisation**

##### **3.3.3.1 Bacterial Selection**

MRSA (NCTC 11939) described in chapter 2 (section 2.2.4) was initially used as a screening tool for all honey samples obtained as one of the aims of this research was to detect novel chemicals for the treatment of hospital acquired infections. The purity of overnight cultures was assessed

using the steak plate method and Gram stain microscopy analysis (chapter 2, section 2.3.5 and 2.3.6).

*B. subtilis* (ATCC 6633) and *B. subtilis* (ATCC 39090) described in chapter 2 (section 2.2.4) were used when activity was detected following the neutralisation of hydrogen peroxide. *B. subtilis* (ATCC 6633) is believed to be susceptible to non-peroxide activity but shows resistance to low levels of peroxide (Kwakman *et al.*, 2010). The previously described maximum level of hydrogen peroxide detected in honey is 1–2 mM/L, a level too low to have an effect on *B. subtilis* (Bang *et al.*, 2003). *B. subtilis* (ATCC 39090) the non-sporogenic mutant was tested against honeys which showed activity once the hydrogen peroxide had been removed to detect any antibacterial activity which was masked by spore formation. *B. subtilis* is also sensitive to Defensin-1 and was originally used for its detection in honey (Kwakman *et al.*, 2010).

### 3.3.3.2 Agar Diffusion Optimisation Assay

To identify the optimal media with which to determine antibacterial activity of honeys the following commercially available agar media were examined (all purchased from Oxoid, Basingstoke, UK); Iso-sensitest agar (ISO), Nutrient agar (NA), Tryptone soya agar (TSA), Lysogeny agar (LB) and Mueller-Hinton agar (MH). Each media was made as per the manufacturers' instructions.

The agar diffusion assay was carried out as described below (section 3.3.3.3) using four different honey samples (Table 3.1), the zones of inhibition were measured and recorded. These samples also have a wide range of water content and pH readings and were collected from different locations across the UK (Table 3.1). The antibacterial activity is predicted based on findings in literature which describe the activity of local honeys and Manuka (Kwakman *et al.*, 2011b).

Table 3. 1: Honey samples used in the agar diffusion optimisation studies

Honey ID	Honey Origin	Water content (g/ 100g)	pH reading	Predicted antibacterial activity
H12	SA44 5EE - Llandysyl	17.6	3.75	Peroxide based
H15	CF32 9QB – Bridgend	23.0	3.92	Peroxide based
H51	TR19 6HX - Cornwall	22.0	4.54	Peroxide based
H53	Holland and Barrett 10+ Manuka honey	17.2	3.76	MGO based

### 3.3.3.3 Agar Diffusion Assay

An agar diffusion assay was employed to screen for antibacterial activity and to determine the effect of the successive neutralisation of antimicrobial compounds. The agar diffusion method was adapted from a punch plate assay previously used to investigate the antimicrobial activity of honey (Boorn *et al.*, 2010, Allen *et al.*, 1991). A bacterial suspension, at OD<sub>600</sub> of 0.05 was prepared from logarithmic phase cultures of the test bacteria in LB broth and was diluted to obtain a concentration of 1x10<sup>6</sup> CFU/mL for testing. A sterile cotton wool swab was used to inoculate each agar plate which was then left to dry at room temperature for 15 mins. To provide individual test wells each inoculated 20 mL agar plate was punched 3 times using a cork borer, which had been previously sterilised using 95% ethanol, to produce a 6mm diameter circular well. Subsequently 50 µL of neat honey was added to the wells and the plates were incubated overnight at 37°C. After incubation the diameter of the zones of inhibition were measured to the nearest millimetre using a calibrated calliper (Fisher) and the final value was calculated by subtracting the diameter of the well (Manyi-Loh *et al.*, 2010). Three wells were punctured on each plate, and each plate was repeated in triplicate. Averages of the independent mean values were documented.

### 3.3.3.4 Broth Microdilution Optimisation Assay

To identify the ideal concentration with which to determine antibacterial activity of the honey samples a range of honey concentrations (50%-0%) were made using sterile water and tested against MRSA (NCTC 11939) in a 96-well titre plate (Fisher). To determine MIC and MBC values the method described below (section 3.3.3.5) were used. Honeys in table 3.2 were examined. This experiment was undertaken as the potency of hydrogen peroxide containing honeys is known to increase as the honey is diluted due to the activation of glucose oxidase, the enzyme which catalyses the production of hydrogen peroxide (Brudzynski *et al.*, 2011).

Table 3. 2: Honey samples used in the broth dilution optimisation studies

*Their origin, water content, pH reading and predicted mechanism of antibacterial activity.*

Honey ID	Honey Origin	Water content (g/ 100g)	pH reading	Antibacterial activity
H3	SA33 5NE - Bancyfelin	18.2	3.67	Peroxide based
H6	SA43 2PQ - Ceredigion	17.0	4.33	Peroxide based
H53	Holland and Barrett 10+ Manuka honey	17.2	3.76	MGO based

A Manuka honey sample H53 was once more included as an example of a honey with non-peroxide based antibacterial activity. Samples H3 and H5 were used as indicators of peroxide activity (Table 3.2). The honeys were tested for activity against MRSA. Each experiment was tested in triplicate to account for any variation or experimental error.

### **3.3.3.5 Broth Microdilution Assay**

All 220 honey samples were diluted to 25% and 50% in sterile deionised water. An overnight culture of bacteria was resuspended to OD<sub>600</sub> 0.05 in LB, the culture was diluted to obtain a concentration of 1x10<sup>6</sup> CFU/mL suspension in sterile water. Then 100 µL of this bacterial suspension was mixed with 100 µL of honey. This resulted in final inoculum concentrations of 5 x 10<sup>5</sup> CFU/mL in each well, the concentration recommended for susceptibility testing (CLSI, 2012). Inoculated plates were incubated at 37 °C on a thermo scientific shaking incubator at 3 g for 24 h (Max<sup>Q</sup>Mini4450).

After 24 h wells were checked for growth by comparing the OD<sub>600</sub> of each well to the negative and positive control wells and an MIC was calculated. Negative control wells contained 50 µL of sterile LB broth while positive control wells contained 50 µL of LB broth inoculated with bacteria. The MBC was determined by sub-culturing 10 µL aliquots from each individual well in triplicate onto MH agar. After overnight incubation at 37 °C bacterial growth was examined in order to assess cell viability. All experiments were performed in triplicate and sugar and phenol controls were also included in each assay.

### **3.3.4 Standardisation of Positive Controls**

#### **3.3.4.1 Phenol Control**

The antibacterial activity of Manuka honey is determined with reference to a standard curve constructed using different concentrations of phenol. Using this system the activity of a honey is represented by UMF® Manuka number which is equivalent to the corresponding phenol concentration (% w/v) (Allen *et al.*, 1991). To provide a standard against which to compare the activity of different honey samples a similar approach was adopted. Solutions of 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10% w/v phenol (Sigma) were prepared in distilled water as standards (Allen *et al.*, 1991), and tested against MRSA and *B. subtilis* using the agar diffusion assay on LB agar. In brief, 50 µL of each phenol solution was placed into the wells and tested against MRSA and the two *B. subtilis* strains as previously described (section 3.3.3.3).

These concentrations were also tested against MRSA in the broth microdilution assay. For the broth assay an overnight culture of bacteria (OD<sub>600</sub> 0.5) in LB was serially diluted to obtain a 1x10<sup>6</sup>

CFU/mL suspension. A total of 100  $\mu$ L bacterial inoculum was mixed with 100  $\mu$ L of each phenol solution. After overnight incubation, 10  $\mu$ L samples were sub cultured onto MH agar and growth was recorded. From the agar diffusion assay a phenol equivalence (% w/v) of honey was determined from a standard curve created from the range of concentrations tested. Each standard was tested in triplicate. Once made up the phenol solutions were stored at 4 °C in the dark for a maximum of 1 month to prevent the degradation.

#### **3.3.4.2 Thymol Control**

Thymol is a plant derived antibacterial component which has been identified in thyme honey and was included in the agar diffusion assay as a second positive control. Thymol is an example of a plant-derived antibacterial compound which has been isolated from honey. By using a thymol control any experimental effects on a honey-derived antibacterial compound can also be assessed. Thymol is also a natural pesticide used to protect bees from the Varroa mite and bacterial infections (Piasenzotto *et al.*, 2002). Synthetic thymol (Sigma) was tested at the following concentrations; 0.01, 0.1, 1 and 10% (w/v) in distilled water using the agar diffusion assay (section 3.3.3.3), zones of clearing were recorded and a standard curve was created.

#### **3.3.4.3 Sugar Control**

A solution with a sugar composition that is representative of the honey was prepared. This was achieved by dissolving 1.5 g sucrose, 7.5 g maltose, 40.5 g fructose and 33.5 g glucose (all Sigma) in 17 mL sterile de-ionized water (Cooper *et al.*, 2002b). This allowed the osmotic/sugar effect to be assessed and a negative control to be implemented.

#### **3.3.5 Characterisation of Antibacterial Activity in Honey**

Once the agar diffusion and broth microdilution assays had been optimised and positive and negative controls had been selected the aim was to determine the source of antibacterial activity. The antibacterial activity of honey is due to a number of different factors working individually or synergistically (Kwakman *et al.*, 2010). To determine the cause of the antibacterial activity of each honey sample the following methods were implemented. Compounds were neutralised sequentially to determine the basis of their antibacterial effects. The complete experimental plan can be better understood from flow diagram (Figure 3.1).

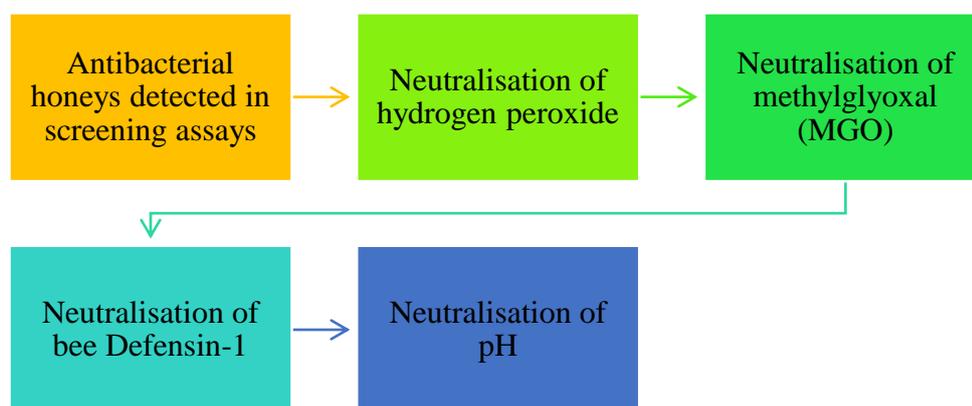


Figure 3. 1: Methodologies for the neutralisation of known antibacterial compounds in honey

### 3.3.5.2 Neutralisation of Hydrogen Peroxide

For each honey sample the agar diffusion assay was repeated with the addition of a minimum of 0.25% (w/v) catalase (Sigma) (Allen *et al.*, 1991). 0.02 g of catalase was added to each 5 mL of honey. The treated honey was incubated in the dark at room temperature for a minimum of 2 h before it was used for subsequent experiments. The antibacterial activity of the treated honey was assessed against MRSA and both strains of *B. subtilis*, and the results were converted to a phenol equivalent value (% w/v) (Allen *et al.*, 1991). To further investigate the peroxide mechanism of honey action the activity of a range of hydrogen peroxide concentrations in water and sugar suspensions was assessed against MRSA and *B. subtilis*.

#### Hydrogen Peroxide Assay

The antibacterial activity of a range of hydrogen peroxide concentrations against MRSA and *B. subtilis* were determined to assess the relative resistance of each stains and to ascertain if there was a concentration-dependent relationship between hydrogen peroxide and each bacterium (Brudzynski *et al.*, 2011). It was determined if the antibacterial activity of hydrogen peroxide differed when the compound was suspended in artificial honey (section 3.3.4.3) or distilled water. Also, as a control it was determined whether 0.25% (w/v) catalase would neutralise 1029 mM/L hydrogen peroxide.

A 1029 mM/L (35%) hydrogen peroxide solution (Fisher) was serially diluted (1/10) in a 96-well micro plate; 20  $\mu$ L hydrogen peroxide to 180  $\mu$ L distilled water for each dilution. The bactericidal effect of each dilution was assessed using the agar diffusion assay as previously described. Briefly 50  $\mu$ L of each dilution was tested against MRSA and *B. subtilis* in triplicate on agar and zones of clearing were recorded. This procedure was repeated and the water was replaced by artificial sugar as the diluent to determine whether sugar had any affect the bactericidal activity of hydrogen peroxide.

### **3.3.5.3 Neutralisation of Methylglyoxal (MGO)**

L- Glutathione reduced solution (0.023 g) (Sigma) was added to 5 mL honey to achieve a final concentration of 15 mM. The subsequent conversion of methylglyoxal (MGO) to S-D-lactoyl-glutathione was initiated by addition of 0.5 U/mL (7.85 µL) glyoxalase I (Sigma) (Kwakman *et al.*, 2010). The agar diffusion assay (section 3.3.3.3) was employed to determine if neutralisation had been achieved, and any antibacterial activity remained.

### **3.3.5.4 Neutralisation of Bee Defensin-1**

The activity of Defensin-1 was inactivated by adding sodium polyanetholsulfonate (SPS) (Sigma), at a final concentration of 0.025% (w/v) (Kwakman *et al.*, 2010). SPS neutralize cationic bactericidal components, this concentration was performed following a recommended protocol (Kwakman *et al.*, 2010). In brief, 0.25 mg of SPS was added to every 1 mL of honey tested to remove peptides. Antimicrobial activity was tested using the agar diffusion assay (section 3.3.3.3).

### **3.3.5.5 Neutralisation of Free Acidity**

To neutralise the antibacterial effect of low pH 0.01M NaOH was used to adjust the pH of honey solution to pH 8.3 as recommended by the International Honey Commission (Bogdanov *et al.*, 1999). A pH meter (Hanna) was calibrated using standardised solutions at pH 3.0, 7.0 and 9.0. In total 10g of honey was dissolved in 75 mL sterile distilled water in a 250 mL beaker and placed on a magnetic stirrer (Bogdanov *et al.*, 1999). Drop by drop 0.1M NaOH was added until a reading of 8.3 was recorded from the immersed pH probes. Activity was subsequently assessed using the agar diffusion assay (section 3.3.3.3).

## **3.4 Results**

### **3.4.1 Characterisation of Honey**

To gauge the physiochemical properties of the honey samples screened the pH and water content was determined for all 220 honey samples collected (chapter 2, table 2.1).

#### **3.4.1.1 pH Analysis**

The pH of each honey was determined using a pH meter. The mean values ranged from 3.29 to 5.10 (Appendix C).

#### **3.4.1.2 Water Content**

The water content of each honey was determined by measuring its refractive index using a refractometer. Each honey sample was analysed on two separate occasions. The mean values of

the 220 honeys tested ranged from 1.4985 to 1.4790 (Appendix C). Using a conversion table (Appendix B) generated by the International Honey Commission (2009) the minimum and maximum water content was determined as 15.20g/100g and 23.00g/100g respectively.

### 3.4.2 Development of Controls for Honey Screening Assay

#### 3.4.2.1 Standardisation of Phenol Positive Control

Standard curves were constructed using varying concentrations of phenol in both agar diffusion and broth microdilution assays. The diameter of the zone of clearing was measured to the nearest millimetre and 6 mm was deduced to account for the diameter of the well. Each honey was tested a minimum of three times and means were calculated figures 3.2 illustrates the relationship between phenol concentration and the size of the zone of inhibition using an LB agar diffusion assay for three different bacterial isolates. For each bacterial isolate a strong correlation was observed between phenol concentration and antibacterial activity meaning that a phenol-equivalent value could be determined for a particular level of antibacterial activity. For this reason a phenol concentration of 5% was included in all agar diffusion assays to serve as a reference positive control.

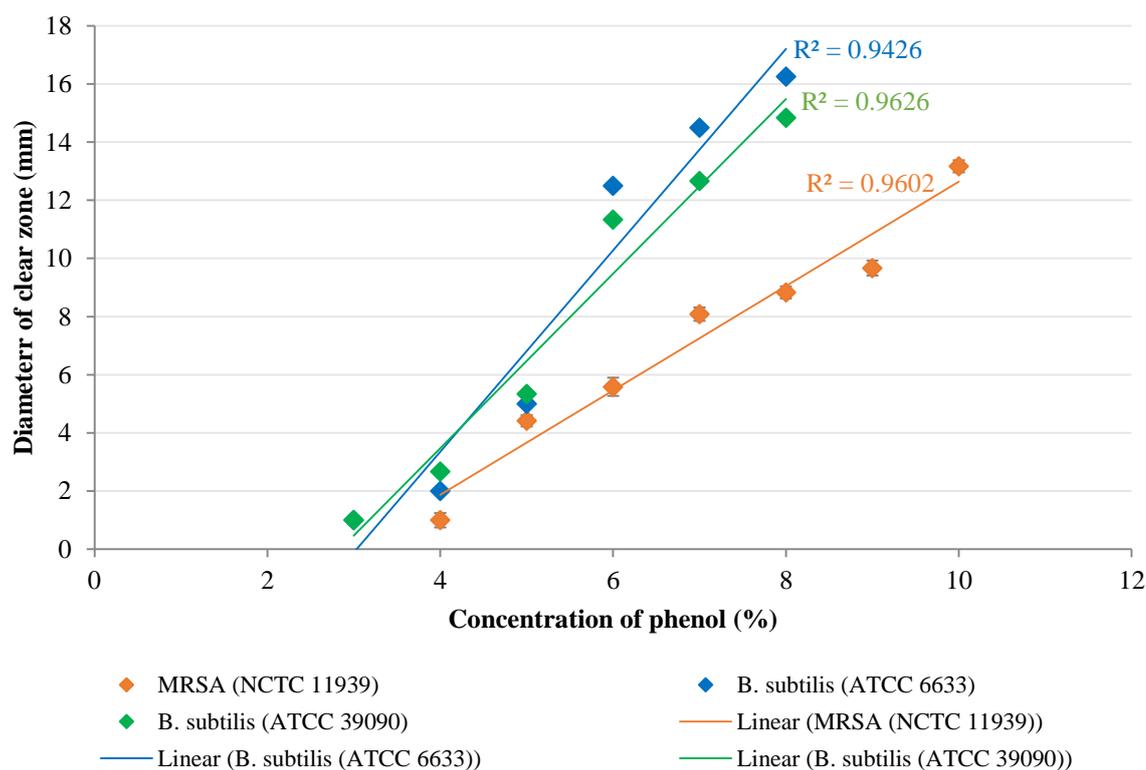


Figure 3. 2: Phenol MRSA (NCTC 11939), B. subtilis (ATCC 6633) and B. subtilis (ATCC 39090) standard curve

Curve representing the relationship between phenol concentration and antibacterial activity. The assay was performed using an agar well diffusion assay on LB agar. (Error bars = SE; n = 3).

To determine the effect of phenol concentration on antibacterial activity in broth a microdilution based assay was employed. Following overnight incubation in the presence of difference concentrations of phenol, 10  $\mu$ L aliquots of the test bacteria, 3 aliquots per phenol dilution were sub cultured onto LB agar which was incubated overnight and examined for the presence of growth. An MBC was recorded at a phenol concentration of 5% and therefore 5% was employed as a positive control in all subsequent broth based assays.

### 3.4.2.2 Standardisation of Thymol Control

A standard curve of the antibacterial activity of different concentrations of thymol for different species of bacteria was constructed using the same agar based diffusion assay which was employed for phenol. Based on the guidelines suggested by Cohen (1988), a strong correlation ( $r^2 = 0.97$ ) between thymol concentration and antibacterial activity against MRSA was detected (Figure 3.3) (Cohen, 1988). Based on this data thymol at a concentration of 0.1 % (w/v) was employed as a positive control.

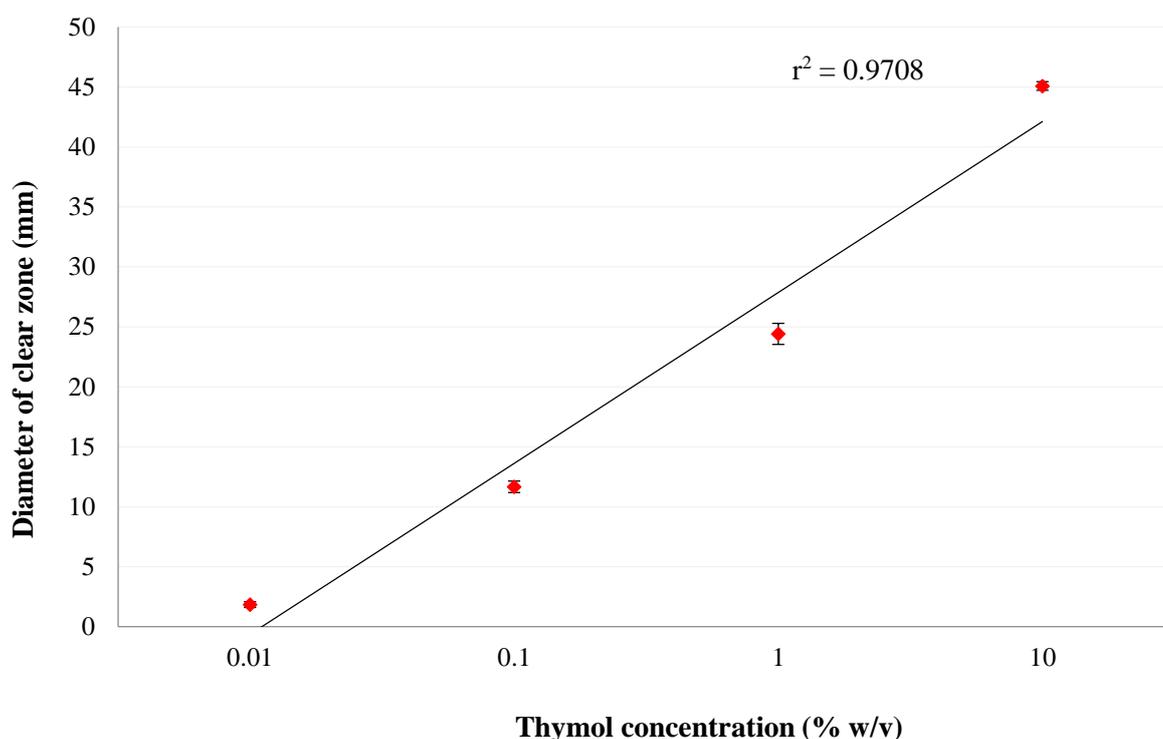


Figure 3. 3: Thymol MRSA (NCTC 11939) standard curve

Curve representing the relationship between thymol concentration and antibacterial activity against MRSA 11939. The assay was performed using an agar well diffusion assay on LB agar. (Error bars = SE;  $n = 3$ ).

Both strains of *B. subtilis* were screened using the same approach but there were no detectable zones of clearing suggesting that *B. subtilis* is resistant to the levels of thymol used in this study. Thus thymol was only used as a positive control in agar diffusion assays against MRSA.

If the diameter of the zone generated by phenol or thymol varied significantly from that of the expected value during the agar diffusion assay the experiment was repeated. Values were calculated based on the equation of the line (Figures 3.2). In the case of phenol this was 10 mm (4 mm across once the 6mm zone of clearing is deducted) and for thymol this was 17 mm zone (11 mm across once the 6 mm zone of clearing is deducted) against MRSA. Against *B. subtilis* phenol was 13 mm (7 mm across once the 6 mm zone of clearing is deducted). This was to ensure the results were not affected by environmental or experimental fluctuations.

### 3.4.2.3 Standardisation of Artificial Sugar Control

To account for the antibacterial osmotic effect of honey, a sugar syrup (1.5 g sucrose, 7.5 g maltose, 40.5 g fructose and 33.5 g glucose) was included as a negative control in the agar diffusion assay (Cooper *et al.*, 1999). Each assay was repeated in triplicate and an image was captured (Figure 3.4). While the sugar failed to generate a clear zone of inhibition for MRSA it did cause a double zoning effect in which the density of bacterial growth differed between the zones. It was hypothesised that this phenomenon is the result of sugar induced osmotic stress. A different phenotype was observed for *B. subtilis*; both isolates showed increased growth surrounding the sugar loaded well (Figure 3.4). Further investigations beyond the scope of this study are required to understand the cause of this phenomenon.

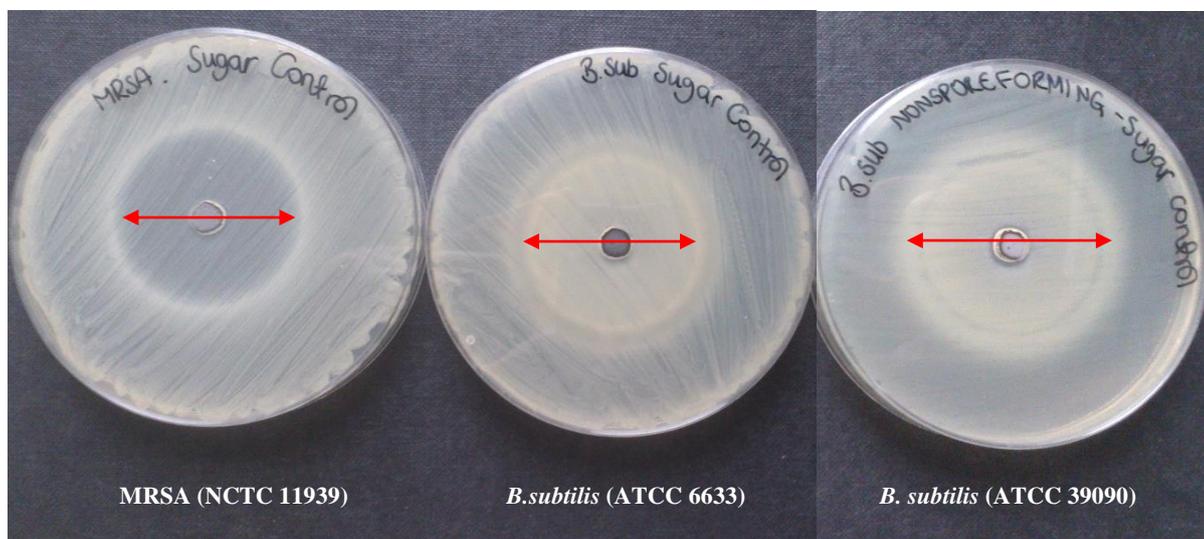


Figure 3. 4: Effect of 100% sugar solution on the growth of MRSA and *B. subtilis*

Assay was performed on LB agar. For the MRSA, *B. subtilis* 6633 and *B. subtilis* 39090 a double zoning effect can be clearly seen. For *B. subtilis* an increase in growth is visible. RED arrows indicate the area effected by the sugar diffusion.

### 3.4.3 Development of an Antimicrobial Screening Assay for Honey

#### 3.4.3.1 Optimisation of the Agar Diffusion Assay

An agar diffusion assay adapted from Boorn *et al.*, (2010) was used to identify the honey samples which possessed antibacterial activity (Boorn *et al.*, 2010). Media composition is known to influence the activity of antibacterial compounds against bacteria and for this reason the impact of the following commercially available agar formulations (ISO, NA, TSA, LB and MH) on the sensitivity of test bacteria to honey was determined.

#### 3.4.3.2 Media Selection for Agar Diffusion Assay - MRSA

For media optimisation honey samples which had hydrogen peroxide and MGO based antibacterial activity were used (Table 3.1). The honeys were tested for activity against MRSA (NCTC 11939) using the media described above. Each medium was tested in triplicate to account for any variation in the sizes of the zones of clearing. Phenol and thymol were run as positive controls. While all of the honey samples demonstrated some level of antibacterial activity, the biggest zones of inhibition were seen with H12, H15 and H51 on LB agar (Figure 3.5).

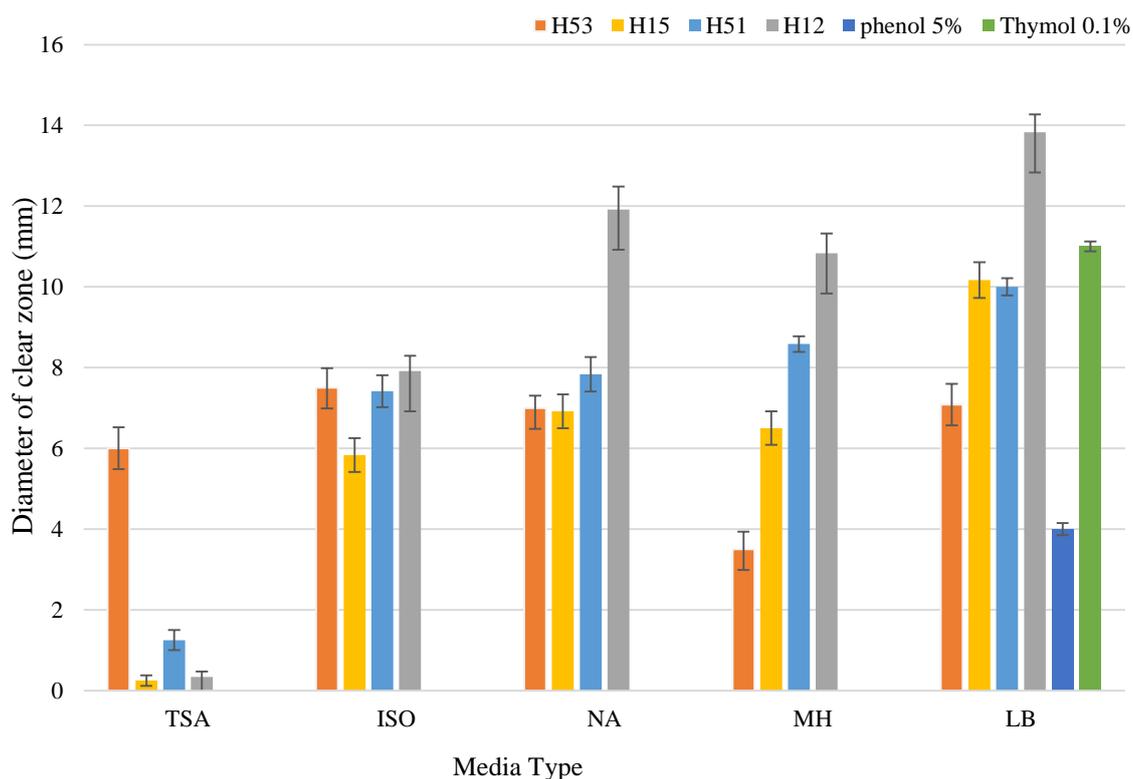


Figure 3. 5: The effect of media composition on the antibacterial activity of honey samples against MRSA (NCTC 11939)

Determined using an agar diffusion assay. H53 represents the Manuka sample (Error bars = SE; n = 3).

The Welsh honey samples showed higher levels of antimicrobial activity than Manuka honey (H53). To determine if the differences seen in the zones of inhibition on the different agar media were statistically significant differences a non-parametric Kruskal Wallis test was employed and there was a significant difference between all the honeys on the different media tested ( $n = 60$ ;  $p < 0.001$ ;  $r = 0.47$ ). A Bonferroni-Dunn's test highlighted the differences across the honey samples on different media.

In summary based on figure 3.5;

The zones of inhibition for H12 on LB were significantly bigger ( $p < 0.001$ ) than on TSA and ISO  
The zones of inhibition of H15 on LB were significantly bigger ( $p < 0.05$ ) than on MH, NA, TSA and ISO

The zones of inhibition of H51 on LB were significantly bigger ( $p < 0.05$ ) than on NA, TSA and ISO

The zones of inhibition of H53 on LB were significantly bigger ( $p < 0.001$ ) than on MH

The results obtained using TSA agar suggest that this media is inhibitory to the antibacterial activity of three of the four honey samples, H12, H15 and H51. These samples have high levels of peroxide activity (detected in 3.4.5). The Manuka honey H53 differed in that its antibacterial activity was not significantly affected by TSA when compared to the other media. This confirms that Manuka has a different mechanism of action compared to the other honey samples. With the Manuka honey there was only a significant difference ( $p < 0.001$ ) in antibacterial activity on LB and MH agars. It therefore appears that TSA contains compounds which inhibit the activity of H12, H15 and H51 and that MH contain compounds, which inhibit the activity of Manuka honey against MRSA. On the basis of these results it was decided to use LB agar in all subsequent agar diffusion assays for MRSA.

#### **3.4.3.3 Media Selection for Agar Diffusion Assay – *B. subtilis***

It was next determined if media composition had a similar impact on the sensitivity of spore forming ATCC 6633 and non-sporing ATCC 39090 strains of *B. subtilis*, to the four honey samples. It can be seen that only Manuka honey (H53) demonstrated measurable antibacterial activity against the spore-forming strain of *B. subtilis* (Figure 3.6). A Kruskal Wallis test revealed a significant ( $X^2 = 20.545$ ;  $n = 30$ ;  $z = -2.074$ ;  $p < 0.001$ ;  $r = 0.38$ ) difference when comparing the different media. The Bonferroni-Dunn's test showed there was a significant difference in zone diameter between LB and TSA ( $p < 0.001$ ) and between NA and TSA ( $p < 0.05$ ). These results differ from those observed with MRSA were it was MH which inhibited the activity of Manuka honey against MRSA, However the activity was higher on LB agar.

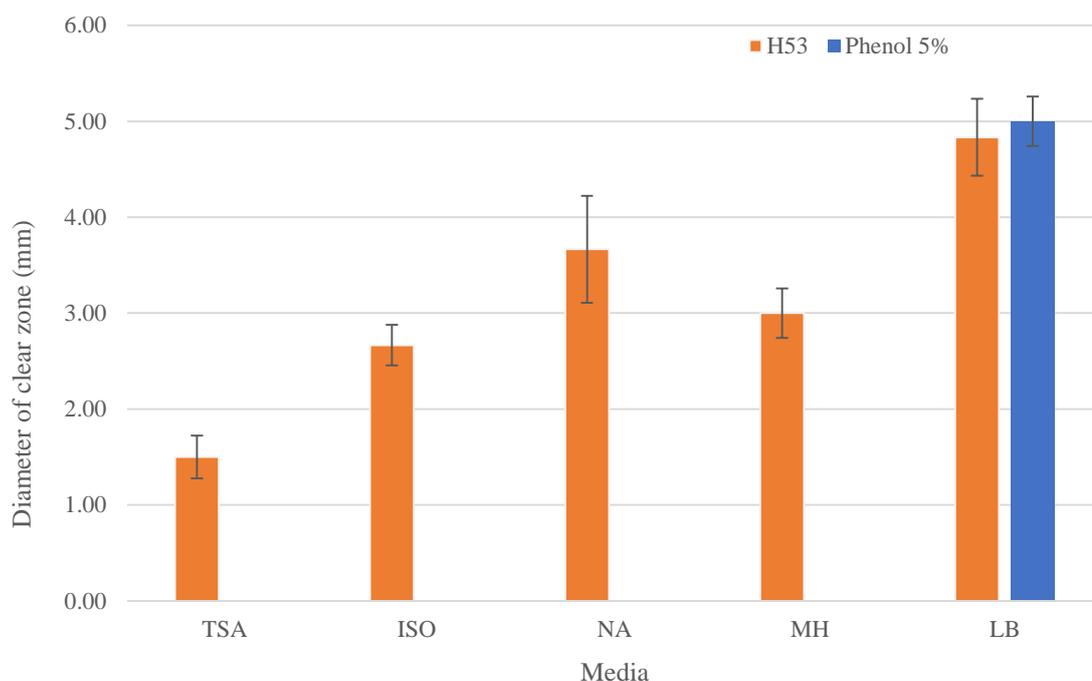


Figure 3. 6: The effect of agar composition on the antibacterial activity of H53 (Manuka) against *B. subtilis* (ATCC 6633).

Activity was determined using an agar diffusion assay and the following media; MH, TSA, ISO, NA and LB. (Error bars = SE;  $n = 3$ ).

To determine the sensitivity of a non-spore forming variant of *B. subtilis* (ATCC 39090) when cultured under the same test conditions. This strain appears to be more sensitivity to honey than its spore forming counterpart (Figure 3.7). A Mann-Whitney U test was performed to compare the activity of Manuka (H53) against the *B. subtilis* isolates 6633 ( $Md = 9$  mm,  $n = 36$ ) and 39090 ( $Md = 13.5$  mm,  $n = 36$ ). Manuka is significantly ( $U = 285$ ;  $z = -4.125$ ;  $p = 0.001$ ;  $r = 0.68$ ) more active against the non-spore forming bacteria compared to the spore former, on all five media tested.

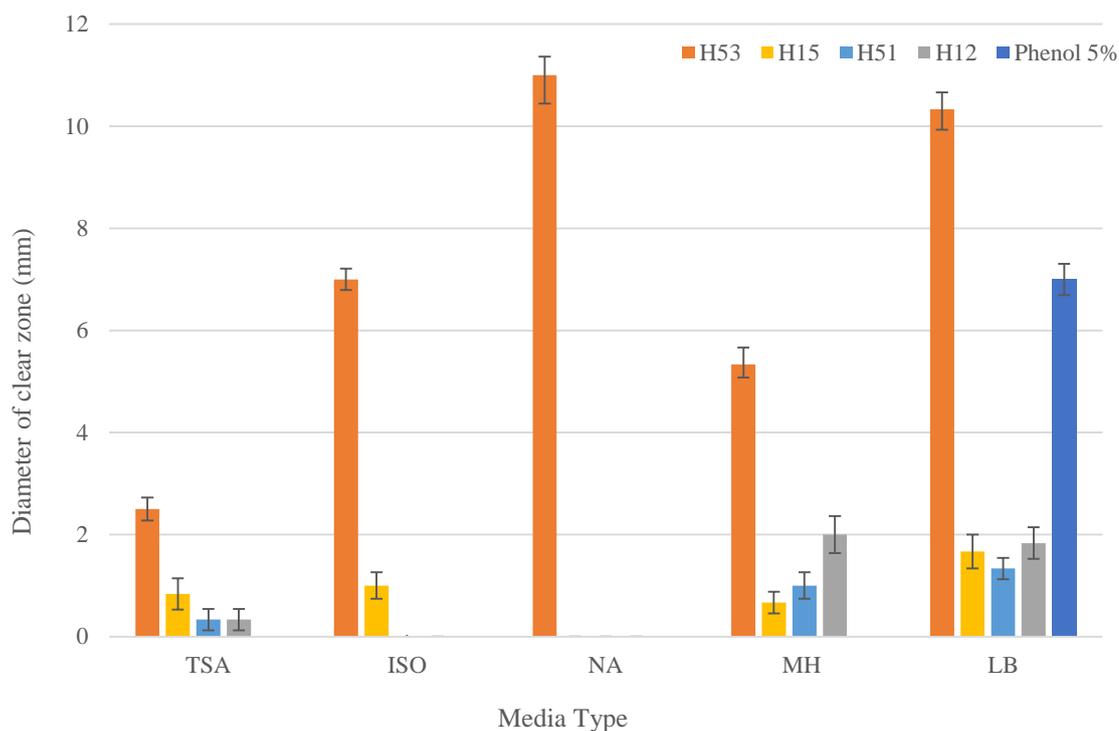


Figure 3. 7: The effect of agar composition on the antibacterial of honey against *B. subtilis* (ATCC 39090).

The activity of honey samples H12, H15, H51 and H53 was determined using an agar diffusion assay and the following media; MH, TSA, ISO, NA and LB. (Error bars = SE; n = 3).

The result of these experiments suggests that LB agar is a more appropriate agar with which to detect the antibacterial activity of honey and for this reason it was used in all subsequent studies presented in this thesis.

#### 3.4.3.4 Optimisation of Broth Dilution Assay

Assay optimisation was performed on the broth microplate dilution assay. LB broth was used based on the findings of the agar diffusion assay optimisation process and to keep methods consistent. A broth dilution assay was performed as described previously (section 3.3.3.4) with a range of honey concentrations (50%-0) in LB broth to determine the range of honey concentrations to use in the screening assay.

Based on literature the optimal dilution of honey for hydrogen peroxide activation is 30% (Kwakman *et al.*, 2011a). H3 was most effective at the lowest dilution followed by H53 (Manuka) and then H6. The MBC for the two peroxide samples H3 and H6 was 10% v/v and 30% v/v respectively (Table 3.3). The MBC of 20% v/v produced by the Manuka sample is believed to be due to the presence of MGO.

*Table 3. 3: The MIC and MBC of different concentrations of honey against MRSA.*

*Activity was determined using a broth based method using LB. Each experiment was repeated in triplicate and an average result was determined. Growth (+) and no-growth (-)*

<b>Honey sample</b>	<b>Honey Concentration</b>	<b>Detection of growth</b>	<b>MIC</b>	<b>MBC</b>
H6	50%	-	30%	30% (v/v)
	40%	-		
	30%	-		
	20%	+		
	10%	+		
	0%	+		
H3	50%	-	10%	10% (v/v)
	40%	-		
	30%	-		
	20%	-		
	10%	-		
	0%	+		
H53 (Manuka)	50%	-	20%	20% (v/v)
	40%	-		
	30%	-		
	20%	-		
	10%	+		
	0%	+		
Phenol	5%	-		

As the aim of this research was to detect plant derived compounds and not hydrogen peroxide activity, honey at dilutions of 25% and 50% (v/v) were employed to determine the antibacterial activity of the entire sample collection. Phenol at a concentration of 5% was included as a reference positive control for each series of experiments.

### **3.4.4. Determination of the Antibacterial Activity of Honey Samples**

#### **3.4.4.1 Agar Diffusion Assay**

The antibacterial activity of 220 honey samples was determined using an optimised LB agar diffusion assay (section 3.3.3.3) with MRSA as the indicator strain (Appendix C). The standard errors of each honey tested were calculated to determine the variation between results. To allow comparison between assay results the mean diameter of three replicates was expressed as a phenol % (w/v) equivalent using the phenol standard curve equation ( $y = 1.7946x - 5.3125$ ) for MRSA (section 3.4.2.1). Having been converted to phenol % (w/v) antibacterial activity ranged from 3.5% (no activity) to 13.5% (Appendix C).

Phenol (5%) and thymol (0.1%) were included in each assay run to serve as reference positive controls. A sugar control well was also included in each assay run but on no occasion did it demonstrate any antibacterial activity. The results from the agar diffusion assay were summarised

(Table 3.4). The majority (88%) of samples showed some level of antibacterial activity against MRSA.

*Table 3. 4: A summary of the antibacterial activity of 220 honey samples expressed as a phenol equivalent.*

*Tested on LB agar against MRSA (11939). A 5 % phenol control was used throughout and each honey was tested in triplicate and an average results was determined*

<b>Activity expressed as phenol equivalent (% w/v)</b>	<b>Number of samples with antibacterial activity</b>
>10	61
3.5-10	133
<3.5 (undetectable levels)	26

#### **3.4.4.2 Broth based dilution assay**

The antibacterial activity of 220 honey samples was determined using a broth based dilution assay (section 3.3.3.3) with MRSA as the indicator strain (Appendix C). Activity of a particular honey was determined by assaying the sample in triplicate and the results were summarized (Table 3.5). It was found that 68% of the 220 honey samples tested demonstrated no antibacterial effect on MRSA. 20% of the honey samples had MBC values of <25% showing good levels of antimicrobial activity, 3 of these samples killed at 25% but not 50% which would be due to the production of hydrogen peroxide upon dilution. Hydrogen peroxide is produced by glucose oxidase enzyme upon dilution of honey (Kwakman *et al.*, 2011). In total 32% of the honey samples showed some level of activity at 25% and/or 50%.

*Table 3. 5: The MBC of different concentration of honey against MRSA.*

*Activity was determined using a broth based method using MH. Each experiment was repeated in triplicate and an average result was determined*

<b>MBC (% v/v)</b>	<b>Number of samples with antibacterial activity</b>
<25%	45
Between 25% and 50%	25
>50% (No activity)	150

By combining the results of the two antibacterial assays 26 honey samples were identified which failed to demonstrate any detectable antibacterial activity against MRSA. These samples were

excluded from subsequent experiments. The remaining samples were further analysed to determine the nature of their antibacterial activity.

### 3.4.5 The Antibacterial Activity of Hydrogen Peroxide in Honey

Hydrogen peroxide is a major antibacterial factor in honey (Cooper and Wheat, 2008). To determine the sensitivity of MRSA and *B. subtilis* ATCC 6633 to hydrogen peroxide different concentrations of the compound were added to water and artificial honey (Cooper *et al.*, 2002b) and then tested for their antibacterial activity using the agar diffusion assay (section 3.3.3.3). A concentration-dependent effect was seen for both bacteria (Figure 3.8).

A significant difference was observed when comparing sensitivity of MRSA ( $Md = 49$  mm,  $n = 9$ ) to *B. subtilis* ATCC 6633 ( $Md = 23$ mm,  $n = 9$ ) using a Mann Witney U test in water ( $U < 0.001$ ;  $n = 18$ ;  $z = -3.616$ ;  $p < 0.001$ ;  $r = 0.85$ ), MRSA being the most sensitive. The same was observed in artificial sugar ( $U < 0.001$ ;  $n = 12$ ;  $z = -2.918$ ;  $p < 0.01$ ;  $r = 0.84$ ) when comparing MRSA ( $Md = 41$  mm,  $n = 6$ ) compared to *B. subtilis* ATCC 6633 ( $Md = 18$  mm,  $n = 6$ ). In summary MRSA was significantly more sensitive to hydrogen peroxide than *B. subtilis* in both water and sugar.

There were statistically significant differences in the antibacterial activity of hydrogen peroxide against both test bacteria when the compound was administered in water and artificial honey solution. Against MRSA a Mann Witney U test highlighted a significant difference ( $U < 0.001$ ;  $n = 15$ ;  $z = -3.208$ ;  $p = 0.01$ ;  $r = 0.78$ ). Similarly for *B. subtilis* a significant difference ( $U < 0.001$ ;  $n = 15$ ;  $z = -3.237$ ;  $p = 0.01$ ;  $r = 0.86$ ). These results imply that sugar content of the honey has an adverse effect on the antibacterial potency of hydrogen peroxide.

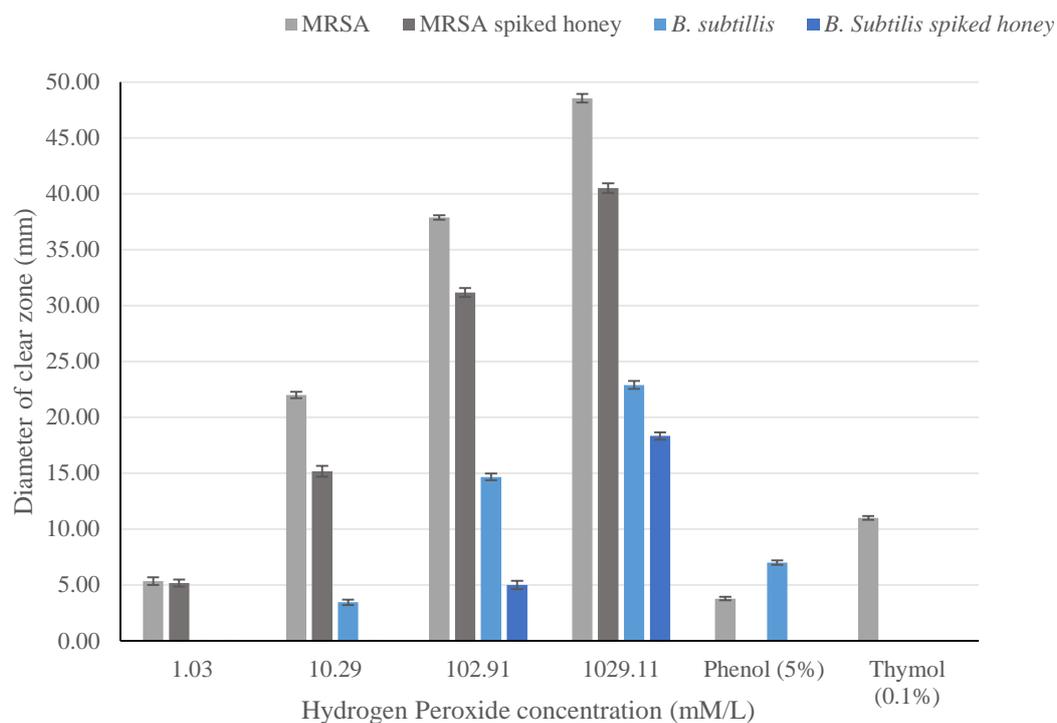


Figure 3. 8: The sensitivity of MRSA and *B. subtilis* (ATCC 6633) to hydrogen peroxide

Tested across a range of concentration when the agent was suspended in water or artificial honey. Phenol (5%) and thymol (0.1%) were tested against MRSA. Error bars show standard error of the zones of clearing for a mean of triplicate assays.

To determine if a honey samples possess antibacterial activity which was not due to the presence of hydrogen peroxide a method capable of neutralising the activity of this compound was implemented. It has been reported that catalase at a concentration of 0.25% (w/v) was able to neutralise the hydrogen peroxide content of honey (Cooper *et al.*, 2002b). To determine if this concentration was sufficient to neutralise the antibacterial activity of the compound in water and artificial honey samples were treated with 0.25% (w/v) of catalase and screened for antibacterial activity against MRSA using our agar diffusion assay. Treatment with catalase neutralised antibacterial activity at all of the concentrations of Hydrogen peroxide tested (Figure 3.9) which included a concentration of 1029 mM/L which is considerably higher than the maximum level (1–2 mM/L) previously detected in honey (Bang *et al.*, 2003).

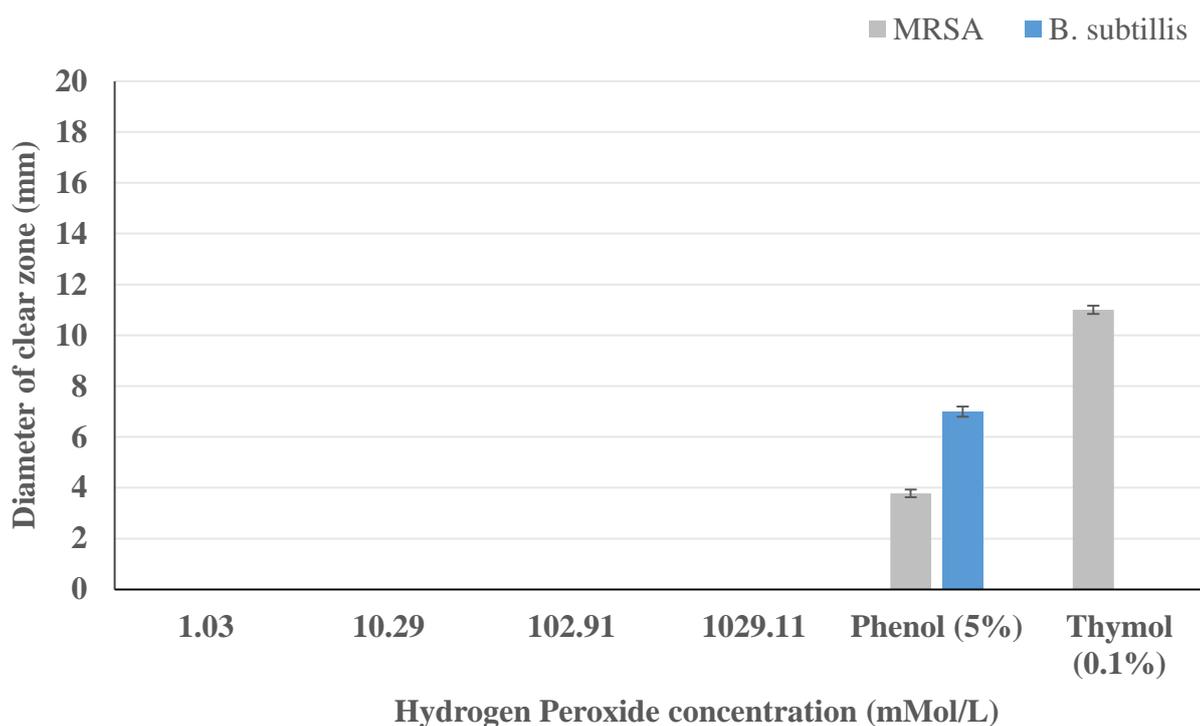


Figure 3. 9: Catalase treated hydrogen peroxide at a range of concentrations on an agar diffusion assay.

The double zoning osmosis effect described in section 3.4.2.3 was the only phenotypic effect that remained for the majority of honey samples following the neutralisation of hydrogen peroxide activity.

### 3.4.6 Neutralisation of Known Antibacterial Factors

#### 3.4.6.1 Neutralisation of Hydrogen Peroxide

The antibacterial activity of honey may be due to a number of factors which include the production of hydrogen peroxide. To determine if the honey samples which had demonstrated antibacterial activity in the screening assays owed their activity to the production of hydrogen peroxide, samples were mixed with 0.25% (w/v) catalase and then screened for antibacterial activity against MRSA and *B. subtilis* using the agar diffusion assay described above.

Of the 194 honey samples tested only 13 retained antibacterial activity against MRSA following treatment with catalase (Table 3.6). The phenol equivalent dropped after the treatment of catalase for all the honeys tested against MRSA, the activity only reduced slightly for the manuka samples (H53, H54 and H52). This activity was expected as Manuka honey is known to possess antibacterial activity which is independent of hydrogen peroxide (Kwakman *et al.*, 2011b). This suggests the honey samples have both peroxide activity and non-peroxide based activity which required further investigation.

Table 3. 6: The antibacterial activity of honey samples following the neutralisation of hydrogen peroxide against MRSA.

The antibacterial activity of thirteen honey samples treated with catalase (before and after) screened against MRSA and spore and non- spore forming isolates of *B. subtilis*. Activity determined using an agar diffusion assay. The level of activity of individual honeys has been converted to a phenol equivalent (% w/v). Samples highlighted in red were Manuka honeys and (-) represents no zone of inhibition. All experiments were run in triplicate and standard error was calculated. Sorted in descending order based on MRSA antibacterial effect after catalase treatment.

Honey Sample	Antibacterial activity phenol equivalent (% w/v) (SE)	
	MRSA (NCTC 11939) Before catalase treatment	MRSA (NCTC 11939) After catalase treatment
H53	7.42 (0.40)	7.05 (0.22)
H54	6.94 (0.19)	6.95 (0.17)
H25	8.95 (0.28)	6.49 (0.40)
H52	7.09 (0.24)	6.16 (0.48)
H24	7.51 (0.32)	5.70 (0.23)
H201	7.33 (0.17)	4.82 (0.21)
H180	5.00 (0.22)	4.49 (0.18)
H150	9.88 (0.23)	3.87 (0.25)
H116	7.19 (0.29)	3.87 (0.50)
H57	8.02 (0.26)	3.70 (0.38)
H169	13.27 (0.57)	3.59 (0.23)
H72	3.61 (0.27)	3.56 (0.23)
H51	9.37 (0.40)	3.52 (0.25)
Sugar Control	-	-
Phenol (5%)	5.05 (0.08)	5.05 (0.13)
Thymol (0.1%)	9.02 (0.12)	9.00 (0.11)

These 13 honeys were also screened against the *B. subtilis* strains to determine the presence of non-peroxide antibacterial activity (Table 3.7). Values were again converted to a phenol equivalent % (w/v) which was calculated based on the phenol standard curve equations  $y = 3.4643x - 10.512$  for *B. subtilis* 6633 and  $y = 3.0048x - 8.554$  for *B. subtilis* 39090 (section 3.4.2.1). The three Manuka samples had an antibacterial effect on both *B. subtilis* strains due to non-peroxide activity.

Table 3. 7: The antibacterial activity of honey samples following the neutralisation of hydrogen peroxide against *B. subtilis*.

The antibacterial activity of thirteen honey samples treated with catalase against spore and non- spore forming isolates of *B. subtilis*. Activity determined using and agar diffusion assay. The level of activity of individual honeys has been converted to a phenol equivalent (% w/v). Samples highlighted in red were Manuka honeys and (-) represents no zone of inhibition. All experiments were run in triplicate and standard error was calculated.

Honey Sample	Antibacterial activity phenol equivalent (% w/v) (SE)	
	<i>B. subtilis</i> (ATCC 6633)	<i>B. subtilis</i> (ATCC 39090)
H53	5.98 (0.23)	8.53 (0.12)
H54	0.00 (0.00)	4.07 (0.17)
H25	6.16 (0.13)	8.02 (0.15)
H52	5.00 (0.14)	7.05 (0.38)
H24	5.93 (0.22)	8.25 (0.19)
H201	4.49 (0.13)	5.00 (0.22)
H180	6.02 (0.19)	7.93 (0.15)
H150	-	5.37 (0.22)
H116	-	-
H57	-	4.17 (0.17)
H169	-	-
H72	-	-
H51	-	-
Sugar Control	-	-
Phenol (5%)	5.51 (0.15)	6.07 (0.19)
Thymol (0.1%)	-	-

Four honey samples; H25, H24, H201 and H180 demonstrated antibacterial activity (phenol equivalent >4.49% w/v) against MRSA and both *B. subtilis* strains following the addition of catalase, suggesting that they contain additional antibacterial compounds. Three of these samples (H180, H25 and H24) were all extracts from one hive in Aberdovey, west Wales (LL36 9EW) and H201 is an extract from a hive in Southampton (SO45 5AW). Some honey samples (H116, H169, H72 and H51) had no effect on the *B. subtilis* strains highlighting the high levels of resistance when compared to MRSA.

These results suggest that antibacterial activity of the natural honey samples analysed in this study was predominantly due to the production of hydrogen peroxide. Only 13 samples demonstrated non-hydrogen peroxide mediated antibacterial activity and thus were subjected to further analysis.

### 3.4.6.2 Neutralisation of Methylglyoxal

Methylglyoxal (MGO) is known to be responsible for the antibacterial activity of Manuka honey (Mavric *et al.*, 2008). To determine if the 13 honey samples which had demonstrated antibacterial

activity following neutralisation of hydrogen peroxide activity contained MGO they were treated with L- glutathione and glyoxalase I, solutions which neutralise MGO activity (Kwakman *et al.*, 2011b). Samples were then screened for activity against MRSA and *B. subtilis* using the agar diffusion assay (section 3.3.3.3).

As expected the 3 samples of Manuka honey (H52, H53 and H54) lost their activity following neutralisation (Table 3.8). Of the remaining UK samples only four, H24, H25, H180 and H201 retained antibacterial activity following treatment. Three of these samples from Aberdovey (H24, H25, and H180) and a sample from Southampton (H201) were the only samples to show non-peroxide activity against both *B. subtilis* strains. These results suggest that the antibacterial activity of the remaining honey samples is due to the presence of MGO or compounds which were affected by the neutralisation process.

*Table 3. 8: The antibacterial activity of honey samples following the neutralisation of MGO against MRSA and B. subtilis.*

*Activity was determined using an agar diffusion assay. The level of activity of individual honeys has been converted to a phenol equivalent (% w/v). Samples highlighted in red were Manuka and (-) represents no zone of inhibition. All experiments were run in triplicate and standard error was calculated.*

Honey Sample	Phenol equivalent (% w/v) (SE)		
	<b>MRSA (NCTC 11939)</b>	<b><i>B. subtilis</i> (ATCC 6633)</b>	<b><i>B. subtilis</i> (ATCC 39090)</b>
<b>H53</b>	-	-	-
<b>H54</b>	-	-	-
H25	4.98 (0.18)	6.14 (0.13)	7.98 (0.14)
<b>H52</b>	-	-	-
H24	6.02 (0.23)	5.94 (0.12)	8.15 (0.09)
H201	5.79 (0.91)	4.52 (0.15)	5.00 (0.22)
H180	5.51 (0.34)	5.98 (0.19)	7.90 (0.13)
H150	-	-	-
H116	-	-	-
H57	-	-	-
H169	-	-	-
H72	-	-	-
H51	-	-	-
Sugar Control	-	-	-
Phenol (5%)	5.05 (0.11)	5.50 (0.11)	6.08 (0.17)
Thymol (0.1%)	9.02 (0.13)	-	-

### 3.4.6.3 Neutralisation of Bee Derived Defensin-1 Antibacterial Activity

Defensin-1 is a known antibacterial factor which has been detected in honey (Kwakman *et al.*, 2010). This neutralisation step was performed to ensure the Defensin-1 peptide was not contributing to the antibacterial activity of any of the samples. This was performed following a protocol already described in literature (Kwakman *et al.*, 2010).

To determine if Defensin-1 was responsible for the activity detected in the four honey samples its activity was neutralised. The zones of inhibition were unaffected by the addition of SPS (Table 3.9).

*Table 3. 9: The antibacterial activity of honey samples against MRSA and B. subtilis following neutralisation of Defensin-1.*

*Activity was determined using an agar diffusion assay. The level of activity of individual honeys has been converted to a phenol equivalent (% w/v). (-) represents no zone of inhibition. Phenol, thymol and sugar were included as controls. All experiments were run in triplicate and standard error was calculated.*

Honey sample	Phenol equivalent (% w/v) (SE)		
	MRSA (NCTC 11939)	<i>B. subtilis</i> (ATCC 6633)	<i>B. subtilis</i> (ATCC 39090)
H24	5.98 (0.23)	5.94 (0.12)	7.84 (0.09)
H180	5.56 (0.19)	5.88 (0.09)	7.92 (0.13)
H201	5.79 (0.19)	4.50 (0.15)	5.00 (0.18)
H25	5.00 (0.14)	6.03 (0.15)	7.96 (0.14)
Sugar control	-	-	-
Phenol (5%)	5.05 (0.11)	5.52 (0.11)	6.10 (0.19)
Thymol (0.1%)	9.05 (0.11)	-	-

### 3.4.6.4 Neutralisation of Acidity

The antibacterial activity of all of the samples tested (H24, H25, H180 and H201) was inactivated by increasing the pH to 8.3 using 0.1M Sodium hydroxide. As the pH of these four honey samples were within the normal range (H24 – 4.13, H25 – 3.99, H180 - 3.55 and H201 - 4.22 from Appendix C) and this activity was not detected by any other samples, further investigations were performed on these honeys. The stability and activity of acidic and phenolic compounds are effected by pH (Friedman and Jürgens, 2000), it is therefore possible that a combination these factors are responsible for the activity of H24, H25, H180 and H201.

In conclusion, using the optimised screening assay 88% of the honeys screened showed some level of antibacterial activity against MRSA. Of these, 13 honey samples retained their activity following catalase treatment for the neutralisation of hydrogen peroxide. The activity of these samples was multifactorial. Three manuka samples retained their activity until the MGO was neutralised. There was no evidence of the antibacterial peptide in H24, and following the neutralisation of all known antibacterial compounds four honey samples had detectable activity (H24, H25, H180 and H201). The activity subsided following the neutralisation of pH, suggesting the antibacterial compounds may have an acidic nature however, further investigation is required for full characterisation.

### 3.5 Discussion

Numerous studies have shown that honey has a broad spectrum of antibacterial activity against different bacteria, as summarised by Carina *et al.* (2014). While the antibacterial activity of honey against microorganisms including MRSA and *B. subtilis* is well known the factors responsible for this activity are not always clear (Boorn *et al.*, 2010, Jenkins *et al.*, 2011). While the antibacterial activity of medical grade honey including Manuka has been well described, the same cannot be said for the majority of locally produced honeys. The lack of robust standardised methods to quantify the antimicrobial activity of honey and the compounds responsible for its antimicrobial activity, has made it difficult to compare the results of the various published studies and to fully characterise the antimicrobial profile of individual honey samples.

The emergence of antibiotic resistance has renewed interest in honey as a source of novel antimicrobial compounds (Kwakman *et al.*, 2010). The full range of plant derived compounds in Welsh honey has yet to be determined and the frequency with which they occur in different honeys is affected by the diversity of the flora surrounding the hive (Kaškonienė and Venskutonis, 2010, Kwakman and Zaat, 2012, Brudzynski *et al.*, 2012). The main aim of this chapter was to develop a screening assay which could be used to detect honey samples with activity against clinically significant bacteria such as MRSA having eliminated all known antibacterial compounds.

In this study two assays were developed which were optimised to determine the growth media with which to detect the antimicrobial activity of honey. Media composition is known to affect the activity of certain classes of antimicrobial compounds. MH is the agar of choice for the susceptibility testing of antibiotics in the United States of America as it has been found to be compatible with most antibiotics and supports the growth of a large number of microorganisms (CLSI, 2012). It was found that LB produced significantly larger ( $p < 0.05$ ) zones of inhibition than MH against MRSA for two honey samples one of which owed its activity to the presence of MGO.

In the UK the media of choice for antimicrobial susceptibility testing is ISO agar as recommended by the British Society for Antimicrobial Chemotherapy (BSAC, 2015). As was the case for MH significantly larger ( $p < 0.05$ ) zones of inhibition were observed for three out of the four test honeys against MRSA on LB when compared to ISO agar.

It is known that the composition of the growth media can adversely affect the antimicrobial activity of a compound either by affecting the growth characteristics of the bacteria or by directly interacting with the test compound. Trace metals may cause oxidation of polyphenols through Fenton reactions (Dai and Mumper, 2010). Casein is known to affect the potency of antimicrobial tea polyphenols (Bourassa *et al.*, 2013). Many broths and solid media preparations for

antibacterial testing and culturing contain casein or components of casein, such as casein hydrolysate, casein peptones and other digests of casein proteins. ISO, TSA and MH agar all contains between 2.0 - 1.5 % (w/v) of casein components. LB does not contain casein and trace metals which are known to interfere with polyphenols and was subsequently used for the agar diffusion assay.

Cooper *et al.*, (2008) investigated the antibacterial activity of 139 Welsh honey samples against *S. aureus* using an agar diffusion bioassay and found that antibacterial activity was due to the production of hydrogen peroxide; there was no evidence of non-peroxide mediated activity (Cooper and Wheat, 2008). TSB was combined with NA in the Cooper study, this may explain the low level of activity observed (Cooper and Wheat, 2008).

To determine if the results observed with the different culture media were specific to a particular bacterial species the effect of media composition was examined and phenotypic characteristics on the antibacterial activity of honey for *B. subtilis*. In this study *B. subtilis* was specifically used to confirm the presence of a non-peroxidase antimicrobial compounds in honey. As was the case for MRSA the largest zone of inhibition for *B. subtilis* (ATCC 6633) was observed on LB agar against the Manuka honey sample (H53). Unlike MRSA no activity was seen against the other honey samples on any of the test media suggesting that this bacteria may be insensitive to the low levels of hydrogen peroxide generated by these samples, which would correspond to literature (Chen *et al.*, 1995).

This insensitivity is based on the production of enzymes which counter oxidative stress and protect the vegetative bacteria from hydrogen peroxide, the PerR regulon regulates the response (Naclerio *et al.*, 1995). The PerR repressor senses peroxide stress and PerR:Fe senses hydrogen peroxide specifically. *B. subtilis* subsequently produce the major vegetative catalase (*katA*) during log phase growth, which can neutralise hydrogen peroxide (Faulkner *et al.*, 2012). The bacterium also encodes several peroxide stress genes including *mrgA*, which induces a DNA-binding protein that protects *B. subtilis* cells from peroxidase attack (Chen *et al.*, 1995). The bacterium when present in its spore form shows increased resistance to the action of hydrogen peroxide (Brudzynski *et al.*, 2011).

Honey is a known source of the spore forming bacteria including *Clostridium botulinum* which can cause infant botulism (Tanzi and Gabay, 2002). To determine the contribution of spore formation to honey resistance the sensitivity of a variant of *B. subtilis* which lacked the ability to form spores was determined. As expected *B. subtilis* ATCC 39090, a non-spore forming variant showed increased sensitivity to honey, when compared to the sporulation proficient ATCC 6633 suggesting that spore formation contributes to resistance.

Broth microdilution assay optimisation was also successfully performed to ensure honey samples were screened effectively and no hydrophobic molecules were missed, whilst taking into consideration of the dilution effect of hydrogen peroxide (Brudzynski *et al.*, 2011). MBC values were determined using the broth assay. Only 32% of honey samples were effective on the broth dilution assay compared to 88% of the samples tested on the agar diffusion assay. Dilutions of honey were performed for the broth assay (50% and 25%) whereas for the agar diffusion assay it was possible to use neat honey. With 68% of the honey sample having no effect on the MRSA it is clear to see that the 50% concentration of honey tested in the broth assay was not as effective as the neat honey used in the agar diffusion assay. There is a dilution effect associated with both methods however, it appears that the more potent the antibacterial activity of the honey, the more it can be diluted and still retain its inhibitory activity. On the basis of these optimisation studies LB agar and broth was selected as the media of choice for the large scale screening studies.

The agar diffusion assay was utilised for the neutralisation studies as higher levels of sensitivity were obtained. MRSA was utilised as the study aims to look at the effect of honey on healthcare associated pathogens. These methods were robust, effective and simple to implement for the mass screening of honey 220 samples. The osmotic effect and pH of honey is known to contribute to its antibacterial activity. The water levels (15.20g/100g to 23.00g/100g) and pH values (3.29 to 5.10) of all 220 honey samples examined were similar to the results reported by others (Molan, 1992, Bogdanov *et al.*, 1999); suggesting that other factors probably account for the levels of antibacterial activity observed.

In this study 88% of donated honey samples demonstrated antibacterial activity against MRSA. The highest level of activity was the equivalent to a UMF® rating of 13.5, which is a value higher than that seen in many commercial Manuka samples. Cooper and Wheat (2008) investigated the antibacterial activity of 139 Welsh honey samples against *S. aureus* using an agar diffusion bioassay and found that antibacterial activity was due to the production of hydrogen peroxide (Cooper and Wheat, 2008). In another study 477 honey samples, derived from various regions of Australia were investigated (Irish *et al.*, 2011). It was found that 16.8% samples of the samples screened had non-peroxide activity however this was mainly attributed to *Leptospermum* spp. (Irish *et al.*, 2011).

The honey samples which demonstrated antibacterial activity in this study were subjected to a series of characterisation and neutralisation assays to determine the basis of their activity. Using an approach previously employed by Kwakman and colleagues to identify the presence of defensin-1 in honey the activity was assessed (Kwakman *et al.*, 2010). Hydrogen peroxide was initially investigated as it has been identified as a major antibacterial component of honey

(Brudzynski *et al.*, 2011). To determine if hydrogen peroxide was responsible for the antibacterial activity honeys samples were treated with catalase. Following neutralisation 13 local Welsh honeys retained their antibacterial activity against MRSA, suggesting the presence of non-peroxidase based compounds.

The sensitivity of MRSA and both *B. subtilis* strains to hydrogen peroxide were determined and a dose-dependent response was observed (Figure 3.8). As previously discussed the levels of hydrogen peroxide in honey is not sufficient to inhibit *B. subtilis*, but higher concentrations overcome its defence and stress mechanisms and zones of inhibition were observed. MRSA was significantly ( $p < 0.001$ ) more sensitive to hydrogen peroxide than *B. subtilis* ATCC 6633. This finding concurs with an earlier study which reported that MRSA is more sensitive to hydrogen peroxide than *B. subtilis* ATCC 6633 (Kwakman *et al.*, 2010).

It was observed that the suspension of hydrogen peroxide in sugar reduces the antibacterial effect against both MRSA and *B. subtilis*, when compared directly to water. A high sugar content may enhance the growth conditions for MRSA and *B. subtilis* due to the high level of sugars which can be utilised as a carbon source (Brudzynski *et al.*, 2011). It may also be a physical effect, with the higher viscosity sugar reducing the diffusion efficiency of the peroxide. It is well known that hydrogen peroxide has harmful effects on many bacterial cells but honey compounds such as catalases, polyphenols, Maillard reaction products, and ascorbic acid can lower the oxidative stress to cells and may have a protective effect against hydrogen peroxide (Brudzynski, 2006). Brudzynski *et al.*, (2011) concluded that the bacteriostatic efficacy of hydrogen peroxide differ significantly from that of honey based hydrogen peroxide, possibly due to synergistic effects, highlighting the need to investigate other antimicrobial compounds (Brudzynski *et al.*, 2011).

Those samples which demonstrated antibacterial activity following exposure to catalase were next treated to neutralise the possible impact of MGO and bee defensin-1. As expected the three commercial Manuka honey sample lost all detectable antibacterial activity following neutralisation of MGO (Kwakman *et al.*, 2011b). Defensin-1, which is effective against Gram positive bacteria, has been detected in different natural honey samples. The neutralisation of defensin-1 was performed based on previous literature (Kwakman *et al.*, 2010). Major royal jelly protein (MRJP) are multifunctional protein which assists the differentiation of honey bee larvae into queens. Proteins known as Jelleins are produced by the processing of MRJP-1 and have demonstrated antimicrobial activity against *S. aureus*, *B. subtilis* and *E. coli* (Romanelli *et al.*, 2011). MRJP-1 has been previously detected in honey (Majtan *et al.*, 2014) however, the antibacterial effects of this protein within a complex honey matrix has yet to be described. To fully characterise and assess the antibacterial effects of these proteins with the honey samples tested further analysis would be required. Immunoblotting could be performed, Proteins would

be separated by SDS-PAGE, transferred onto nitrocellulose membranes and detected using an anti-rabbit secondary antibody (Kwakman *et al.*, 2010). Following successive neutralisation steps only four honey samples retained antimicrobial activity. Three of these samples, H24, H25 and H180 were obtained from a single hive in Aberdovey while the fourth sample, H210, came from a hive in Southampton. On the basis of these results it can be concluded that these honey samples may contain antibacterial phytochemicals.

Titration of pH reduced the bactericidal activity of honey to a level identical to that of sugar solution (Figure 3.4), the low pH may be inhibitory to pathogenic bacteria. These findings correlate to the study by Kwakman *et al.*, (2010) who revealed an antibacterial effect caused by low pH having neutralised all other known active compounds (Kwakman *et al.*, 2010). No correlation has been found previously when investigating the relationship between pH and antimicrobial activity (Molan, 2009b). When honey gets diluted in culture media or serum the pH makes a minor contribution to antibacterial activity (Molan, 2009b). This antibacterial activity of honey may be partly due to acidity due to gluconolactone/gluconic acid. Honey contains a number of acids other acids which may be contributing to the low pH; these include amino acids and organic acids which vary considerably. The non-peroxide activity detected in the Aberdovey samples (H25, H25 and H180) and Southampton sample (H201) differ to all the other natural honey samples tested warranting further investigation.

In conclusion the optimisation of the screening assays was performed to improve the level of sensitivity of standard screening assays. In total, 220 honey samples were assessed for antibacterial activity against the clinically relevant pathogen MRSA. The screening of 220 honey samples yielded four samples (3 from Aberdovey and 1 from Southampton) which contained potentially novel antibacterial compounds. These samples will be subjected to detailed analysis using the methods and techniques described in subsequent chapters to identify both their chemical and floral profile.

## Chapter 4

# **IDENTIFYING THE BOTANICAL CONSTITUENTS OF HONEY – A DNA METABARCODING APPROACH**

## 4.1 Chapter Introduction

### 4.1.1 Traditional Approaches of Pollen Analysis

Honey is produced by honey bees (*Apis mellifera*) from the nectar and secretions collected from flowers surrounding the hive. The medicinal properties of honey is partly dependent on the plant composition, it is therefore important to characterise the contributing flora. The spectrum of pollen within honey indicates the plants visited by bees during its creation. As described in chapter 1 the traditional approach to ascertain the floral composition of honey is melissopalynology; the morphological examination of pollen under a light microscope which was proposed by the International Commission of Bee Botany (ICBB) in 1978 (Louveaux *et al.*, 1978).

Melissopalynology requires considerable skill to identify the plants based on pollen morphology and must be performed by an expert (Khansari *et al.*, 2012). Some plants can be difficult to distinguish using pollen morphology, for example species of Campanulaceae and Poaceae exhibit few unique morphological features (Khansari *et al.*, 2012). Within the Rosaceae individual species can show high levels of pollen grain morphological variation making characterisation difficult (Hebda and Chinnappa, 1990). In spite of these limitations melissopalynology remains a valuable tool for the identification of many plants within honey (Song *et al.*, 2012).

To further improve the accuracy and efficiency of plant characterisation the use of specific chemical marker compounds has been proposed (Figure 4.1) (Kaškonienė and Venskutonis, 2010).

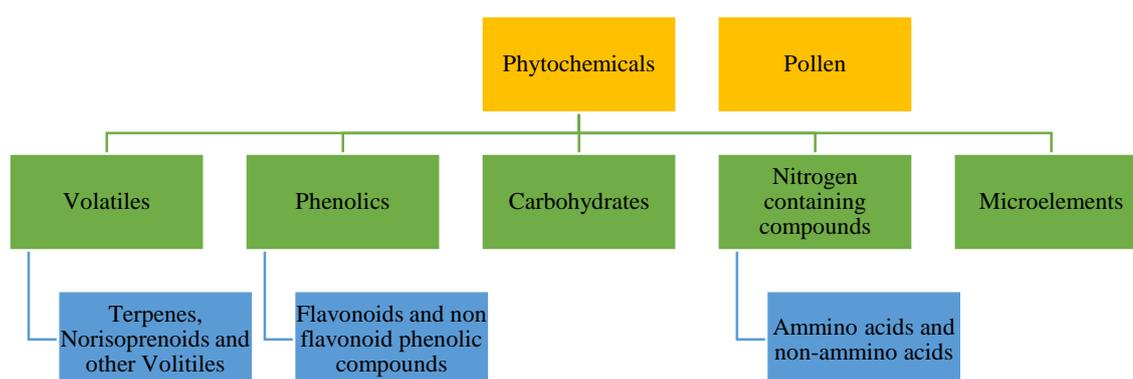


Figure 4. 1: Floral markers for the characterisation of honey.

Honey is a complex mixture; to date, hundreds of compounds have been identified by means of different analytical techniques including gas chromatography-mass spectrometry (GC-MS), high performance liquid chromatography (HPLC) and solid phase micro-extraction (SPME) (Montenegro

*et al.*, 2009). A complex mixture of phenolic acids and flavonoids contribute to honeys chemical composition (Molan, 1998).

#### **4.1.2 Molecular Biology for Honey Characterisation**

Developments in NGS platforms means that DNA metabarcoding can be used for pollen characterisation. Bulk samples can be processed, producing thousands of reads from one amplicon for simultaneous species identification (Glenn, 2011). The Genome Sequencer (GS) instrument developed by 454 Life Science was introduced in 2005. DNA sequencing experienced revolutionary changes which for the first time challenged the supremacy of the dideoxy method (Sanger and Coulson, 1975, Mardis, 2013).

The extraction of DNA from pollen within the honey has been more recently investigated in order to obtain higher DNA yield with sufficient purity for downstream processing. Guertler *et al.*, (2014) created an automated DNA extraction method which improved DNA yield and quality, but required the use of high-cost instrumentation (Guertler *et al.*, 2014). Soares *et al.*, (2015) investigated honey sample pre-treatments and commercial DNA extraction kits and found that a combination of mechanical disruption and the Wizard DNA extraction kit gave the best results (Soares *et al.*, 2015). A range of extraction methods including Wizard, CTAB and DNeasy kits (Qiagen) have all been shown to be suitable for the extraction of amplifiable DNA (Soares *et al.*, 2015, Jain *et al.*, 2013, Waiblinger *et al.*, 2012).

Laube *et al.*, (2010) demonstrated that with a prior knowledge of the plant species likely to be present in the honey, real-time polymerase chain reaction (PCR) could be used to characterise the plants in a honey sample (Laube *et al.*, 2010). DNA metabarcoding is a useful tool for the identification of mixed samples since standard, internationally agreed markers are used. Plant specific DNA markers which are present in pollen are targeted and DNA barcoding can be used for the identification of unknown species in complex samples. Valentini *et al.*, (2010) first employed a DNA metabarcoding approach based on the *trnL* plastid region and next generation sequencing (NGS) to identify previously unknown plants in two honey samples (Valentini *et al.*, 2010). Bruni *et al.*, (2015) amplified the *rbcL* and *trnH-psbA* plastid markers to identify the floral composition of honey from the Italian Alps (Bruni *et al.*, 2015). However, their method used cloning to sequence individual amplicons, which places limits on the depth of sequencing that can be achieved.

High Throughput Sequencing (HTS) technologies are an emerging and widely adopted tool for characterization of mixed DNA samples. The number of samples and sequence reads from a single experiment is vastly greater than the 96 obtained with modern capillary electrophoresis-based Sanger sequencers (Metzker, 2010). NGS technologies including Roche 454, Ion Torrent and

Illumina sequencing platforms are currently being utilised in metabarcoding (Taberlet *et al.*, 2012, Yoccoz, 2012, Kajtoch, 2014, Mardis, 2013). HTS reduces processing time, increases the level of species discrimination and does not require the high level of taxonomic expertise required for melissopalynology (Schnell *et al.*, 2010, Valentini *et al.*, 2010, Jain *et al.*, 2013, Bruni *et al.*, 2015).

454 sequencing is a technology based on pyrosequencing and oil emulsion PCR (Figure 4.2) (Zhang *et al.*, 2011). Adapter-ligated DNA fragments are bound to beads and suspended in an oil droplet containing PCR reagents (Margulies *et al.*, 2005). PCR amplification results in DNA coated beads carrying millions of copies of a unique DNA template which can then be sequenced within the 454 Genome Sequencer FLX instrument (Roche Applied Science). When complementary nucleotides are added, light signals are produced with every successful base pairing which are captured by the CCD camera in the instrument (Margulies *et al.*, 2005, Zhang *et al.*, 2011).

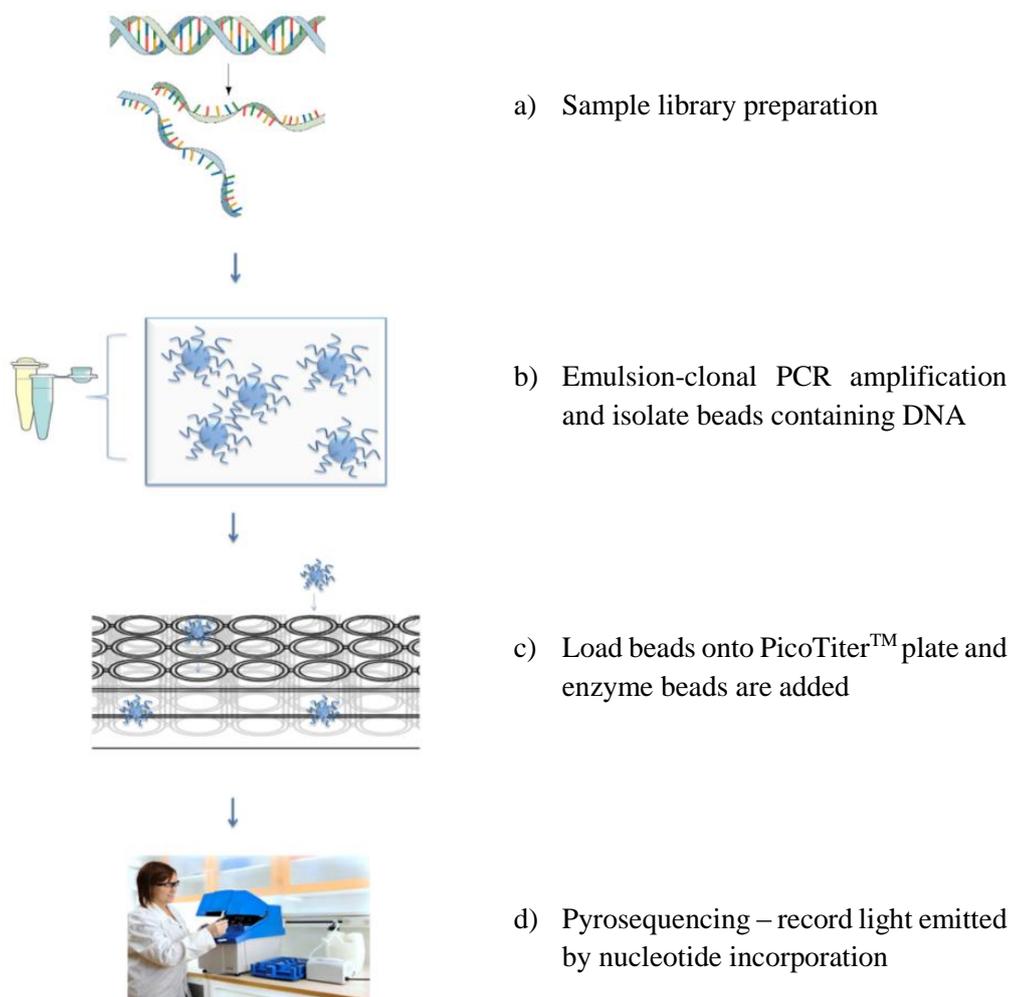


Figure 4. 2: Pyrosequencing (454) process overview

a) A purified DNA library is produced b) Emulsion-based clonal amplification is performed on micron-sized beads c) Pyrosequencing-based sequencing by synthesis is then performed on the beads (d) Light is detected as nucleotides are incorporated

The Illumina platform offers significantly increased depth and coverage compared to the Roche 454 platform. Illumina sequencing yields a higher throughput at a lower cost relative to 454 sequencing (Luo *et al.*, 2012). Illumina sequencing by synthesis (SBS) approach also lowers per-base error rate and improves sequence quality. In Illumina DNA sequencing single DNA fragments are amplified in dense clusters on a hollow flow cell channel slide (Zhang *et al.*, 2011). Solid-phase bridge amplification generates several million dense clusters of double-stranded DNA. After amplification and excitation, the emitted fluorescence from each cluster is captured and the first base is identified (Zhang *et al.*, 2011). This is repeated and sequence of bases in a fragment is determined, one base at a time. These methods do not require cloning of DNA fragments which eliminates any bias and significantly reduces the labour cost.

HTS technologies especially Roche 454 and Illumina sequencing platforms have been successfully used in applications ranging from the composition of microbial (Nelson *et al.*, 2014, Poretsky *et al.*, 2014), freshwater (Hajibabaei *et al.*, 2011) and fungal communities (Blaalid *et al.*, 2012, Větrovský and Baldrian, 2013); diet analysis (Pompanon *et al.*, 2012, Shehzad *et al.*, 2012, Kajtoch, 2014) to biodiversity assessments (Ji *et al.*, 2013, Gibson *et al.*, 2014).

Limitations to using NGS to determine the species composition of mixed samples include sample viability, DNA extraction efficiency, PCR amplification biases and sequencing errors (Sinclair *et al.*, 2015, Shokralla *et al.*, 2012, Pompanon *et al.*, 2012). A major limitation to the characterisation of mixed samples is the comprehensiveness and universality of the reference database against which that the resulting sequences are compared (Taberlet *et al.*, 2012). This study relies on the fact that 98% of the native flowering plants of Wales have been DNA barcoded using the *rbcL* DNA barcode marker (de Vere *et al.*, 2012), providing a much more comprehensive reference library against which to compare the DNA sequences too.

The plants visited by the bees can be characterised using these NGS and metabarcoding techniques. In this study the floral composition of eleven honey samples provided by domestic beekeepers in Wales and England (UK) were analysed. Melissopalynology and DNA analysis was performed on H24 and H201, which showed high levels of antimicrobial activity in chapter 3 (Table 3.8), to determine the plants which contribute to the making of these samples. DNA metabarcoding using the *rbcL* DNA barcode marker, 454 pyrosequencing, Illumina and melissopalynology are compared.

## 4.2 Chapter Aims and Objectives

The aim of this chapter is to develop a method capable of identifying the plant species which have contributed to the making of a sample of honey.

- Use melissopalynology and DNA metabarcoding techniques to identify the plants which contribute to the making of natural honey with high levels of antimicrobial activity in chapter 3.
- Optimise the DNA extraction and amplification from plant material extracted from natural honey.
- Perform DNA metabarcoding using 454 next generation sequencing approach to characterise plant derived DNA extracted from honey samples.
- Perform Illumina sequencing of plant derived DNA extracted from honey for further characterisation.
- Compare all three techniques; melissopalynology, 454 and Illumina sequencing.
- Determine the plants which will be subjected to further chemical analysis in chapter 5.

## 4.3 Methods and materials

### 4.3.1 Honey Samples Analysed

Eleven honey samples, including a repeat of a sample from Aberdovey were selected for analysis (Table 4.1). Samples were chosen based on hive location and antibacterial activity detected in chapter 3 (Table 3.8). Particular attention was paid to samples H24, H25 and H201 which had previously demonstrated non-peroxidase antibacterial activity in chapter 3 (Table 3.8) suggesting that they contained plant derived antibacterial compounds.

*Table 4. 1: Honey samples analysed using melissopalynology and NGS technologies*

*The location of the eleven honey samples analysed and which samples were analysed using which techniques.*

<b>Honey ID</b>	<b>Hive location</b>	<b>Co-ordinates (Lat/Long)</b>	<b>Microscopy analysis</b>	<b>454 analysis</b>	<b>Illumina analysis</b>
H23	Reidiog, Llansannan, Wales	53.171426, - 3.610821	✓	✓	✓
H24 and H25	Aberdovey, Gwynedd, Wales	52.587166, - 4.083243	✓	✓	✓
H26	Llandysul, Ceredigion, Wales	52.071726, - 4.387493	✓	✓	✓
H31	Cwmpengraig, Carmarthenshire, Wales	51.840725, - 4.157574	✓	✓	–
H36	Coytrahen, Bridgend, Wales	51.564926, - 3.602989	✓	✓	✓
H44	Penderyn, Aberdare, Wales	51.776733, - 3.534182	✓	✓	✓
H107	East Cowes, Isle of Wight, England	50.735613, - 1.26929	✓	✓	✓
H114	Dryslwyn, Carmarthenshire, Wales	51.876283, - 4.105855	✓	✓	✓
H160	Aberystwyth, Dyfed, Wales	52.373164, - 4.064016	✓	✓	✓
H201	Hythe, Southampton, England	50.861388, - 1.411488	✓	–	✓

In addition ten other the honey samples were successfully analysed on the Illumina run (Appendix E).

### 4.3.2 Traditional Pollen Analysis

The traditional approach of characterising the plants which contribute to the making of a honey sample is melissopalynology. Melissopalynology requires an expert to examine pollen under a light microscope and identify the plant species present. To compare the traditional methods with the DNA metabarcoding approach pollen counts and microscope analysis were performed.

#### 4.3.2.1 Pollen Counts

Pollen counts were performed on the eleven honey samples (Table 4.1) using a haemocytometer. In total 5 g of honey was dissolved in 5 ml of distilled water and centrifuged for 10 mins at 3000 g (MSE, Mistral 1000, London, UK) and supernatant was discarded until 2ml of liquid remained (Shubharani *et al.*, 2012). A 10  $\mu$ l aliquot of this liquid was added to the haemocytometer chamber and the number of grains in each square (1 mm x 1 mm) of the chamber was counted under oil emersion at 100X (Olympus DP10). Each honey sample was analysed on three separate occasions and the number of pollen grains within 1 g of honey was calculated. The following equation was used:

$$\text{Average cell number in 1 square (1 mm x 1 mm)} \times 10^4 = \text{pollen grain number per ml}$$

$$(\text{Pollen grain per ml} \times 2 \text{ ml}) \div 5 \text{ g} = \text{pollen grains per 1 g of honey}$$

#### 4.3.2.2 Melissopalynology

The eleven honey samples (Table 4.1) were sent to the National Pollen and Aerobiology Research Unit at the University of Worcester to be analysed by a palynology expert. Microscopy analysis was performed to characterise the pollen present in these honey samples. Two separate repeats of the Aberdovey honey (H24 and H25) from the same hive were sent for analysis to determine the discrimination and reproducibility of this method.

The guidelines of the International Honey Commission were adopted (Bogdanov *et al.*, 1999, Louveaux *et al.*, 1978). 40 ml of 0.5% sulphuric acid solution was combined with 2g of honey and incubated in a water bath for 5min at 80 °C. Each sample was filtered (5  $\mu$ m), placed in filter assembly with pump and 500 ml hot distilled water was used to rinse the samples. The filter was washed using 8 ml glacial acetic acid to dehydrate the sample and a centrifuge step was performed at 3000 g for 2 mins. The supernatant was decanted, mixed with 1ml acetolysis mixture and placed in a water bath at 80°C for 12 mins.

The centrifugation step was repeated; the sample was resuspended in 1ml glacial acetic acid and centrifuged again. Three drops were mounted onto a microscope slide and examined using a microscope at x400 or x1000 magnification. A minimum of 300 grains were characterised, unidentifiable grains were also noted. All eleven honeys were tested in this manner, including two repeats H24 and H25 which were independently analysed.

#### 4.3.3 DNA Extraction Protocol

Total DNA was extracted from 10 g of honey using a protocol developed from published methods adapted for use within the current study (Guertler *et al.*, 2014, Cheng *et al.*, 2007, Valentini *et al.*,

2010, Laube *et al.*, 2010, Schnell *et al.*, 2010, Soares *et al.*, 2015). Each of the honeys (Table 4.1) was extracted four times so that DNA was recovered from a total of 40 g of honey. This extraction process was repeated with two different samples of honey from Aberdovey (H24 and H25) to investigate repeatability. DNA was extracted using an adaption of the DNeasy Plant Mini kit extraction protocol (Qiagen). Samples were placed in a sterile centrifuge tube and ultrapure water (Sigma) was added until the meniscus reached 30 ml. Samples were placed in a water bath at 65 °C for 30 mins with occasional shaking. Samples were then centrifuged (Sorvall RC-5B) for 30 mins at 27,000 g. All centrifugation steps were performed at room temperature (15-25 °C); any further centrifugations were carried out using a Heraeus® Pico 17 microcentrifuge (Thermo Scientific, UK).

The supernatant from the separation was discarded and the pellet was resuspended in 400 µL of AP1 from a DNeasy plant mini kit (Qiagen) and 80 µL of proteinase K (1 mg/ml) (Sigma). The mixture was vortexed and incubated for a further 10 mins at 65 °C in a water bath to allow the chemical breakdown of the pollen coat. Sample material was then disrupted using the TissueLyser II (Qiagen) for 4 mins at 30 Hz with 3 mm tungsten carbide beads. Following these adjustments the remaining steps were carried out according to the DNeasy Qiagen protocol, excluding the use of the QIAshredder and the second wash stage. The DNA was stored at -20 °C until required for subsequent analysis.

#### **4.3.4 Next Generation Sequencing (NGS) - 454 Analysis**

##### **4.3.4.1 Honey Samples Analysed - 454**

Ten of the honey samples from table 4.1 were selected for DNA metabarcoding analysis using the 454 approach. The non-peroxide antibacterial activity demonstrated by sample H201 was not detected until after the 454 run had been completed and thus was not included in the analysis. Two repeats of the honey from Aberdovey (H24 and H25) were included in the run as these samples had demonstrated the greatest antibacterial activity in chapter 3 (Table 3.8).

##### **4.3.4.2 Polymerase Chain Reaction (PCR) Amplification – 454**

DNA was amplified using the *rbcL* DNA barcode marker region (CBOL Plant Working Group *et al.*, 2009). Two rounds of PCR were performed, firstly to amplify the *rbcL* region and then to attach unique 5 bp tags so that different samples could be separated bioinformatically after sequencing. Ten different honey samples, labelled with unique adaptor tags, were investigated. The primer pairs shown in table 4.2 were used to amplify the *rbcL* region (de Vere *et al.*, 2012), to which adaptor ‘tails’ had been added. Unique DNA tags were attached to each pair to ensure sequences could be credited to the correct honey sample after the 454 run.

Table 4. 2: The primers used for the amplification of the *rbcL* region.

Primer Name	Primer Direction	Primer Concentration	Primer sequence
<i>rbcLaf-t</i>	Forward	10 $\mu$ M	GACGATGAGTCCTGAGGTATGTCACCA CAAACAGAGACTAAAGC
<i>rbcLr590-t</i>	Reverse	10 $\mu$ M	GACGATGAGTCCTGAGGTAGTCCACCG CGTAGACATTCAT

(de Vere et al., 2012)

First round PCR was performed in a final volume of 25  $\mu$ L using the following reagents: 12.5  $\mu$ L PCR 2x Biomix (Bioline), 0.5  $\mu$ L of each primer (5  $\mu$ M), 1.0  $\mu$ L BSA (10  $\mu$ M) and 8.0  $\mu$ L molecular grade water (Sigma). A 22.5  $\mu$ L volume of master mix was added to each PCR tube which contained 2.5  $\mu$ L of extracted DNA. The reaction was performed in a thermal cycler MJ Mini (Bio-Rad Laboratories, Hercules, USA) using the following conditions: initial denaturing at 95  $^{\circ}$ C for 2 mins, followed by 30 cycles of 95  $^{\circ}$ C for 2 mins, 50  $^{\circ}$ C for 90 secs, 72  $^{\circ}$ C for 40 secs with a final extension at 72  $^{\circ}$ C for 5 mins and 30  $^{\circ}$ C for 10 secs (de Vere *et al.*, 2012). A negative water control was run for each PCR performed.

The PCR reaction products were analysed on a 1% agarose gel. 130 ml of 1xTAE buffer was combined with 1.3 g of agarose (Sigma). The agarose mixture was heated in a microwave on full power (800W) for 3 mins. Having allowed the mixture to cool to touch, 3  $\mu$ L of gel stain (CyberSafe) was added. The molten agarose and CyberSafe mix was put in a gel support, combs were added and bubbles were coaxed to one side. A combined aliquot of sample DNA (4  $\mu$ L) and loading buffer (2  $\mu$ L) were loaded onto the gel, one sample per well. A Bioline hyper ladder (4  $\mu$ L), and negative water control was also included on each gel. The gels were run for 20 mins at a constant 120V. Gels were visualised under a chemidoc (MP system, Biorad) with the presence of visible bands confirming successful amplification.

The products were visualised on a 1% agarose gel and those producing the brightest bands were diluted by 1/2000, medium by 1/1000 and faint by 1/500. 2.5  $\mu$ L of the diluted product was used as the template for the second round of PCR.

For the second round of PCR the following reagents were used: 12.5  $\mu$ L of 2x Biomix (Bioline), 1.0  $\mu$ L of a unique 5bp tag with adapter sequences (10  $\mu$ M), 1.0  $\mu$ L of BSA (10  $\mu$ M) and 8.0  $\mu$ L of molecular grade water. A 22.5  $\mu$ L volume of master mix was added to each PCR tube which contained 2.5  $\mu$ L of diluted DNA from the first PCR. The thermocycle protocol was repeated (de Vere *et al.*, 2012) but modified to run for 15 cycles. The second PCR product was also visualised on a 1% agarose gel to confirm the presence of amplified DNA. Each sample was amplified twice using this procedure and the resulting products from all the PCR runs were then pooled and purified using a QIAquick PCR purification kit following manufacturers' instructions (Qiagen).

#### **4.3.3.3 High Throughput Sequencing - 454**

A total of 90 µL of pooled DNA was sent for Roche/454 GS FLX Titanium pyrosequencing at the University of Pennsylvania using a ¼ plate.

#### **4.3.3.4 Data Analysis - 454**

Sequences were sorted according to the identity of their 5 bp tag into the different honey samples. Sequences were assessed for quality and length using scripts written in the Python programming language (Appendix D). Any sequences where the 5 bp tag and the entire primer sequence could not be found were removed. The tag and primer sequences were then trimmed and sequences with a read length of 250 bp or less after trimming were discarded.

A local reference database of *rbcL* sequences was created by downloading all *rbcL* sequences available on GenBank. This included 98% *rbcL* coverage of Welsh native flowering plants uploaded to GenBank as part of the Barcode Wales Project (de Vere *et al.*, 2012). Each sequence resulting from the honey samples was compared to the reference database using Megablast. The species with the top bit score for each sequence was retained. When multiple top hits occurred with all species belonging to the same genus then the result was given to genus. If top hits belonged to multiple genera within the same family then a family level designation was made. Sequences blasting to multiple families were considered to be unknown. Identification results for each honey sample were tallied and then filtered so that only species found within the UK were returned. Stace (2010) and Cubey and Merrick (2014) were used as references for plants occurring in the UK either as natives, aliens or in horticulture or agriculture (Stace, 2010, Cubey and Merrick, 2014). If a species was not recorded within the UK then the sequence was designated to genus. To reduce results arising from amplification or sequencing errors taxa recorded from less than 10 sequences for that honey sample were removed from further analysis (Coissac *et al.*, 2012).

#### **4.3.5 Next Generation Sequencing (NGS) - Illumina Analysis**

##### **4.3.5.1 Illumina Analysis Trial Run**

Optimisation of the Illumina run was necessary as this process had not been previously performed on honey DNA. A trial Illumina run was performed and the details of the initial run are described in Appendix E. A full Illumina run was subsequently performed based on these findings.

##### **4.3.5.2 Honey Samples Analysed – Illumina**

To further optimise the DNA metabarcoding of honey the methods described in the 16s metagenomics sequencing library preparation guide were trialled (Illumina, 2013). The honey

samples highlighted in table 4.1 (except H31) were analysed using Illumina technology. H31 was not included in the Illumina run due to low levels of characterisation success in the 454 run.

#### 4.3.5.3 Polymerase Chain Reaction (PCR) Amplification – Illumina

Two rounds of PCR were used to incorporate *rbcL* universal primers and Illumina index tags. The Illumina 16S Metagenomic Sequencing Library Preparation protocol was used and adapted to target the *rbcL* plant region for Illumina analysis. Illumina overhang adapter sequences were appended to the primer pair sequences for compatibility with Illumina index and sequencing adapters. Amplicon primers were designed and ordered (Sigma); *rbcL* locus-specific sequences were added to the Illumina adapters to target the *rbcL* region. The primer pairs shown in table 4.3 were used for amplification (de Vere *et al.*, 2012). A second PCR was performed to add unique Illumina index to ensure sequences could be credited to the correct honey sample after the Illumina run.

During the optimisation process a primer was used which targeted a shorter region of the *rbcL* gene (*rbcLr506*) (Appendix E). This increased the amplification success rate and was therefore used in subsequent experiments.

Table 4. 3: Illumina overhang adapters (bold) added to the locus-specific primers to target the *rbcL* gene region.

Primer Name	Primer Direction	Primer Concentration	Primer sequence
<i>rbcLaf</i>	Forward	10 µM	<b>TCGTCGGCAGCGTCAGATGTGTATAAGA</b> <b>GACAGATGTCACCACAAACAGAGACTAAA</b> <b>GC</b>
<i>rbcLr506</i>	Reverse	10 µM	<b>GTCTCGTGGGCTCGGAGATGTGTATAAG</b> <b>AGACAGAGGGGACGACCATACTTGTTCA</b>

#### First-round PCR

The amplicon PCR was performed using the following reagents: 12.5 µl PCR Biomix (Bioline), 0.5 µl of each primer, 9.0 µl molecular biology grade water (Sigma). A 22.5 µl volume of master mix was added to each PCR tube which contained 2.5 µl (5 ng/µl) of extracted DNA, PCRs were run at a total volume of 25 µl. The PCR cycle used the following conditions: 95 °C for 3 mins, then 45 cycles of 95°C for 30 secs, 50 °C for 1.5 mins, 72 °C for 30 secs. This was then followed by 72 °C for 5 mins and 30 °C for 10 secs (de Vere *et al.*, 2012). The number of cycles was increased from the protocols recommended 25 cycles to 45 due to the low success rate obtained from 25 cycles during the optimisation process. A negative water control was run for each PCR performed.

The PCR reaction products were analysed on a 1% agarose gel as previously described (section 4.3.4.2). Amplified samples were taken to Aberystwyth University (IBERS) for further processing. An AMPure XP bead clean-up was performed following Illumina instructions to remove free primer and primer-dimers (Illumina, 2012).

### **Second-round PCR**

The index PCR was designed so that the amplified sequences could be tagged for identification. The Nextera XT Index Primers were added using a 16s Metagenomics Library Preparation Guide. For the second PCR the following reagents were used: 25 µl 2x KAPA HiFi HotStart ReadyMix, 5 µl of each Nextera index primer and 10 µl H<sub>2</sub>O. A 45 µl volume of master mix was added to each PCR tube which contained 5 µl of diluted DNA from the first PCR. The PCR cycle used the following conditions: 95 °C for 3 mins, then 8 cycles of 95°C for 30 secs, 55 °C for 30 secs, 72 °C for 30 secs. This was then followed by 72 °C for 5 mins (Illumina, 2013). An AMPure XP bead clean-up was performed to purify the DNA samples. The index PCR product was also visualised on a 1% agarose gel to confirm the presence of amplified DNA as previously described (section 4.3.4.2).

### **4.3.5.4 High Throughput Sequencing - Illumina**

Illumina libraries were prepared and pooled following the 16s Metagenomics Library Preparation Guide in IBERS at Aberystwyth University (Illumina, 2012). Libraries were quantified using a fluorometric Qubit analyser which uses dsDNA binding dyes to calculate concentration. Samples were diluted to a final concentration of 4 nM. Diluted DNA from each library was then pooled. Libraries were denatured using NaOH, diluted with hybridization buffer and heat denatured following manufacturers' instructions. Samples were cleaned using band capture on the Sage Science BluePippin system. Sequences were run on the MiSeq system using v3 reagents, a 10% PhiX control was used.

### **4.3.5.5 Data Analysis - Illumina**

Paired 2x300 bp Illumina MiSeq reads were produced using the Illumina Nextera library protocol (Illumina, 2013). The raw Illumina data was demultiplexed and converted to sample fastq files using Illumina bcl2fastq software (version 1.8.3). Read quality was assessed using FastQC. Overlapping pairs of reads were merged using Flash (version 1.2.11) with a maximum overlap set to 200 bases, minimum overlap 10 bases and maximum mismatch density 0.25. Three quality control steps were carried out on the merged reads using Trimmomatic. The Nextera paired end library sequences were trimmed from the 3' ends (using Nextera paired end library sequences supplied by Trimmomatic). A 3 base crop of the 5' end of the reads was carried out to resolve base bias identified by FastQC, and a 3' crop when a the mean quality in a four base sliding

window falls below a phred score of 20. Sequences shorter than 500 bp after trimming were discarded. Fastq reads were converted into unique fasta reads using FASTA/Q Collapser in the FASTX-Toolkit (version 0.0.13)

Sequences were polled and subsequently blasted using Megablast against the Genbank database. Results with the highest bit score were attributed to the unknown DNA sequences. If more than one result had the same bit score, if they were for the same genus then the result was given to genus level. If more than one genus came up then these are listed as unknown and could not be characterised. Any result that appeared less than 10 times were removed to eliminate the effects of PCR and sequence error. Of the remaining results samples were filtered so that they only contain species recorded in the UK, either native, alien or in horticulture. Any results that were not present in the UK were renamed as unknown and were subsequently uncharacterised or characterised to genus level.

## 4.4 Results

### 4.4.1 Traditional Pollen Analysis

#### 4.4.1.1 Pollen Counts

The number of pollen grains in each honey was determined by means of haemocytometer analysis (Figure 4.3), so that results could be estimated on a per pollen grain basis. It is not always possible to fully characterise a honey sample using melissopalynology if low amounts of pollen grains are present.

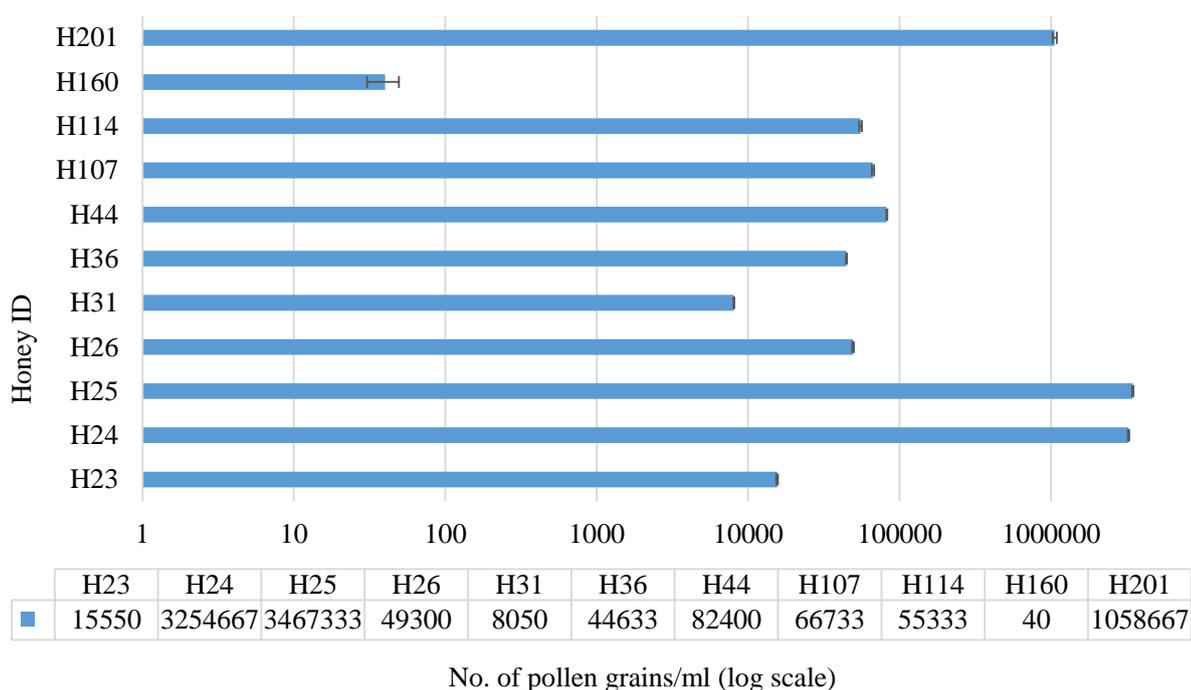


Figure 4. 3: Total number of pollen grains identified in honey samples

Counted using a haemocytometer. Mean of three repeats (error bars = SE).

The highest number of pollen grains were recorded in samples H24, H25 and H201 which demonstrated the highest levels of non-peroxide activity in chapter 3 (Table 3.8). Only 40 grains/ml was counted in H160 suggesting there may be insufficient pollen for analysis by melissopalynology.

#### 4.4.1.2 Melissopalynology

Melissopalynology was performed on all eleven honey samples and approximately 300 grains were characterised in each sample. The majority (98-100%) of pollen grains could be identified to species, genus or family level (Table 4.4). The number of taxa detected ranged from 8 to 31 highlighting the diverse range of pollen grains found within natural honey.

Table 4. 4: Summary of melissopalynology results

Honey ID, the number of pollen grains characterised or unknown and the % that were successfully identified to family, genus or species level. The number of pollen grains only detected once which were representative of one taxa (singletons).

Honey ID	No. of pollen grains detected	No. of unknown pollen grains	No. identified to family, genus or species level (%)	No. of taxa found	No. of singletons
H23	293	3	98.0	13	3
H24	337	3	97.8	31	17
H25	362	8	100.0	28	13
H26	306	2	99.1	9	2
H31	338	8	98.4	18	4
H36	346	7	97.6	20	10
H44	305	4	99.0	16	6
H107	317	0	97.6	8	1
H114	367	6	98.7	12	6
H160	206	5	99.3	10	3
H201	339	3	97.8	23	8

Within the microscopy analysis a high number of taxa were represented by single pollen grain. Single pollen grains occur regularly with microscope analysis and are often responsible for the high levels of diversity. In total 55% of the taxa identified in H24 were represented by singletons and for samples H114 and H36 50% of their taxa diversity was represented by singletons (Table 4.4).

Scanning electron microscopy (SEM) was also performed on H24 following standard SEM protocol to further investigate the pollen grains found within this honey sample (Figure 4.4).

A total of 64 plant taxa from 40 plant families were recorded from the eleven honeys using melissopalynology (Figure 4.5). The most commonly occurring family detected across the honeys was the Rosaceae. Within Rosaceae, *Malus* was abundant in all eleven honeys and *Rubus* was recorded in 82% of the samples. Fabaceae was also detected in all the honey samples, with the exception of H107. Other important taxa are *Impatiens glandulifera* (64%) and *Brassica*/Brassicaceae (73%), which is likely to be oil seed rape (*Brassica napus*).

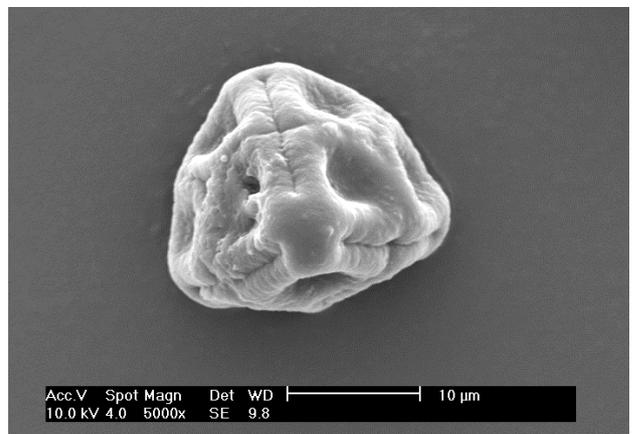
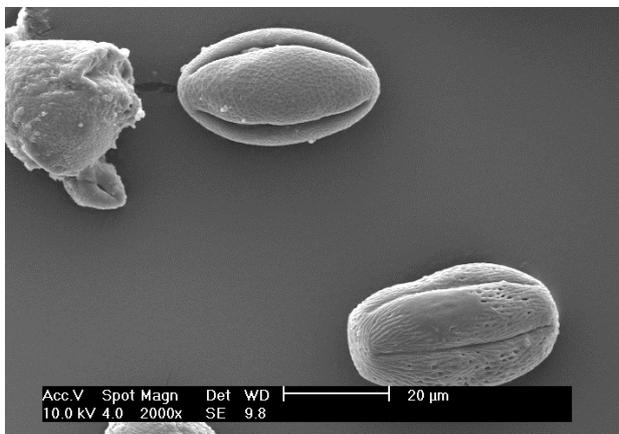
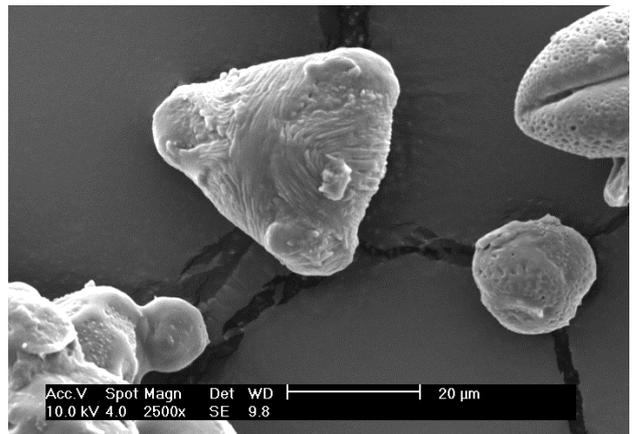
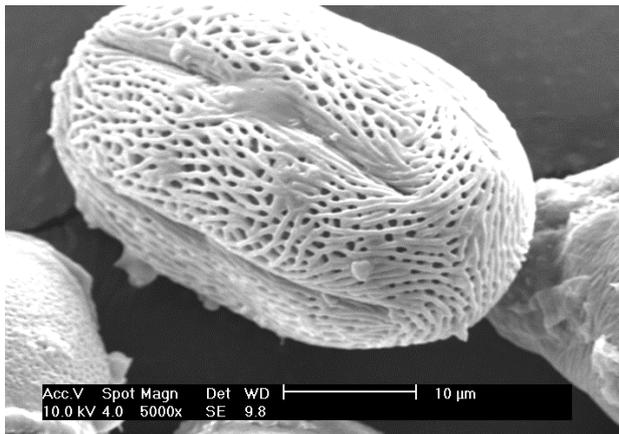
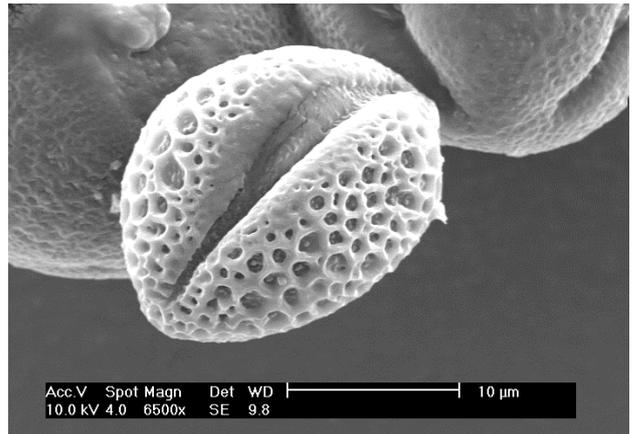
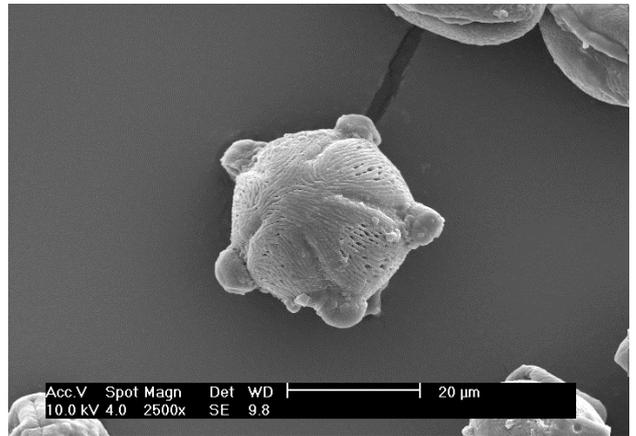
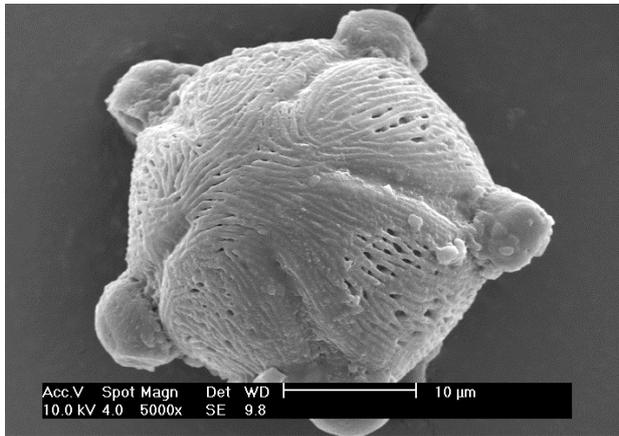


Figure 4. 4: SEM images of H24

Order	Family	Taxa	H23	H24	H25	H26	H31	H36	H44	H107	H114	H160	H201
<b>Apiales</b>	<b>Apiaceae</b>	<b>Apiaceae</b>	1				1	1					6
Apiales	Araliaceae	Hedera helix		1	1		5						
Aquifoliales	Aquifoliaceae	Ilex aquifolium		1					1				1
Asparagales	Amaryllidaceae	Allium		1									2
Asparagales	Asparagaceae	Hyacinthoides non-scripta			1				1				
Asparagales	Asparagaceae	Muscari		1									1
Asterales	Asteraceae	Bellis						5			1		
Asterales	Asteraceae	Cirsium	6										
Asterales	Asteraceae	Taraxacum		1	1			1	2				
Asterales	Campanulaceae	Campanula		1	1								
Asterales	Campanulaceae	Legousia											1
<b>Boraginales</b>	<b>Boraginaceae</b>	<b>Boraginaceae</b>		1									
Boraginales	Boraginaceae	Borago officinalis									1		
Boraginales	Boraginaceae	Echium		2									
Brassicales	Brassicaceae	Arabis											1
Brassicales	Brassicaceae	Brassica	71			6			137	101			
<b>Brassicales</b>	<b>Brassicaceae</b>	<b>Brassicaceae</b>	8		2	6	26	5		2		3	
Caryophyllales	Caryophyllaceae	Paronychia			2								
Dipsacales	Caprifoliaceae	Lonicera			1								
Dipsacales	Caprifoliaceae	Sambucus nigra	1		1								5
Dipsacales	Dipsacaceae	Knautia arvensis		1									
Ericales	Balsaminaceae	Impatiens glandulifera	1	5			5	94	19		166	6	
<b>Ericales</b>	<b>Ericaceae</b>	<b>Ericales</b>							2				1
Ericales	Ericaceae	Calluna vulgaris			1								
Ericales	Ericaceae	Erica			2								2
Fabales	Fabaceae	Acacia		1									
<b>Fabales</b>	<b>Fabaceae</b>	<b>Fabaceae</b>		5	1		5				1	15	6
Fabales	Fabaceae	Lotus		105			2	37	1		32		
Fabales	Fabaceae	Trifolium	174	14	12	138	61	20			70	157	17
Fagales	Betulaceae	Betula					1					1	
Fagales	Betulaceae	Corylus avellana			1				1				
Fagales	Fagaceae	Castanea sativa		34				114					
Fagales	Fagaceae	Quercus							2				
Lamiales	Lamiaceae	Lamium								2			
Lamiales	Oleaceae	Ligustrum		1	1								
Lamiales	Orobanchaceae	Pedicularis		1									
Lamiales	Plantaginaceae	Veronica		1									
Lamiales	Scrophulariaceae	Buddleja			2		1		4				4
Laurales	Lauraceae	Persea		1									
Malpighiales	Euphorbiaceae	Euphorbia		1									
Malpighiales	Euphorbiaceae	Mercurialis		1									
Malpighiales	Hypericaceae	Hypericum			9								
Malpighiales	Salicaceae	Salix		9	18		14	4	88	80	1	1	25
Malvales	Cistaceae	Cistus							1				
Malvales	Malvaceae	Tilia		1									
<b>Myrtales</b>	<b>Myrtaceae</b>	<b>Myrtaceae</b>	6		6	4	3		5		1		5
Myrtales	Onagraceae	Oenothera			2			1					
Pinales	Taxaceae	Taxus baccata						1					
<b>Poales</b>	<b>Poaceae</b>	<b>Poaceae</b>	3				2	1					
Ranunculales	Papaveraceae	Papaver		1	1		1						
Ranunculales	Ranunculaceae	Helleborus						1					
Ranunculales	Ranunculaceae	Ranunculus			1			1			1		1
<b>Rosales</b>	<b>Rhamnaceae</b>	<b>Rhamnaceae</b>		13									1
Rosales	Rosaceae	Filipendula	11		15	1	20	3			4	1	18
Rosales	Rosaceae	Malus	3	54	183	53	118	1	32	94	53	4	94
Rosales	Rosaceae	Potentilla					4						
Rosales	Rosaceae	Prunus			18				4	6			13
<b>Rosales</b>	<b>Rosaceae</b>	<b>Rosaceae</b>	2	54	42	6	13	21	1	31		3	114
Rosales	Rosaceae	Rubus	3	12	9	89	52	26			30	10	8
<b>Rosales</b>	<b>Urticaceae</b>	<b>Urticaceae</b>						1					
Sapindales	Rutaceae	Skimmia		3	19								9
Sapindales	Sapindaceae	Acer		4	1	1		1					1
Sapindales	Sapindaceae	Aesculus hippocastanum								1			
Saxifragales	Crassulaceae	Sedum		3									

Figure 4. 5: Summary of plant taxa detected through pollen analysis in eleven honey samples.

Number of pollen grains detected in each honey sample was recorded. Conditional formatting highlights the taxa detected; high (green) to low (yellow).

#### 4.4.2 Next Generation Sequencing - 454 analysis

The DNA from the ten honey samples were extracted and subjected to 454 sequencing. All of the honeys were repeatedly extracted 4 times and amplified using distinct adaptor tags. Sample H201 was not included in the 454 run as its antimicrobial properties had not yet been discovered. A total of 51,131 sequences over 250bp in length could be attributed to tagged sequences of *rbcL* using the 454 pyrosequencing approach (Table 4.5). Of these reads 47512 (93%) could be characterised to family, genus or species level. The number of taxa detected ranged from 5 (H31) to 24 (H24).

The percentage of successful reads characterised ranged from 86.2% (H24) to 95.5% (H44). One sample, H31, had a very low number of reads that were assigned to taxa (149), sequence quality was also slightly lower for H31 (Table 4.5). Of the other honeys the number of identifiable reads ranged from 3745 (H23) to 8097 (H44). With the exception of H31 the sequence quality were very similar with an average QV of 28. A minimum of 80% of reads had a QV greater than 20 across the ten honey samples (Table 4.5).

Table 4. 5: Summary of 454 results

Number of DNA sequences returned from 454 analysis and their quality, the number of sequences which could not be characterised and the subsequent % success rate from the total number of sequences obtained and the total number of different taxa.

Honey ID	Mean Length (SD)	Mean QV (SD)	Mean % reads with QV >20	Reads >250 bp	No. of unknown reads	No. identified to family, genus or species level (%)	No. of taxa detected
H23	385 (71)	28 (2)	84 (7)	3922	177	95.5	10
H24	389 (72)	28 (2)	84 (6)	4612	636	86.2	24
H25	385 (72)	28 (2)	83 (7)	5286	347	93.4	22
H26	387 (70)	28 (2)	83 (7)	4649	220	95.3	11
H31	328 (60)	26 (2)	80 (7)	168	19	88.7	5
H36	383 (71)	28 (2)	84 (6)	7575	724	90.4	21
H44	376 (71)	28 (2)	84 (6)	8564	467	94.5	18
H107	387 (70)	28 (2)	84 (6)	6862	357	94.8	18
H114	388 (71)	28 (2)	85 (6)	4325	123	97.2	12
H160	391 (69)	28 (2)	84 (6)	5168	549	89.4	19

The plant species identified within the ten different samples can be seen in figure 4.6. A total of 48 plant taxa from 25 plant families were recorded from the ten honeys using 454 pyrosequencing (Figure 4.6). The most commonly occurring family detected across the honeys was the Rosaceae family. Rosaceae *Rubus* was detected in 90% of the samples and *Filipendula* in 80%. The Fabaceae family was also detected in all the honey samples, with *Trifolium* occurring in 90% of the honeys. The Asteraceae family was more frequently detected in DNA analysis than

microscopy, being found in seven honey samples compared to four with microscopy. *Taraxacum officinale* was the most important species detected within this family. *Impatiens glandulifera* (40%) and *Brassica/ Brassicaceae* (70%) were also detected.

Order	Family	Taxa	H23	H24	H25	H26	H31	H36	H44	H107	H114	H160
Apiales	Apiaceae	Angelica sylvestris						47				
<b>Apiales</b>	<b>Apiaceae</b>	<b>Apiaceae</b>	13									
Apiales	Araliaceae	Hedera helix									14	
Asparagales	Asparagaceae	Hyacinthoides non-scripta		476	127				12	63		
Asparagales	Asparagaceae	Muscari		122								
<b>Asterales</b>	<b>Asteraceae</b>	<b>Asteraceae</b>	30	42	89			177	529		40	
Asterales	Asteraceae	Cirsium	33					234			46	
Asterales	Asteraceae	Hypochaeris radicata						194				
Asterales	Asteraceae	Senecio							1397			
Asterales	Asteraceae	Sonchus		16	10				17			
Asterales	Asteraceae	Taraxacum		324	569			94	1940	67		
Brassicales	Brassicaceae	Brassica	2777	271		644		474	20	195		728
<b>Brassicales</b>	<b>Brassicaceae</b>	<b>Brassicaceae</b>		76								28
<b>Caryophyllales</b>	<b>Caryophyllaceae</b>	<b>Caryophyllaceae</b>							70			
Ericales	Balsaminaceae	Impatiens glandulifera						2974	12		327	32
<b>Fabales</b>	<b>Fabaceae</b>	<b>Fabaceae</b>		10	16	16		40	16		24	1489
Fabales	Fabaceae	Lotus						95				
Fabales	Fabaceae	Trifolium	679	751	235	1227	46	615	12		2191	1799
Fabales	Fabaceae	Ulex	18	18						93		14
Fagales	Betulaceae	Alnus glutinosa				522						
Fagales	Betulaceae	Corylus avellana				340						
Fagales	Fagaceae	Castanea sativa		36	24			136	172	33	64	
<b>Fagales</b>	<b>Fagaceae</b>	<b>Fagaceae</b>		19	85			32	110	53		
Fagales	Fagaceae	Quercus		404	944			424	3715	1129	31	81
Gentianales	Rubiaceae	Galium		682								
Lamiales	Oleaceae	Ligustrum						186				10
Malpighiales	Hypericaceae	Hypericum						129				
Malpighiales	Salicaceae	Salix		10	86				12	168		20
Malpighiales	Violaceae	Viola				236						15
Myrtales	Onagraceae	Chamerion angustifolium	71		98			99				
Myrtales	Onagraceae	Oenothera						12				
Pinales	Cupressaceae	Juniperus								30		
Pinales	Pinaceae	Pinus								64		
<b>Poales</b>	<b>Poaceae</b>	<b>Poaceae</b>			55							30
Polypodiales	Woodsiaceae	Athyrium				1038						
Ranunculales	Ranunculaceae	Ranunculus							10			
Rosales	Rosaceae	Crataegus		62	138				12	683		10
Rosales	Rosaceae	Filipendula	37	25	191	82	15	173			12	81
Rosales	Rosaceae	Malus		31	28	12	12		14	775		16
Rosales	Rosaceae	Prunus		43	1019					953		20
Rosales	Rosaceae	Rosa	11	22	11	17		26			50	13
<b>Rosales</b>	<b>Rosaceae</b>	<b>Rosaceae</b>		155	545		10	13	27	1564		26
Rosales	Rosaceae	Rubus	76	353	485	295	66	677		10	1295	190
Rosales	Rosaceae	Sorbus		17	31					28		
Sapindales	Rutaceae	Skinmia		11								
Sapindales	Sapindaceae	Acer			12					564		17
Sapindales	Sapindaceae	Aesculus hippocastanum			141					33		
Solanales	Solanaceae	Solanum									108	

Figure 4. 6: Summary of plant taxa detected through 454 analysis in ten honey samples.

Number of sequences obtained from each honey sample was recoded. Conditional formatting highlights the taxa detected; high (green) to low (yellow).

Another interesting observation was the presence of non-flowering plants detected with the DNA analysis, but not the microscopy. *Juniperus* and *Pinus* were detected in H107 and DNA from the fern *Athyrium* was found in high levels in honey H26. The DNA detected from these species may

be from pollen and spores, but may also be from other plant material which could provide an explanation for their absence from microscopic analysis.

H24 was the honey sample which demonstrated the highest levels of non-peroxide activity of all of the samples when antimicrobial screening was performed. The analysis of H24 revealed the dominance of three taxa; woodruff (*Galium odoratum*), bluebell (*Hyacinthoides non-scripta*) and dandelion (*Taraxacum officinale*).

#### **4.4.3 Next Generation Sequencing - Illumina Results**

The Illumina 16S Metagenomic Sequencing Library Preparation protocol using adapted *rbcL* primers was used to analyse the DNA extracted from 19 honey samples (Illumina, 2013). The analysis of the honeys samples were repeated at least twice. Results of the repeats were combined to give a detailed list of the plants detected using the Illumina approach.

A summary of the ten honey samples (Table 4.1) can be seen in figure 4.7. The H31 sample was not included due to low level of amplification success which was achieved during the 454 analysis suggesting that insufficient DNA was present.

A total of 659780 sequences over 250 bp in length could be attributed to tagged sequences of *rbcL* using the Illumina sequencing approach (Table 4.6). Of these reads 572077 (87%) could be characterised to family, genus or species level. The percentage of successful reads characterised ranged from 79.6% (H24) to 100% (H44) across the ten honey samples. Two samples, H25 and H44, had a very low number of reads that were assigned to taxa; 3848 and 5021 respectively. The number of taxa detected for both H25 and H44 were subsequently low, with only 4 and 2 taxa characterised, these two samples were considered to have failed. Of the other honeys the number of identifiable reads ranged from 25,446 (H114) to 171,708 (H160) and the number of taxa detected ranged from 14 (H107) to 23 (H26).

Table 4. 6: Summary of Illumina results

Number of DNA sequences returned from Illumina analysis, the number of sequences which could not be characterised and the subsequent % success rate from the total number of sequences obtained and the total number of different taxa.

Honey ID	Reads >250 bp	No. of unknown reads	No. identified to family, genus or species level (%)	No. of taxa detected
H23	48846	9970	79.6	19
H24	38174	1783	95.3	18
H25	3935	87	97.8	4
H26	117503	25458	78.3	23
H36	34529	2433	93.0	15
H44	5021	0	100.0	2
H107	88426	12705	85.6	14
H114	25701	255	99.0	13
H160	204173	32465	84.1	21
H201	93472	2547	97.3	15

The plant species identified within the ten different samples can be seen in figure 4.7. A total of 53 plant taxa from 23 plant families were recorded from the ten honeys using Illumina NGS. Within the Asteraceae family *Taraxacum officinale* was detected in 90% of the samples. The Rosaceae and Fabaceae families were commonly detected across the honeys; *Prunus* and *Trifolium* were detected in 80% of the honeys. *Impatiens glandulifera* (30%) and Brassicaceae/*Brassica* (60%) were also detected.

As previously described, H24 and H201 were the honey samples which demonstrated the highest levels of non-peroxide activity of all of the samples with the antimicrobial screening assay. The analysis of H24 and H201 revealed the dominance of dandelion (*Taraxacum*) in both honey samples.

Order	Family	Taxa	H23	H24	H25	H26	H36	H44	H107	H114	H160	H201
Asparagales	Amaryllidaceae	Allium				90						
Asparagales	Asparagaceae	Hyacinthoides non-scripta		440								
Asparagales	Asparagaceae	Scilla				1245						
Asterales	Asteraceae	Bellis										5473
Asterales	Asteraceae	Centaurea	97	486			276					
Asterales	Asteraceae	Cirsium	65	13		400	66					17
Asterales	Asteraceae	Crepis				130						116
Asterales	Asteraceae	Hypochaeris					5075					3885
Asterales	Asteraceae	Lactuca										111
Asterales	Asteraceae	Leucanthemum				12						
Asterales	Asteraceae	Solidago	40			131	34					
Asterales	Asteraceae	Taraxacum officinale	7321	17722	11	41693	98		663	1569	519	55223
Brassicales	Brassicaceae	Brassica				725	43		25	61	1969	
Brassicales	Brassicaceae	Brassica napus			83	1346	3245		5449	126	11700	
Brassicales	Brassicaceae	Brassica oleracea				103			604	48	21079	
Brassicales	Brassicaceae	Erysimum					23					
Cornales	Hydrangeaceae	Philadelphus										61
Dipacales	Adoxaceae	Sambucus / Viburnum	99	41		548			16		334	
Ericales	Balsaminaceae	Impatiens glandulifera	13665				12127			2098		
Ericales	Ericaceae	Calluna vulgaris					2754					
Escalloniales	Escalloniaceae	Escallonia	28			38						
Fabales	Fabaceae	Fabaceae	10								29372	
Fabales	Fabaceae	Genista										337
Fabales	Fabaceae	Lathyrus				3211						
Fabales	Fabaceae	Ononis	163								201	
Fabales	Fabaceae	Trifolium	33	14		138	23	28		941	2180	727
Fabales	Fabaceae	Trifolium pratense								1100		3204
Fabales	Fabaceae	Trifolium repens	2156	779		1527	2666	4993		10616	91206	9152
Fabales	Fabaceae	Ulex	1408	1251								4432
Fabales	Polygalaceae	Polygala		24								
Fagales	Fagaceae	Castanea sativa	67				98					
Fagales	Fagaceae	Quercus	32	10							36	
Lamiales	Lentibulariaceae	Pinguicula?										2652
Laurales	Lauraceae	Persea				76			13			
Liliales	Liliaceae	Lilium				106						
Magnoliales	Magnoliaceae	Magnolia							15			
Malpighiales	Hypericoideae	Hypericum	25									
Malpighiales	Salicaceae	Salix	3559						4364			
Poales	Poaceae	Agrostis capillaris									30	
Poales	Poaceae	Arrhenatherum elatius									53	
Poales	Poaceae	Festuca								44	37	
Poales	Poaceae	Holcus lanatus								1043		
Polypodiales	Athyriaceae	Athyrium filix-femina				902						
Rosales	Rosaceae	Cotoneaster		26					2119		169	
Rosales	Rosaceae	Crataegus monogyna		7155					17998		238	
Rosales	Rosaceae	Filipendula ulmaria		165	27						780	128
Rosales	Rosaceae	Malus		206		209			31182		723	
Rosales	Rosaceae	Prunus	8522	1781	3727	23550			11712	65	40	5407
Rosales	Rosaceae	Rosa	18	128		1178	75			232	549	
Rosales	Rosaceae	Rubus fruticosus	1568	6124		13869	5493			7503	10300	
Rosales	Rosaceae	Sorbus		26					601		193	
Sapindales	Sapindaceae	Acer							960			
Saxifragales	Saxifragaceae	Heuchera / Astilbe				818						

Figure 4. 7: Summary of plant taxa detected through Illumina analysis in ten honey samples.

Number of sequences obtained from each honey sample was recorded. Conditional formatting highlights the taxa detected; high (green) to low (yellow).

#### 4.4.4 Comparison of Melissopalynology, 454 and Illumina

The aim was to compare the sensitivity and discriminatory power of the three methods. In total, seven honeys from table 4.1 were analysed using all three techniques. Of these seven honeys 98% of the pollen grains were characterised using melissopalynology to family, genus or species level. A high percentage of sequences could be identified with the two metabarcoding techniques; 93% and 91% for 454 and Illumina respectively. This marginally lower percentage attributed to

Illumina was due to the data processing of the results. The ‘unknown’ taxa from the 454 analysis were manually analysed and characterised to family level if two or more species were identified from the same family (highlighted in grey in figure 4.7). Due to the large amounts of data obtained from the Illumina analysis this was not feasible, so the number of ‘unknown’ taxa was subsequently slightly higher.

A number of the taxa detected using microscopy but not DNA analysis were represented by single pollen grains. Within H24, 15 different taxa were represented by single pollen grains which were not detected using the DNA barcoding techniques. Using microscopy 55% of the taxa identified in H24 were represented by lone pollen grains (Figure 4.7). 50% for the taxa detected in samples H114 and H36 respectively were represented by singletons which were not detected using the two DNA techniques.

#### 4.4.4.1 The Taxa Similarity across the Melissopalynology, 454 and Illumina

To allow a fair comparison, from this point onwards, any family level categorisation and taxa represented by lone pollen grains with microscopy were removed. This was done as the analysis of the Illumina results did not involve the categorisation of unknown taxa to family level, by removing these results a comprehensive comparison can be made. Singletons are responsible for a low proportion of the pollen grains within a honey sample and DNA metabarcoding is not suitable for detecting the taxa present in low levels, lone pollen grains were subsequently removed for fair analysis across the three techniques. The results obtained from the seven samples which had been analysed using all three methods were compared (Table 4.7). The number of taxa in the microscope analysis now ranged from 4 (H26 and H160) to 11 (H24). The number of taxa detected ranged from 8 (H23) to 19 (H24) for 454 and 13 (H114) to 23 (H26) for Illumina (Table 4.7).

*Table 4. 7: Summary of counts and number of taxa detected from seven honey samples using melissopalynology, 454 and Illumina*

<b>Honey ID</b>	<b>No. of pollen grains</b>	<b>No. of reads 454</b>	<b>No. of reads illumina</b>	<b>No. of Taxa microscopy</b>	<b>No. of taxa 454</b>	<b>No. of taxa Illumina</b>
H23	268	3702	38866	6	8	18
H24	245	3674	36391	11	19	18
H26	286	4413	92045	4	10	23
H36	303	6589	32096	8	17	15
H107	283	4888	75721	5	16	14
H114	355	4138	25446	6	10	13
H160	177	3046	142336	4	15	20

Order	Family	Taxa	H23			H24			H26			H36			H107			H114			H160				
			M	R	I	M	R	I	M	R	I	M	R	I	M	R	I	M	R	I	M	R	I		
Apiales	Apiaceae	Angelica sylvestris											0.71												
Apiales	Araliaceae	Hedera helix																0.34							
Asparagales	Amaryllidaceae	Allium								0.10															
Asparagales	Asparagaceae	Hyacinthoides non-scripta					12.96	1.21								1.29									
Asparagales	Asparagaceae	Muscari					3.32																		
Asparagales	Asparagaceae	Scilla									1.35														
Asterales	Asteraceae	Bellis											1.65												
Asterales	Asteraceae	Centaurea			0.25										0.86										
Asterales	Asteraceae	Cirsium	2.24	0.89	0.17								0.43	3.55	0.21			1.11							
Asterales	Asteraceae	Crepis											0.14												
Asterales	Asteraceae	Hypochaeris												2.94	15.81										
Asterales	Asteraceae	Leucanthemum													0.01										
Asterales	Asteraceae	Solidago			0.10										0.14										
Asterales	Asteraceae	Sonchus					0.44																		
Asterales	Asteraceae	Taraxacum officinale				18.84	8.82	48.70				45.30	1.43	0.31		1.37	0.88				6.17			0.36	
Boraginales	Boraginaceae	Echium				0.82																			
Brassicales	Brassicaceae	Brassica	26.49	75.01			7.38		2.10	14.59	0.79		7.19	0.13	35.69	3.99	0.03			0.24		23.90	1.38		
Brassicales	Brassicaceae	Brassica napus									1.46			10.11			7.20			0.50				8.22	
Brassicales	Brassicaceae	Brassica oleracea									0.11					0.80			0.19					14.81	
Brassicales	Brassicaceae	Erysimum																							
Dipacales	Adoxaceae	Sambucus / Viburnum			0.25			0.11																0.23	
Ericales	Balsaminaceae	Impatiens glandulifera				35.16	2.04						31.02	45.14	37.78						46.76	7.90	8.24	3.39	1.05
Ericales	Ericaceae	Calluna vulgaris													8.58										
Escalloniales	Escalloniaceae	Escallonia			0.07																				
Fabales	Fabaceae	Lathyrus																							
Fabales	Fabaceae	Lotus				42.86							12.21	1.44						9.01					
Fabales	Fabaceae	Ononis			0.42																			0.14	
Fabales	Fabaceae	Trifolium	64.93	18.34	0.08	5.71	20.44	0.04	48.25	27.80	0.15	6.60	9.33	0.07						19.72	52.95	3.70	88.70	59.06	1.53
Fabales	Fabaceae	Trifolium pratense																							
Fabales	Fabaceae	Trifolium repens																					4.32		
Fabales	Fabaceae	Ulex		0.49	3.62		0.49	3.44									1.90						0.46		
Fabales	Polygalaceae	Polygala						0.07																	
Fagales	Betulaceae	Alnus glutinosa									11.83														

Figure 4.8: Summary of plant taxa detected through melissopalynology (M), 454 (R) and Illumina (I) analysis in nine honey samples

Converted to percentage of total pollen grain count or number of sequences. Conditional formatting highlights the taxa detected; high (green) to low (yellow).



The taxa similarity across the melissopalynology, 454 and Illumina was compared for the seven honey samples (Table 4.8). The level of similarity between microscopy and 454 ranged from 24-56%. Between 454 and Illumina it ranged from 21-48% (Table 4.8). The lowest level was seen between microscopy and Illumina with similarity ranging from 12-27%, these two techniques are the furthest apart in the level of discrimination possible. Many plants were characterised to family level with microscopy whereas many different plant families were characterised to species level with Illumina, resulting in a divergence of results. DNA methods generate a greater degree of discrimination and it is subsequently difficult to directly compare with microscopy. The similarity across the three techniques subsequently ranged from 10-17% (Table 4.8).

Table 4. 8: Comparison of the three different techniques

The number of taxa which matched for 2/3 of the techniques, and the % similarity. The number of taxa detected in all three techniques and the overall % similarity

Number of matches detected	H23	H24	H25	H26	H36	H114	H160
Microscopy and 454	5	6	4	4	6	4	4
No. of taxa	9	24	17	10	19	12	15
% similarity	56	25	24	40	32	33	27
454 and Illumina	5	12	8	6	9	4	9
No. of taxa	21	25	22	27	23	19	26
% similarity	24	48	36	22	39	21	35
Microscopy and Illumina	3	3	4	4	4	3	3
No. of taxa	21	26	15	23	19	16	21
% similarity	14	12	27	17	21	19	14
Microscopy, 454 and Illumina	3	3	4	4	4	3	3
No. of taxa	22	30	23	27	25	21	26
% similarity	14	10	17	15	16	14	12

Although there are differences in the taxa found between the techniques, the dominant floral components of the honeys do appear to be detected with all three methods although the proportions of them differ considerably (Figure 4.8). Dominant plant families showed good levels of similarity. There are 26 taxa that appear with >20 % abundance across all of the honey samples using both DNA metabarcoding and microscopy (indicated with a red outline in figure 4.8). Of these 27 taxa 14 are found using all three methods, at genus level, giving 52% correspondence. Five of these taxa were found in 2/3 of the techniques increasing the correspondence to 70%.

Eight taxa of high abundance are represented by one technique; for two of these taxa 42% and 64% of sequences in H114 and H160 respectively were characterised as *Trifolium repens* (species level), whereas microscope and 454 analysis has lower discrimination and characterised high levels of *Trifolium* (genus level). This highlights the higher level of discrimination of the Illumina

technique. One of these matches was DNA from a fern that would not have been detected in the microscopic analysis. The remaining 5 taxa of high abundance did not match and were only detected in one of the three techniques. The proportion of the taxa within the samples does not correlate a taxa with high abundance using one method may be detected using the other methods but at much lower level. After Spearman's Correlation Analysis and Bonferroni correction for multiple testing only one honey (H23), showed a significant correlation between the proportions of taxa found using microscopy and 454 analysis ( $r = 0.722$ ;  $p < 0.001$ ).

Honeys are described as monofloral if they contain more than 45% pollen from a single species. Of the seven honeys, only one (H160) would be considered monofloral (over 45% clover) if the classification was based on the results of all three methods. In some cases the classification varied depending on method of analysis. Based on Illumina results sample H24 would be classed as monofloral for dandelion. While H114 would be categorised as clover honey based on 454 results it would be classed as Himalayan balsam based on microscopy. Sample H23 would be categorised as oil seed rape based on 454 results but clover honey based on microscopy. Sample H36 is Himalayan balsam according to 454 and clover according to microscopy while H26 is mostly dandelion based on Illumina but clover based on microscopy. These findings suggest a plant profile can be produced, but proportion analysis would require further investigating.

#### **4.4.4.2 Discrimination between Melissopalynology, 454 and Illumina Techniques**

The level and type of discrimination also varies between the three methods. Table 4.9 shows the number of taxa that are detected within each plant family that is represented by multiple taxa, looking across the results of the seven compared honey samples. Of these families Illumina metabarcoding identifies a greater number of taxa for the top five families. For example, within the Asteraceae Illumina DNA detects seven taxa whilst 454 detects four and microscopy finds two. For the Rosaceae family Illumina metabarcoding detects eight taxa whilst 454 detects seven and microscopy detects four. In one family (Betulaceae) 454 detects two taxa but this family was not seen with microscopy and Illumina.

*Table 4. 9: Plant family analysis*

*Number of taxa detected within the families with more than 1 taxa across all seven honey samples. The total number of taxa characterised across the three techniques within each family.*

Order	Family	Total no. of taxa	No. of taxa detected in the family		
			Microscopy	DNA	Illumina
Asterales	Asteraceae	9	2	4	7
Rosales	Rosaceae	8	4	7	8
Fabales	Fabaceae	7	2	3	6
Brassicales	Brassicaceae	4	1	1	4
Poales	Poaceae	4	0	0	4
Fagales	Betulaceae	2	0	2	0
Fagales	Fagaceae	2	1	2	2
Myrtales	Onagraceae	2	0	2	0
Sapindales	Sapindaceae	2	1	2	1
Asparagales	Asparagaceae	3	1	2	2

#### **4.4.4.3 Repeat Sampling Using DNA Metabarcoding and Melissopalynology**

Two of the samples included in the analysis, H24 and H25, were obtained from the same batch of honey enabling the reproducibility of the different analysis methods to be assessed. Only 4 taxa were identified in the Illumina analysis of H25, suggesting the repeat did not amplify successfully. This would need to be repeated to confirm whether it was an experimental error or a consistent finding, subsequently the Illumina results were not included in the comparison.

Melissopalynology and 454 data were compared and a plant profiles of the two repeats (H24 and H25) were produced (Figure 4.9). To compare results effectively singletons and family groups were again removed. 454 DNA analysis detected slightly higher numbers of taxa in each sample, the 454 DNA found 19 and 17 taxa whilst the microscopy found 11 and 12 taxa (Table 4.10). The repeatability of the taxa found was much higher for the 454 DNA metabarcoding compared to the microscope analysis. There was 63% match of the taxa found using DNA metabarcoding compared to 28% with melissopalynology (Table 4.10). Highlighting the reproducibility of the metabarcoding technique compared to microscopy.

*Table 4. 10: Comparison between microscopy and 454 replicated of honey sample H24*

*The number of pollen grains counted or sequences obtained, the number of taxa characterised to Order, family and taxa level across the sample, the number of taxa detected within and shared across the samples and subsequently the % similarity of taxa within the replicates of the two repeats.*

	<b>H24 Microscopy</b>	<b>H25 Microscopy</b>	<b>H24 454</b>	<b>H25 454</b>
Number of reads	245	298	3974	4149
Number of taxa	11	12	19	17
Number of taxa within DNA & microscopy replicates	18		22	
Number of taxa shared within DNA & microscopy replicates	5		14	
Similarity within replicates for DNA & microscopy (%)	28%		63%	

Order	Family	Taxa	Roche 454		Microscopy	
			H24	H25	H24	H25
Apiales	Araliaceae	Hedera helix				
Aquifoliales	Aquifoliaceae	Ilex aquifolium				
Asparagales	Amaryllidaceae	Allium				
Asparagales	Asparagaceae	Hyacinthoides non-scripta	476	127		
Asparagales	Asparagaceae	Muscari	122			
Asterales	Asteraceae	Sonchus	16	10		
Asterales	Asteraceae	Taraxacum	324	569		
Asterales	Campanulaceae	Campanula				
Boraginales	Boraginaceae	Echium			2	
Brassicales	Brassicaceae	Brassica	271			
Caryophyllales	Caryophyllaceae	Paronychia				2
Dipsacales	Caprifoliaceae	Lonicera				
Dipsacales	Caprifoliaceae	Sambucus nigra				
Dipsacales	Dipsacaceae	Knautia arvensis				
Ericales	Balsaminaceae	Impatiens glandulifera			5	
Ericales	Ericaceae	Calluna vulgaris				
Ericales	Ericaceae	Erica				3
Fabales	Fabaceae	Acacia				
Fabales	Fabaceae	Lotus			105	
Fabales	Fabaceae	Trifolium	751	235	14	13
Fabales	Fabaceae	Ulex	18			
Fagales	Betulaceae	Corylus avellana				
Fagales	Fagaceae	Castanea sativa	36	24	34	
Fagales	Fagaceae	Quercus	404	944		
Gentianales	Rubiaceae	Galium	682			
Lamiales	Oleaceae	Ligustrum				
Lamiales	Orobanchaceae	Pedicularis				
Lamiales	Plantaginaceae	Veronica				
Lamiales	Scrophulariaceae	Buddleja				2
Laurales	Lauraceae	Persea				
Malpighiales	Euphorbiaceae	Euphorbia				
Malpighiales	Euphorbiaceae	Mercurialis				
Malpighiales	Hypericaceae	Hypericum				9
Malpighiales	Salicaceae	Salix	10	86	9	18
Malvales	Malvaceae	Tilia				
Myrtales	Onagraceae	Chamerion angustifolium		98		
Myrtales	Onagraceae	Oenothera				2
Ranunculales	Papaveraceae	Papaver				
Ranunculales	Ranunculaceae	Ranunculus				
Rosales	Rosaceae	Crataegus	62	138		
Rosales	Rosaceae	Filipendula	25	191		15
Rosales	Rosaceae	Malus	31	28	54	183
Rosales	Rosaceae	Prunus	43	1019		18
Rosales	Rosaceae	Rosa	22	11		
Rosales	Rosaceae	Rubus	353	485	12	9
Rosales	Rosaceae	Sorbus	17	31		
Sapindales	Rutaceae	Skimmia	11		3	19
Sapindales	Sapindaceae	Acer		12	4	
Sapindales	Sapindaceae	Aesculus hippocastanum		141		
Saxifragales	Crassulaceae	Sedum			3	

Figure 4. 9: List of taxa detected for replicate analyses of honey sample H24 and H25 using DNA metabarcoding and melissopalynology.

Number of pollen grains or sequences obtained from each honey sample was recorded. Conditional formatting highlights the taxa detected; high (green) to low (yellow).

## 4.5 Discussion

Understanding the floral composition of honey has many applications, this study utilises DNA metabarcoding which targets the universal *rbcL* region and next generation sequencing (NGS) technology for floral characterisation. This novel application allows a comparison to be made between melissopalynology, 454 and Illumina techniques. The floral profile of H24, H25 and H201 was determined to characterise the plants which contribute to the making of these active honey samples.

Pollen grains vary in shape, size and pollen wall composition and this is likely to effect DNA extraction efficiency for different species (Borg and Twell, 2011). Pollen counts were performed and the honey samples with high levels of pollen were the samples which showed novel antibacterial activity in chapter 3 (Table 3.8). In this study successful DNA extraction was performed which allowed PCR amplification to be performed. The DNA analysed for DNA metabarcoding often comes from plastid markers, such as *rbcL*, *trnL* or *psbA-trnH* (Valentini *et al.*, 2010, Hiiesalu *et al.*, 2012, Kajtoch, 2014, Galimberti *et al.*, 2014, Bruni *et al.*, 2015, Kraaijeveld *et al.*, 2015). Within most plant species chloroplasts are maternally inherited but the pollen grain contains plastids within the vegetative cell that can be targeted. The number of plastids varies with different species and also the maturity of the pollen grain (Borg and Twell, 2011). Nevertheless studies on DNA extracted from pollen have shown excellent ability to amplify plastid markers over a wide range of species confirming the presence of sufficient DNA (Valentini *et al.*, 2010, Galimberti *et al.*, 2014, Bruni *et al.*, 2015, Kraaijeveld *et al.*, 2015).

Once the DNA is extracted PCR biases can lead to some taxa being preferentially amplified (Shokralla *et al.*, 2012). In order to minimise this it is important to use a marker with a high degree of universality across a broad range of taxonomic groups (Taberlet *et al.*, 2012). The *rbcL* DNA barcode marker was used as it has been shown to have the highest universality of all markers that have been proposed for DNA barcoding plants (CBOL Plant Working Group *et al.*, 2009). De Vere *et al.*, (2012) have shown that the *rbcL* primers used in the current study can amplify 98% of the Welsh flora (de Vere *et al.*, 2012). However even with high universality of primers when working on species in isolation, amplification may still be skewed within a multi-template PCR (Pompanon *et al.*, 2012). Some species, especially those in low quantities can be missed out when a mixed sample is amplified (Hajibabaei *et al.*, 2011, Gibson *et al.*, 2014). This is illustrated within the current study where species recorded using microscopy from a single pollen grain are less likely to be detected using DNA metabarcoding. Sequence errors arising during amplification and DNA sequencing will also reduce the ability to correctly assign samples (Coissac *et al.*, 2012).

The Illumina MySeq platform utilised the use of 2x 300 bp kits meaning that the entire *rbcL* barcode region can be amplified with far greater depth and reduced error rates compared to the

454 approach (Luo *et al.*, 2012). This helped reduce some of the limitations of 454 pyrosequencing. Techniques are also being developed that avoid the PCR stage altogether, such as using shotgun sequencing with subsequent recovery of DNA barcode markers or even whole chloroplasts (Taberlet *et al.*, 2012, Ji *et al.*, 2013, Kane *et al.*, 2012, Tang *et al.*, 2014).

Melissopalynology, 454 and Illumina are three effective methods for characterising pollen found within honey samples. Plant profiles were produced for seven honeys using the three techniques. When comparing these seven honeys the plants characterised had a broad taxonomic range, covering 83 taxa from 45 families and 28 orders, with each honey containing between 8 to 31 taxa. Although a wide range of taxa were observed a fairly small number were consistently found in the different honey samples. Some of these frequently occurring species, such as *Taraxacum officinale* (dandelion), *Trifolium* (clover) and *Rubus fruticosus* (bramble) are common UK native plants frequently found in gardens, grasslands and waste ground. *Brassica* is frequently seen and is likely to be either oil seed rape (*Brassica napus*) or garden *Brassica* species grown for food. The non-native invasive species *Impatiens glandulifera* (Himalayan balsam) is also often recorded.

Non-flowering species with juniper and pine were recorded in one honey sample. This may reflect honey bees collecting resin from these conifers in order to make propolis (Wilson *et al.*, 2013). Resin is actively collected from a range of species and combined with wax to make propolis that is deposited within the hive as it has antimicrobial properties (Wilson *et al.*, 2013). Another possibility is that the presence of DNA of conifers as well as from some of the other anemophilous species is due to bees foraging on honeydew. Honey bees collect the exudate from sap-sucking insects as an alternative to nectar. Honeydew collection is often recorded from sap feeding insects feeding on conifers and other anemophilous species (Oddo *et al.*, 2004). An unusual taxon recorded here is DNA belonging to the fern *Athyrium*. This occurs in just one honey sample so could potentially be considered as an anomalous result, but *Athyrium* was also recorded in a commercial honey previously analysed using DNA barcoding (Valentini *et al.*, 2010).

The abundant floral constituents of the antibacterial honeys H24, detected in chapter 3 (Table 3.8), were determined using 454 analysis. *Taraxacum officinale* (dandelion) appeared in high abundance in the majority of the samples tested. Whereas *Galium odoratum* (woodruff) and *Hyacinthoides non-scripta* (bluebell) were found in H24 and they did not feature highly in any of the other honey samples tested. Dandelion belongs to a large genus of perennial herbaceous plants in the family Asteraceae. Dandelion is a common flowering plant which has long been exploited for its medicinal properties (Dias *et al.*, 2014, Schütz *et al.*, 2006). Dandelion extracts have shown high level of antimicrobial activity against many organisms including *B. subtilis* and *E. coli* (Izzo *et al.*, 1995). High levels of dandelion was also detected in H201, the other honey sample with

high level of antibacterial activity in chapter 3, through Illumina analysis and melissopalynology. Woodruff is a flowering perennial plant commonly found in woodland. Extracts from woodruff are known to have wound healing potential, antioxidant and antibacterial activities (Kahkeshani *et al.*, 2013, Izzo *et al.*, 1995). Bluebell is another perennial flowering plant commonly found in hedgerows and woodland. The antibacterial activity of *Hyacinthaceae* extracts has been recently investigated and bluebell is known to be toxic to livestock (Mulholland *et al.*, 2013). These plants are further investigated in chapter 5.

All three techniques detect the dominant constituents of honey but showed differences in some of the taxa and the proportions of these found within the honey sample. This is not surprising as honey is a highly heterogeneous natural product and the sampling strategy adopted for the two methods is different. Melissopalynology uses a starting sample of 2 g of honey whilst the DNA methods adopted here used 40 g of honey in total.

DNA metabarcoding and melissopalynology showed little correspondence in the proportions of different taxa found within honey samples. As a direct count of the number of pollen grains present, melissopalynology provides a more quantitative measure, albeit with a smaller sample size. The inability of DNA metabarcoding to provide quantitative results has been observed in a range of studies (Pompanon *et al.*, 2012, Ji *et al.*, 2013, Kraaijeveld *et al.*, 2015, Richardson *et al.*, 2015). There are many stages in the DNA analysis process where biases can occur that will prevent a quantitative estimation of floral composition.

Differences in the identity of taxa detected reflect biases within both the DNA metabarcoding and microscopic analysis. For some families, for example the Rosaceae and Asteraceae, DNA metabarcoding provides a higher level of resolution compared to melissopalynology. For other families such as the Asparagaceae DNA metabarcoding appears less able to detect species within these groups. A key factor in the ability to identify species using DNA metabarcoding is the quality of the reference database that unknown species are compared to (Taberlet *et al.*, 2012). The Welsh native flora DNA barcoded database was utilised in this study for comprehensive analysis (de Vere *et al.*, 2012). This library is not complete however as all of the non-native and garden flora have not been DNA barcoded meaning that identifications for these species rely on the availability of these groups in GenBank.

Groundtruthing using known distributions of plant species for the geographic area being sampled within can help to improve discrimination (de Vere *et al.*, 2012). The methods used here filters the BLAST results generated so that only plants found growing within the UK, whether native, alien or in horticulture or agriculture are returned.

Melissopalynology as a method for characterising the floral composition of honey also has limitations. Some plant groups are known to be difficult to distinguish and some plants can only be taken to the level of family due to lack of morphological differences in the pollen grains, for example the Poaceae (Kraaijeveld *et al.*, 2015, Galimberti *et al.*, 2014). The major limitation to the microscopic investigation of honey however is the high degree of expertise required to identify the different pollen types. The time associated with identifying each pollen grain puts limitations on the amount of sample that can be screened. Microscopic analysis provides a direct quantification of the number of pollen grains but because only a limited number of pollen grains can be processed it means the coverage of the honey is low.

The repeat sampling of honey H24 and H25 reflects the variability of sampling. Each repeat represents a new sample from the same hive extraction. DNA metabarcoding has a much higher reproducibility with 63% similarity compared to 28% for melissopalynology. This is likely to be a reflection of the greater amount of pollen that can be investigated using the DNA metabarcoding approach.

The value of DNA metabarcoding is that a higher volume of honey can be investigated and the technique does not require the high level of taxonomic expertise required to identify the pollen within the honey using microscopy. Once optimised the technique allows the identification of many samples very quickly. Melissopalynology meanwhile has a much more direct relationship between the numbers of pollen grains of each taxa within the honey providing a more quantitative approach.

An ability to characterise the floral composition of honey is valuable as it allows the characterisation of plants which contribute to the chemical profile of the honey sample, which may be contributing to its antibacterial activity. Melissopalynology and DNA metabarcoding can also provide information on honey bee foraging and provides a method for checking the botanical and geographical origin of commercial honeys.

Melissopalynology provides a tried and tested method for characterisation of the pollen contained within honey. It allows a quantitative estimate of pollen amounts but is limited due to its reliance on a high level of technical expertise in order to identify the different pollen morphologies. DNA metabarcoding provides a new tool for pollen identification. It has the advantage of being able to be used without substantial levels of taxonomic expertise and also allows a greater amount of honey to be analysed increasing its repeatability between samples. All three methods contributed to the characterisation of the abundant floral constituents of the antibacterial honeys H24, H25 and H201. Woodruff, dandelion and bluebell were subsequently selected to be investigated using analytical chemistry in chapter 5.

# Chapter 5

## **CHARACTERISATION OF ANTIBACTERIAL COMPONENTS OF HONEY AND THE PLANTS WHICH CONTRIBUTE TO ITS PRODUCTION**

## 5.1 Chapter Introduction

Honey contains a complex mixture of plant-derived secondary metabolites including phenolic compounds, flavonoids and volatiles, as described in chapter 1, section 1.3. Figure 5.1 highlights the chemical structure of the most commonly occurring honey polyphenols (Manyi-Loh *et al.*, 2011, Kaškonienė and Venskutonis, 2010). The phytochemicals found in honey is dependent on the floral sources visited by the honey bee (Dong *et al.*, 2013). They contribute to the characteristic and health related properties of each honey sample. Honey also contains both enzymatic proteins such as catalase and glucose oxidase and other non-enzymatic compounds such as organic acids, amino acids, proteins,  $\alpha$ -tocopherol, catechins and acids (Meda *et al.*, 2005).

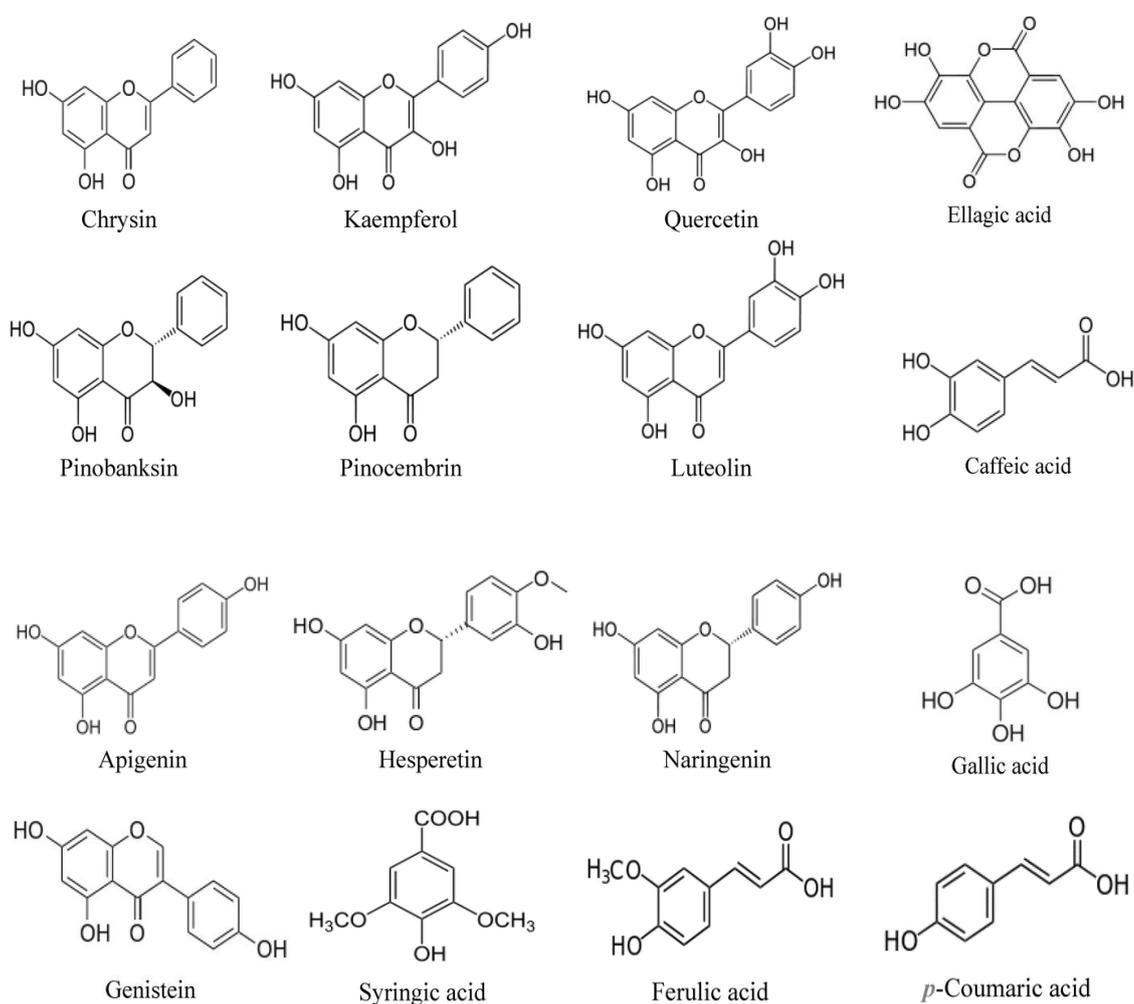


Figure 5. 1: Flavonoid and phenolic acids commonly found in honey

The primary focus of this chapter was to identify non-peroxide compounds isolated from the honey and plant extracts described in chapters 3 and 4 which possess antimicrobial activity (Estevinho *et al.*, 2008).

Gas-chromatography mass spectrometry (GC-MS) and High performance liquid chromatography (HPLC) analysis have been commonly used for the analysis of phenolic compounds in honey (Kaškonienė and Venskutonis, 2010, Pyrzynska and Biesaga, 2009). The analysis of the phenolic acids and flavonoid content of honey is a useful tool for quality control, authentication and classification of floral origin and pharmaceutical research (Campone *et al.*, 2014). To determine the origin of these floral constituents, compounds are extracted from the sample matrix and subjected to analytical separation, various analytical techniques can then be used for characterisation and quantification. By performing extractions on honey and plant material it is possible to determine if antibacterial compounds originate from the plants.

### 5.1.1 Extraction Techniques for Polyphenols

The level of phenolic compounds within natural honey is dependent on the plant or nectar source, the storage of the honey, the conversion of plant compounds by the metabolism of the bee and aerial contaminants (Castro-Vázquez *et al.*, 2008). For the extraction of polyphenols from both honey and plants, a representative sample is first subjected to a series of pre-treatments including homogenisation and filtration to remove solid particles. The elimination of matrix components, mainly sugars and water, is performed to isolate polyphenolic compounds from honey.

Samples are commonly subjected to liquid-liquid extractions (LLE) with organic solvents as honey and plant material comprises a complex mixture of polar and non-polar compounds. Phenolic compounds can be extracted using organic solvents of varying polarities, which allows the non-peroxide compounds to be concentrated (Tura and Robards, 2002). Mid to high polarity solvents such as acetone and methanol can be used to isolated polar constituents (Manyi-Loh *et al.*, 2010). A less-polar solvent such as n-hexane is suitable for the extraction of non-polar extraneous compounds (Stalikas, 2007). Using chemical solvents presents health and safety issues and are considered an expensive method to use (Stalikas, 2007). In recent years other LLE extraction techniques have been explored for polyphenol extraction; microwave-assisted extraction (MAE), steam distillation extraction (SDE), ultrasonic extraction (UE), supercritical fluid extraction (SFE) and pressurized liquid extraction (PLE) which all reduce extraction time and increase yield, however selectivity is reduced compared to solvent extraction and expensive specialist equipment is required (Trusheva *et al.*, 2007, Wang *et al.*, 2004, Smelcerovic *et al.*, 2006).

Solid phase extraction (SPE) can also be used to extract and concentrate plant derived compounds from honey (Pyrzynska and Biesaga, 2009). It avoids the production of artefacts (furan and pyran derivatives) and the need to use costly and toxic solvents (Manyi-Loh *et al.*, 2011). A column packed with Amberlite XAD-2, a non-ionic polymeric resin, can be used to recover 80-90% of phenolic compounds (Tomás-Barberán *et al.*, 1992). Honey samples are mixed with acidified

water and combined with the resin. Adsorbed polyphenols are retained in the matrix as the column is washed with deionised water. The adsorbed compounds are recovered using methanol, which is then evaporated to dryness (Tomás-Barberán *et al.*, 2001). Extracts can be subjected to other commonly used SPE method which utilises Bond Elut octadecyl C18, Oasis HLB and Strata-X columns for the recovery of polyphenols from honey (Dimitrova *et al.*, 2007, Michalkiewicz *et al.*, 2008). In this case analytes are eluted from the column by altering the polarity of the solution in the mobile phase.

### **5.1.2 Antibacterial Activity Guided Separation Techniques**

The phytochemicals present in honey and plant extracts have an effect on the biological activity (Tan and Lim, 2015). Crude extracts uniformly enriched in all compounds of interest can be screened for antibacterial activity. Manyi-Loh *et al.*, (2010) adopted an agar diffusion based method for the qualitative assessment of honey extracts to screen for the presence of antibacterial activity against *H. pylori*. For quantitative analysis the MIC and MBC of the mixed extracts can be determined using an agar diffusion assay and broth microdilution, methods described in chapter 3 (section 3.3.3).

Samples which demonstrated high level of antibacterial activity were subjected to a series of separation techniques to identify the individual compounds responsible for the activity. The principle separation technique used to isolate individual compounds from sample mixtures in natural product chemistry is chromatography (Butler, 2004). Thin layer chromatography (TLC) is the most basic liquid-chromatography application; it is simple, rapid and inexpensive (Fried and Sherma, 1999). Compounds are applied to a solid stationary phase such as silica and analytes are separated based on polarity or size as they are carried up a matrix by a liquid mobile phase. Crude extracts can also be fractionated using silica or Sephadex® column chromatography and this approach represents a useful means of separating individual compounds.

Extracts separated using TLC can be assessed for antibacterial activity using the bioautographic method, which was originally described by Hamburger and Cordel (1987). Bioautographic techniques are used for qualitative research and represent simple and cost effective methods for rapid screening of antibacterial activity. Three variants of the bioautographic method are used to detect antimicrobial compounds, direct TLC, the agar diffusion or contact approach and the immersion or agar overlay approach (Dewanjee *et al.*, 2014).

The direct detection of antibacterial compounds on TLC plates was developed to avoid the effect of agar diffusion and so individual compounds could be tested for activity. In this thesis the direct TLC method of Valgas *et al.* (2007) was adapted and used to characterise chromatographic fractions derived from crude honey and plant extracts. In brief, a standardised suspension of

bacteria is applied to fractionated compounds on a TLC plate and incubated in a humid environment to encourage growth. Clear zones of inhibition and therefore antibacterial activity can be visualised using a dehydrogenase-activity-detecting reagent (Hamburger and Cordel, 1987). Metabolically active bacteria convert the tetrazolium salt (yellow) into an intensely coloured formazan (deep pink) (Dewanjee *et al.*, 2014).

### 5.1.3 Advanced Chromatographic and Electrophoretic Analysis of Polyphenols

The use of chromatographic techniques to construct a fingerprint of the chemical composition of honey has been proposed as a means with which to evaluate and characterise the polyphenol content (Kaškonienė and Venskutonis, 2010). Commonly characterisation is performed using different adaptations of mass spectrometry (MS), high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) (Pyrzynska and Biesaga, 2009, Proestos *et al.*, 2013).

The MS approach has been employed in an attempt to facilitate the characterisation of polyphenolic compounds in honey (Gomez-Caravaca *et al.*, 2006). When coupled with the bioautographic assay it enables individual TLC spots/bands with high levels of antimicrobial activity to be assigned a mass and assessed for purity directly from the TLC plate. To combine the TLC and MS approach for direct analysis of compounds a TLC/MS interface can be utilised. A CAMAG TLC/MS interface available since 2009, allows planar chromatography samples to be run in parallel only on an 'as required' basis (Morlock and Schwack, 2010) (Figure 5.2). A solvent is passed directly over the TLC plate to collect target compounds into the elution head, extracting the compounds from the silica and transferring directly into a mass spectrometer. The interface allows identification and elucidation of unknown substances without the need for complex sample manipulation, such as extraction and derivatisation (Wilson, 1999).

Quantitative HPLC analysis of polyphenols is the most useful analytical technique for characterizing polyphenolic compounds (Gomez-Caravaca *et al.*, 2006, Proestos *et al.*, 2013). During HPLC a crude mixture is carried in the mobile phase and separated in a stationary phase, usually a column of packed C-18 silica. Compounds elute from the system at different times due to different affinities for the stationary phase. Individual peaks representing compounds can be quantified by their absorbance and retention time. Often comparisons are made between the HPLC chromatograms of previously described standards and mixed extractions to identify known compounds (Yao *et al.*, 2004). These techniques have previously been used to detect a number of phenolic compounds including *p*-hydroxybenzoic acid, cinnamic acid, naringenin, pinocembrin, thiophene and N-methyl-D3-aziridine, caffeic acid and chrysin (Estevinho *et al.*, 2008, Manyi-Loh *et al.*, 2010, Proestos *et al.*, 2006). Using a combination of these approaches there is the potential to identify novel, antibacterial plant derived compounds in the honey samples; assisted by comparisons to extracts obtained directly from plant material.

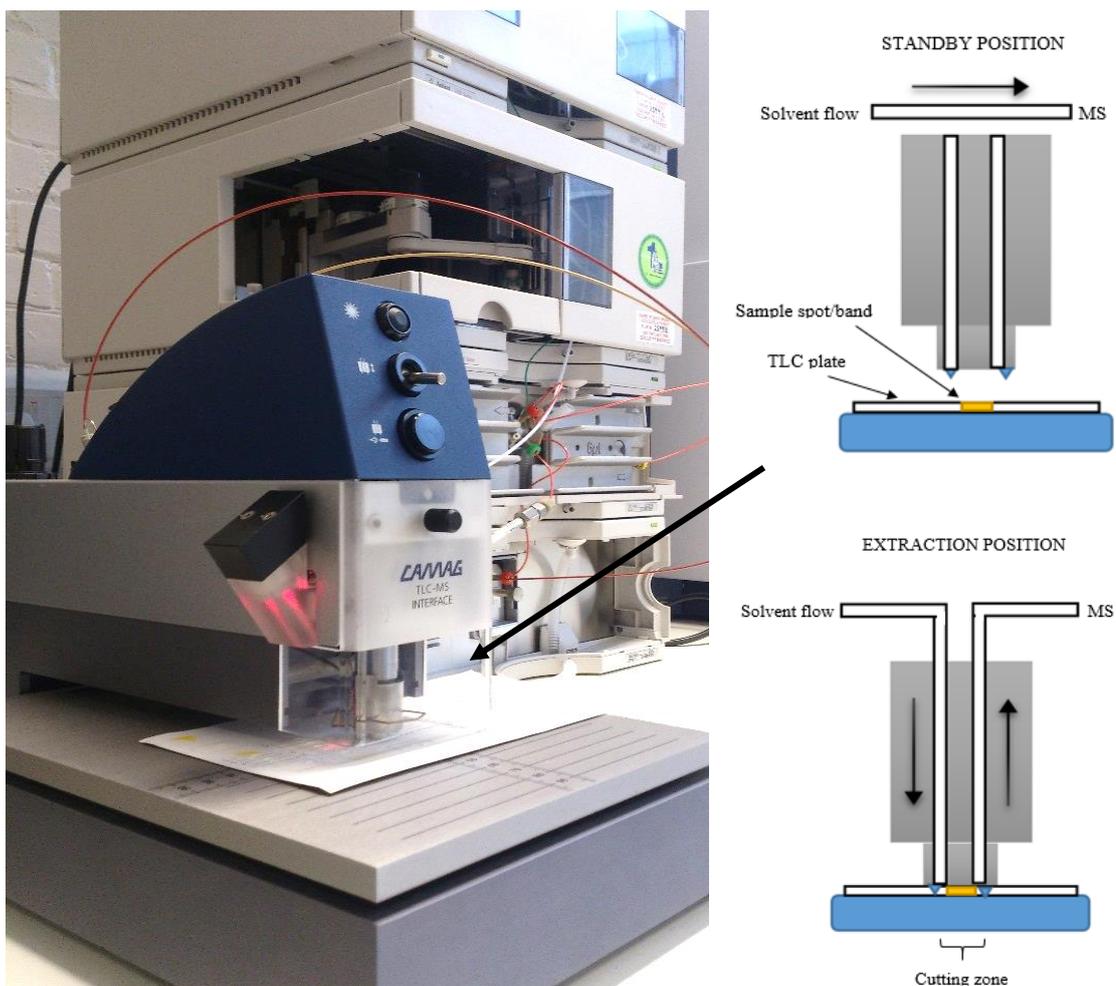


Figure 5. 2: Elution head-based HPTLC-MS: TLC-MS Interface (CAMAG 2009) and solvent pathway in the elution head

Many other chromatography and electrophoresis techniques have been used to detect polyphenols in honey and plant extracts. Liquid chromatography has been coupled with diode array detection and electrospray ionisation mass spectrometry (LC-DAD-ESI/MS) to characterise the flavonoid profiles of Slovenian honey (Bertoncelj *et al.*, 2011) and phenolic compounds in flowers of wild medicinal plants from North-Eastern Portugal (Barros *et al.*, 2012). Ultra-high-performance liquid chromatography (UHPLC) and high resolution mass spectrometry (HRMS) can be used to increase resolution, sensitivity and speed of analysis (Pyrzynska and Biesaga, 2009, Egan *et al.*, 1999). In this study, a number of these techniques were combined and for the separation of phenolic compounds in honey and plant extracts. Analytes from honey and plant extracts (gallium, bluebell and dandelion) have been analysed.

## 5.2 Chapter Aims and Objectives

To identify novel antimicrobial compounds by characterising honey and the plants which contributed to its making.

- To extract plant-derived antibacterial compounds from honey samples which demonstrated antibacterial activity in chapter 3
- To extract antibacterial compounds from flowers (gallium, bluebell and dandelion) identified in chapter 4 as contributing to the making of the antibacterial honey sample H24
- To assess the antibacterial activity of honey and plant crude extracts against MRSA, *E.coli* and *P. aeruginosa*
- To separate individual antibacterial compounds using the bioautographic TLC-overlay assay
- To determine the molecular weight of antibacterial compounds detected using the bioautographic TLC-overlay assay.
- To characterise these compounds using HPLC and to compare honey extracts to plant extracts and standards.

## 5.3 Methods and materials

### 5.3.1 Selection of Honey Samples

The honey samples subjected to organic extractions (H20, H24, H54 and H201) were selected on the basis of their antibacterial activity shown in chapter 3 (Table 3.8). The appearance and antibacterial activity of honey is influenced by the plants visited by the bees (Aljadi and Yusoff, 2002). Honey samples H24 and H201 were shown to retain antimicrobial activity once all known antibacterial factors had been neutralised. H54 is a sample of Manuka honey, and was included to allow comparisons to be made between the active samples and a commercially available antimicrobial honey. Honey H20 is a Welsh heather honey which failed to demonstrate any non-peroxide antimicrobial activity using the initial screening assay (Appendix C). It was included to determine whether solvent extraction and compound concentration could unearth antibacterial compounds which were missed by the screening assays.

### 5.3.2 Selection of Plant Material

The aim of this project was to detect antibacterial compounds in honey, in particular plant derived phenolics and flavonoids. In chapter 4, a novel DNA based approach using 454 and Illumina sequencing identified the plants which contributed to the production of H24, the honey sample which demonstrated the highest levels of non-peroxide activity of all of the samples screened. High levels of *Taraxacum* was also detected in H201. Based on this analysis three plants woodruff (*Galium odoratum*), bluebell (*Hyacinthoides non-scripta*) and dandelion (*Taraxacum officinale*) flowers were screened for the presence of antibacterial compounds.

While pollen from woodruff and bluebell was found in H24 they did not feature highly in any of the other honey samples tested (chapter 4, figure 4.5). Dandelion was also included in the organic solvent extractions due to its high abundance in H24 and across all of the honey samples tested. Samples were collected between May-June from the National Botanic Garden of Wales, Carmarthen, when the spring flowering plants were abundant. Flowers and stems were collected and frozen immediately on site on dry ice. Samples were transported to the lab on site and flowers were quickly removed to avoid oxidation or degradation of any compounds. The flowers of all the plants were subjected to solvent extractions to separate compounds.

### 5.3.3 Bacterial Cultures

The MRSA, *E. coli* and *P. aeruginosa* (chapter 2, section 2.2.4) were used to determine the antibacterial activity of the plant and honey compounds in this chapter. The purity of each overnight culture was checked by the streak plate method and Gram stain microscopy analysis (chapter 2, section 2.3.5 and 2.3.6).

The MRSA was used in the screening assays in chapter 3 and was subsequently used in this chapter as the aim of this thesis is to characterise plant-derived compounds with activity against clinically relevant pathogens. *E. coli* is a Gram negative, opportunistic human pathogen which was used to determine if the extracted compounds demonstrated any antibacterial effect on bacteria in which the cell wall differed markedly from that of *S. aureus*. *P. aeruginosa* is also a Gram negative, opportunistic human pathogen which is inherently more resistant to antibiotics than *E.coli* (Hancock and Speert, 2000).

### 5.3.4 Organic Extractions

Chemical reagents including methanol, acetonitrile, and water were of HPLC grade and were obtained from Fisher scientific or VWR. All other chemicals were of analytical grade, supplied by Sigma. Glassware was obtained from Fisher. Liquid-liquid extractions (LLE) were carried out on honey and plant samples using a range solvents as previously described (Manyi-Loh *et al.*, 2012). Organic solvents; n-hexane, ethyl acetate and methanol were used to extract compounds of different polarities.

For each extraction 25 g of honey was dissolved in a mixture of 150 ml of sterile water and 500 ml of solvent. The samples were agitated overnight at room temperature using a magnetic stirrer (RCT basic, Werke) to enable polar compounds to partition into the organic solvent. The water layer was drawn off and the extraction process was repeated three times, each time over a 72 hr period and the three extracts were subsequently combined.

Following extraction the samples were filtered through a Whatman® size 1 filter (Fisher) to remove any remaining solids. Solvent was then removed using a rotatory evaporator (Büchi Rotavapour R-200) and concentrator (MiVac, Genevac). Samples were dried under vacuum pressure (15 bar) and rotated at 1 g at 35 °C. Round bottom flasks were weighed before and after solvent evaporation using an analytical balance (Mettler Toledo AB54) to determine the weight of the compounds extracted. All extractions were resuspended in their extraction solvent at a concentration of 10 mg/ml. Following extraction compounds were stored at 5 °C in the dark until required.

The same organic extractions were performed on the woodruff, bluebell and dandelion flowers. Flowers which has been removed from their stalks and stored in a – 80 °C freezer were freeze dried to remove excess water. Samples were ground to a fine powder to obtain a homogenous sample using a pestle and mortar. In total 10 g of the dried material was resuspended in 350ml of solvent, and subjected to the same extraction procedure as the honey samples. All extractions were resuspended in their extraction solvent at a concentration of 10 mg/ml for consistency. Extracts were stored at 5 °C in the dark until required.

For each honey (H20, H24, H54 and H201) and plant extract (woodruff, bluebell and dandelion) a 10 mg/ml crude extract in hexane, ethyl acetate and methanol was generated. In total, 21 extracts were used for subsequent antibacterial activity testing and compound identification.

### 5.3.5 Phenolic Compounds Extraction

The non-ionic polymeric resin Amberlite XAD-2 (Sigma - SUPELCO, pore size 9 nm, particle size 0.3-1.2 mm) was used to remove sugars, water-soluble and other non-polar compounds from the methanol extract of the four different honey samples. A method adapted from that described by Kačániová *et al.*, (2011) was utilised, with modifications. Initially the Amberlite resin was cleaned with methanol (500 ml) on a magnetic stirrer for 25 mins between each extraction to guarantee it was free from contamination. The Amberlite was immersed in a 1:1 ratio of deionised water and methanol overnight to allow the resin to swell. Prior to use, the activated Amberlite was washed with (400 mL) deionised water.

A 20 g sample of honey was mixed with 500 ml of acidified water (adjusted to pH 2 with HCl) and filtered to remove solid debris. The Amberlite resin was added to the filtrate and stirred for 1 hr. To remove the sugars the loaded Amberlite was packed into a column and washed with acidified water (400 ml) and subsequently deionized water (400 ml) (pH 5). The phenolic compounds were eluted by adding methanol (300 ml) to the column (Kačániová *et al.*, 2011). Evaporation of the solvent allowed a mass to be determined after which the dry mass was resuspended in methanol or DMSO (Sigma) to a final concentration of 10 mg/ml. Samples were stored in the dark at 4 °C.

### 5.3.6 Antimicrobial Activity of Honey and Plant Extracts

Antibacterial activity was determined using an adaption of a broth based dilution assay (chapter 3, section 3.3.3.5) originally described by Boorn *et al.*, (2010). Extracts were each dissolved in 10 % DMSO and a stock concentration of 25 mg/ml was prepared. Two-fold serial dilutions of the extracts in MH broth were prepared to a final volume of 50 µL. MH is the standard media used for microbial testing (CLSI, 2012). The final extract concentrations ranged from 12.5 mg/ml to 0.39 mg/ml. Vancomycin was tested alongside each experiment (1 mg/ml) in DMSO as a positive control for experiments using MRSA and *E. coli* (French, 2006), tetracycline (0.01 mg/ml) was used against *P. aeruginosa*. A loop of bacterial suspension containing 10<sup>6</sup> CFU/mL was added to each well. Inoculated plates were sealed with a breathable membrane and incubated at 37 °C on a Thermo Scientific shaking incubator at 3 g for 24 h (Max<sup>Q</sup>™Mini4450).

The microbial bactericidal concentration (MBC) was determined by sub culturing 10 µl aliquots from each individual well in triplicate onto MH agar. By incubating the plates overnight and recording viable growth the lethal concentration of extract could be determined.

After overnight incubation, confluent bacterial growth was observed using a 70 % ethanolic solution of iodinitrotetrazolium chloride (INT) (2mg/mL) (Sigma) (Valgas *et al.*, 2007). The MIC was defined as the lowest concentration of honey extract that activated a change in the INT dye from colourless to purple after incubation for 1 hr. All experiments were performed in triplicate; the artificial sugar solution (chapter 3.3.4.3), vancomycin (1 mg/ml) and tetracycline (0.01 mg/ml) were also included in each assay.

### 5.3.7 Thin Layer Chromatography Optimisation

Following solvent extraction (section 5.3.4) the honey and plant compounds ( $n=21$ ) were further separated by thin layer chromatography (TLC) using an adaption of the method of Manyi-Loh *et al.*, (2012). A 40  $\mu$ l sample previously concentrated to 10 mg/ml in organic solvent was gently spotted onto a TLC prep-plate (50 mm x 75 mm, 0.20 mm Silica, Fisher) and the plate was left in a fume hood for 10 mins to allow the solvent to evaporate. The plates were initially run with a range of solvent systems to optimise the mobile phase and therefore the separation of any compounds on the basis of their polarity. Different ratios of the organic solvents hexane, ethyl acetate, methanol and acetone were trialled.

For each extraction ( $n=21$ ) three identical TLC plates were produced and used for:

- I. Visualisation of compounds
- II. The detection of antibacterial compounds
- III. Compound identification using TLC/MS analysis

This method was repeated on three occasions for each extract under the same conditions and retention factor ( $R_f$ ) values were recorded. This process was performed for the analysis of compounds against MRSA. The same process was performed for the analysis of *E.coli* and *P. aeruginosa*, however there was insufficient time for the production and analysis of plate III for these bacteria. Thymol was used in chapter 3 as a positive control and was subsequently used for consistency. Thymol was easy to visually detect and was run on every TLC plate to highlight experimental error.

TLC plate I was viewed under UV light, the silica plate fluoresces UV light, it subsequently glows everywhere except where an organic compound are located. Compounds containing unsaturated groups (carbon-carbon double bonds), *e.g.* a benzene ring are highlighted. To visualise saturated compounds, the TLC plates were viewed using vapour emitted from iodine crystals (Sigma) in a sealed container. Iodine vapour reacts with these compounds or simply sticks to them, highlighting their location. An exposure of 30-40 mins to the iodine vapour was enough to visualise bands and mark their location. Iodine was used as a reversible, non-permanent stain, while vanillin was used to generate a permanent stain. Vanillin reacts with steroids, higher alcohols, phenols, and essential oils producing a range of different colour bands. For example,

aromatic aldehyde, vanillin and the flavonol ring react to form a red adduct (Price *et al.*, 1978). The following method was employed (Valgas *et al.*, 2007). Vanillin spray; 3 g of vanillin (Sigma), 0.5 ml conc. sulphuric acid (Sigma) and 30 ml of methanol (Fisher), was prepared fresh for each TLC plate. Working in a fume hood, the TLC plates were coated with the vanillin mixture and heated to 100 °C with a heat gun, enabling the visualisation of bands.

The Rf values of each spots/band was determined according to the procedure of French and Wild using the following formula (1953):

$$\text{Rf value} = \frac{\text{Distance travelled by the solute (cm)}}{\text{Distance travelled by the solvent (cm)}}$$

### 5.3.8 Bioautographic Method Direct-Variant (Chromatogram Overlay)

A bioautographic method was adapted from Hamburger and Cordel's methods (1987). TLC plate II (section 5.3.7) was overlaid with a suspension of bacteria to determine the presence of antibacterial compounds. Samples ( $n=21$ ) were spotted onto the surface of a TLC plate as described (section 5.3.7) and were eluted to separate components. Following separation, the plates were allowed to air dry to allow solvent evaporation, bands were visualised under the non-invasive UV analysis to ensure separation had taken place.

TLC plates were placed into a sterile petri dish. An overnight culture was prepared as previously described (chapter 2, section 2.3.3). The broth suspension was centrifuged to produce a pellet and was re-suspended in nutrient broth to an  $OD_{600}$  of 0.05. The TLC plate was then covered with bacterial suspension and allowed to dry, a process which was repeated twice (Valgas *et al.*, 2007). The plates were subsequently placed into a humid closed polyethylene box and stored in a vertical incubator (Memmert Ltd, UK) for 24 h at 37 °C. At the end of this period the plates were removed and placed into a laminar flow cabinet. The plates were then sprayed with 2 mg/ml INT (Sigma) after which they were incubated for a further 4 h (Valgas *et al.*, 2007). Zones of clearing were recorded and matched to the position of band visualised with iodine and vanillin on TLC plates run at the same time.

Thymol at varying concentrations (1.25 µg to 20 µg) was spotted onto the TLC plate to act as a control that would give a positive result for antimicrobial activity when overlaid and to provide sensitivity data for the bioautographic technique. Based on the results obtained thymol (5 µg) was used as a positive control for all TLCs.

Metabolically active bacteria can be visualised by a deep pink colour with is the result of the conversion of tetrazolium salt into formazan (Choma and Grzelak, 2011). Zones of inhibition which appear clear against the pink background are symbolic of antibacterial compounds. The zones of growth inhibition on the plates were measured using callipers and the Rf values of

antibacterial analytes were recorded on three separate occasions. This technique has successfully been used by others to indicate inhibitory activity and to further guide separation of target compounds (Valgas *et al.*, 2007, Dewanjee *et al.*).

### **5.3.9 TLC/MS Interface Analysis**

To assign a mass to an unknown antibacterial spots on the TLC plate a TLC/MS interface (CAMAG, Wilmington, North Carolina) was used. Coupling TLC with LC-MS (Bruker microTOF spectrometer - AGILENT 1100) has the ability to identify and elute unknown substances. The TLC-MS interface was connected between the LC module delivering solvent and the MS. TLC plate III (section 5.3.7) containing honey or plant extracts was placed under the interface with the targeted band lying directly under the extraction head. Bands which demonstrated antibacterial activity in the overlay assay (section 5.3.8) were once again matched using UV visualisation and R<sub>f</sub> values and targeted for further analysis. When the head is manually lowered a 4mm wide seal is created to allow solvent passage over the surface of the selected band. Extraction times were optimised using standards to ensure compounds were being detected as they entered the MS machine, the MS was run in positive mode (Morlock and Schwack, 2010).

The interface is directly connected to the LC-MS. Antibacterial compounds can be checked for purity at low concentrations and characterisation can begin. Analysis was performed using an Agilent 1100 series (Agilent Technologies, Palo Alto, USA) system with a series binary pump, microvacuum degasser, series thermostatted column compartment and variable wavelength UV-vis detector. Sample injections were made through an autosampler. Bands identified as antibacterial using the bioautographic assay (section 5.3.8) were assigned an m/z (relative intensity) from the MS chromatogram analysis. Data collection and processing were performed using Bruker software. An Agilent Zorbax SB-C18 column (5 µm particle size, 250 mm × 4.6 mm) (Agilent Technologies, Palo Alto, USA) was used. The tentative identification of compounds was achieved by comparing TLC/MS data to literature, this is a semi-quantitative method and misidentification is a possibility (Gardana *et al.*, 2007). MS data was compared to databases such as SciFinder ([www.scifinder.cas.org](http://www.scifinder.cas.org)) and Chemspider ([www.chemspider.com](http://www.chemspider.com)) to try and match the compound with those previously identified.

### **5.3.10 Extraction of Compounds from a TLC Plate to Recover Compounds**

With the aim of extracting the compounds from the plate a repeat of the TLC (plate III, section 5.3.7) was run on a 200 µm analytical silica gel plate (60 F254, VWR) with the same solvent systems previously used on TLC prep-plates. All of the available sample was used to maximise the recovery of the desired compounds. The silica containing the antimicrobial bands was recovered from the TLC plate and re-suspended in the original solvent. The mixture was then filtered to separate the silica from the compound. This filtrate was placed into a MS vial (Kinesis)

and dried using the rotary evaporator as previously described. A MS vial can be weighed on an analytical balance (Mettler Toledo AB54-S) before and after the addition of the unknown compounds and a mass for the compound determined. Samples were re-suspended to 1 mg/ml in HPLC grade acetonitrile or methanol in preparation for further MS analysis to check purity. Samples were filtered (0.45 µm Whatman) prior to analysis.

### **5.3.11 HPLC Analysis of Honey Extractions**

#### **5.3.11.1 Sample Preparation and Standards**

Standards were purchased based on literature data and commercial availability. The phenolic compounds used for the identification and quantification of compounds in honey were purchased from Sigma: Vial 1 contained ellagic acid, naringenin, p-coumaric acid, kaempferol, chrysin, and syringic acid and Vial 2 contained ferulic acid, caffeic acid, gallic acid, rutin, hesperidin thymol and galangin (Estevinho *et al.*, 2008). Samples and standards were prepared in Cardiff; methanol honey and plant extracts were prepared to 50 µg/ml in HPLC grade methanol. Ethyl acetate and hexane extracts were dissolved to 50 µg/ml in HPLC grade ACN. Standards, honey and plant extracts were then sent to the University of Bath to be analysed using LC-DAD-ESI/MS to characterise phenolic compounds.

#### **5.3.11.2 HPLC Analysis**

LC chromatographic conditions: The column used was an Acquity C18 BEH, 50C1.2 µm column. Mobile phases consisted of ultra-purified water (mobile phase A) and acetonitrile (mobile phase B). Elution was performed at a solvent flow rate of 0.4 mL/min. The composition of the mobile phase in the gradient elution system between 0 and 30 min was as follows: 0 mins 10 % B, 1 min 10 % B, 10mins 80 % B, 11.5 mins > 80 % B, 12 mins 10 % B, 15mins 10 % B. Detection was achieved with a diode array detector and chromatograms were recorded at 260, 290 and 340 nm. The column was maintained at 25 °C. The sample injection volume was 5 µL. Peaks were identified by comparing their retention times of standards.

#### **MS conditions:**

A mass range of 50-850 *m/z* (relative intensity) was assessed in both positive and negative ESI modes. Standards were run at full concentration in both positive and negative ion modes to detect in what polarity, and with what peak area compounds were detected. The standard solution concentration of 16.6 µg/mL and 14.2 µg/mL for standard vials 1 and 2 respectively were analysed.

## 5.4 Results

### 5.4.1 Solvent Extractions

#### 5.4.1.1 Extraction of Crude Material

Based on the diameter of zones of inhibition detected in chapter 3 (Table 3.8) solvent extraction were performed on four honey samples (H20, H24, H54 and H201). Organic extractions of a range of polarities were also performed on three of the dominant plants identified in H24 following 454 DNA analysis (chapter 4, figure 4.5). The samples screened are summarised in table 5.1.

Table 5. 1: Solvent extractions performed – honey and plant extracts

Extraction Number	Natural product	Extraction solvent
E01	H20	Methanol
E02		Ethyl acetate
E03		Hexane
E04	H24	Methanol
E05		Ethyl acetate
E06		Hexane
E07	H54	Methanol
E08		Ethyl acetate
E09		Hexane
E10	H201	Methanol
E11		Ethyl acetate
E12		Hexane
E13	Woodruff	Methanol
E14		Ethyl acetate
E15		Hexane
E16	Bluebell	Methanol
E17		Ethyl acetate
E18		Hexane
E19	Dandelion	Methanol
E20		Ethyl acetate
E21		Hexane

#### 5.4.1.2 Extraction Efficacy of Honey and Plant Compounds

The organic solvents (methanol, ethyl acetate and hexane) were used in the organic extraction method (section 5.3.4). Honey methanol extracts were also subjected to an Amberlite separation to remove residual sugars (section 5.3.5). The total yield from each honey and plant following three extractions in each solvent is displayed in figure 5.3.

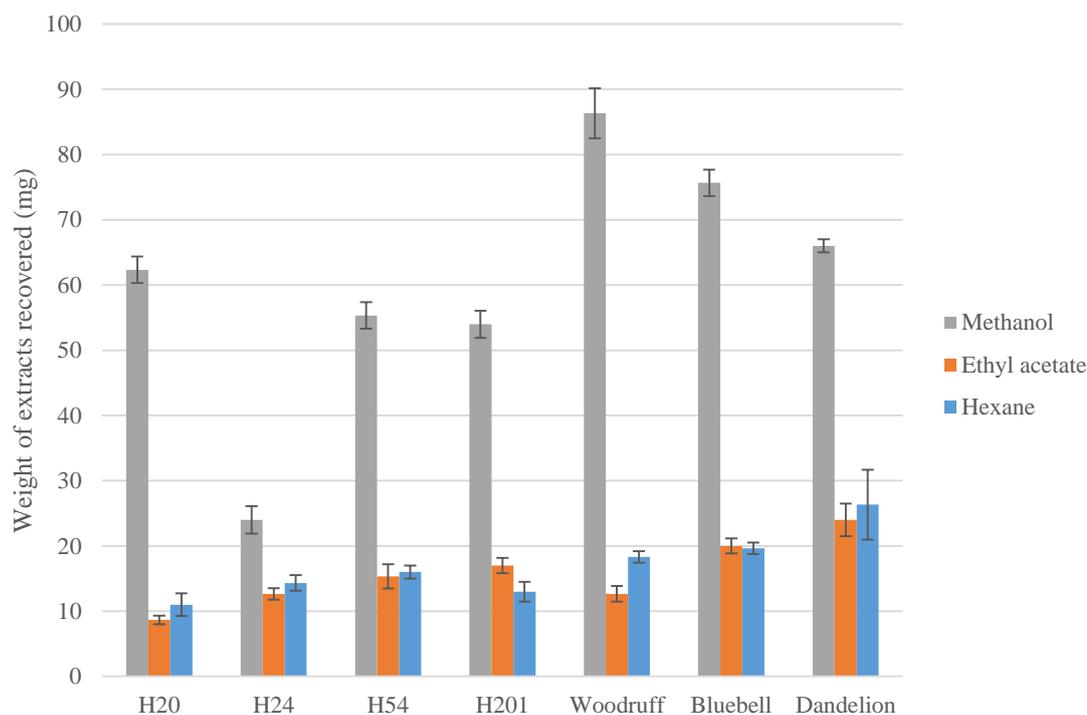


Figure 5.3 Comparison of the total yield (mg) of honey and plant extracts following organic solvent extraction

Extractions were performed over 72 h from 25 g of material after Amberlite separation (representative of 3 separate repeats; error bars =  $\pm$ SE)

The recoverable yield of honey and plant extracts was analysed and upon initial observation of figure 5.3, it is apparent that the recoverable yield from methanol extracts was greater than that from the ethyl acetate and hexane extracts. To determine if there was a statistically significant difference between the total yields of extracted material (mg) using the different organic solvents a Kruskal Wallis analysis was performed using SPSS software. Results indicated there was a statistically significant ( $X^2 = 12.679$ ;  $n = 21$ ;  $z = -2.837$ ;  $p = 0.002$ ;  $r = 0.52$ ) difference between the extraction solvents and total recoverable yield. A Bonferroni test confirmed that methanol extraction yields were significantly higher ( $p < 0.05$ ) than the other two solvents tested. A Mann Whitney U test was performed to compare the yield from the honey and plant extracts. There was no significant difference ( $U = 28$ ;  $n = 21$   $z = -1.849$ ;  $p = 0.064$ ) when comparing the overall yield from the honey extracts to the plant extracts.

## 5.4.2 Antibacterial Activity of Honey and Plant Extracts

### 5.4.2.1 Antibacterial Analysis of Honey Extract

These extracts were screened using an MIC broth dilution assay, an alternative method to using agar diffusion. A broth based method is not dependant on diffusion, allowing all compounds of low polarity to be assessed for activity. To determine MICs of the extracts, the values were

determined both by visual inspection and using the metabolising dye INT. All experiments were performed in triplicate and a geometric mean MIC was calculated.

For statistical analysis values of >12.5 % were converted to the next highest value of 25 % to enable analyses. To compare the difference between the antimicrobial activities of the honey extracts against the three bacteria the MIC values were transformed using a log transformation. This is because the MIC values were obtained by a doubling dilution series and therefore will inherently follow a logarithmic scale, which is incompatible with the statistical tests. Following a log transformation and after assessing the normality of the data sets, it was found that none the data (honey of plant extracts) followed normal distribution, therefore the extracts were analysed using a Kruskal Wallis test and groups were compared using the Bonferroni-Dunn's multiple comparisons test in this section.

A wide range of antibacterial activity was observed between the different honey extracts. The geometric mean of MIC values of the honey extracts ranged from 1.96 mg/ml to >12.5 mg/ml (Table 5.2). The solvent extracts from the four honeys showed variable levels of activity against the MRSA, *E. coli* and *P. aeruginosa*; all the samples inhibited bacterial growth to some extent. The ascending order of susceptibility was MRSA > *E. coli* > *P. aeruginosa*. Vancomycin had an MIC of 1.9 µg/ml against MRSA, 0.5 mg/ml against *E. coli* and an MIC of 2.5 µg/ml for *P. aeruginosa* was obtained with tetracycline, these values remained consistent throughout.

Table 5. 2: Antibacterial activity of honey extracts

The MIC and MBC (mg/ml) of honey extracts determined by broth microdilution (n=3).

Sample	MRSA		<i>E. coli</i>		<i>P. aeruginosa</i>	
	MIC	MBC	MIC	MBC	MIC	MBC
<b>E01</b>	>12.5	>12.5	12.5	12.5	12.5	>12.5
<b>E02</b>	3.13	3.13	6.25	12.5	12.5	12.5
<b>E03</b>	6.25	6.25	7.87	12.5	6.25	12.5
<b>E04</b>	12.5	>12.5	6.25	12.5	12.5	12.5
<b>E05</b>	3.13	6.25	3.13	6.25	3.13	6.25
<b>E06</b>	1.96	3.13	6.25	12.5	6.25	12.5
<b>E07</b>	>12.5	12.5	6.25	12.5	12.5	12.5
<b>E08</b>	6.25	6.25	3.13	3.13	3.13	3.13
<b>E09</b>	6.25	6.25	6.25	12.5	6.25	>12.5
<b>E10</b>	12.5	12.5	3.93	6.25	6.25	6.25
<b>E11</b>	6.25	6.25	6.25	12.5	3.13	6.25
<b>E12</b>	3.13	6.25	6.25	12.5	3.13	12.5

### Activity against MRSA

The highest level of inhibitory activity for MRSA was demonstrated by the hexane extract of H24 (E06), as shown by an MIC of 1.96 mg/ml (Figure 5.4). The lowest level of activity was demonstrated by the methanol extract of all four honeys (E01, E04, E07 and E10) with an MIC of 12.5 mg/ml or higher. The MBC values followed a similar trend with E06 and E02 showing the highest level of bactericidal activity and E01 and E04 showing the lowest. Ethyl acetate and hexane extracts showed a greater level of antibacterial activity compared to methanol extracts.

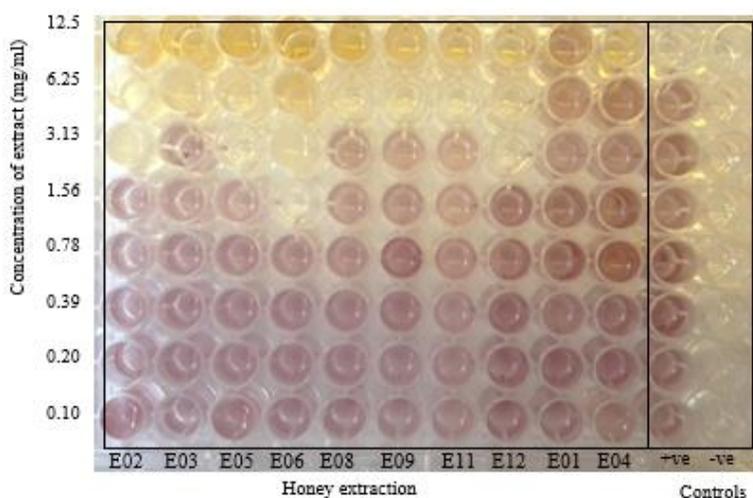


Figure 5. 4: MIC in a 96 well plate against MRSA

Plate contains different honey extractions at a range of concentrations incubated with MRSA for 24 h at 37 °C and sprayed with INT (2 mg/ml) (Positive control = vancomycin 1 mg/ml, Negative control = broth only).

### Activity against *E. coli*

The broth assay was performed against *E. coli* (Figure 5.5). The ethyl acetate samples demonstrated the highest level of inhibitory activity, as highlighted by a MIC values of 3.13 mg/ml obtained for the ethyl acetate extract of H24 (E05) and the ethyl acetate extract of H54 (E08). An MIC values of 6.25 mg/ml were obtained for both ethyl acetate extracts of H20 (E02) and H201 (E11). Interestingly the methanol extract of H201 (E10) also had a MIC of 3.93 mg/ml. The methanol extract of H20 (E01) has the lowest level of antibacterial activity with and MIC of 12.5 mg/ml. All honey extracts tested inhibited or killed the *E.coli* with a concentration of 12.5 mg/ml or less.

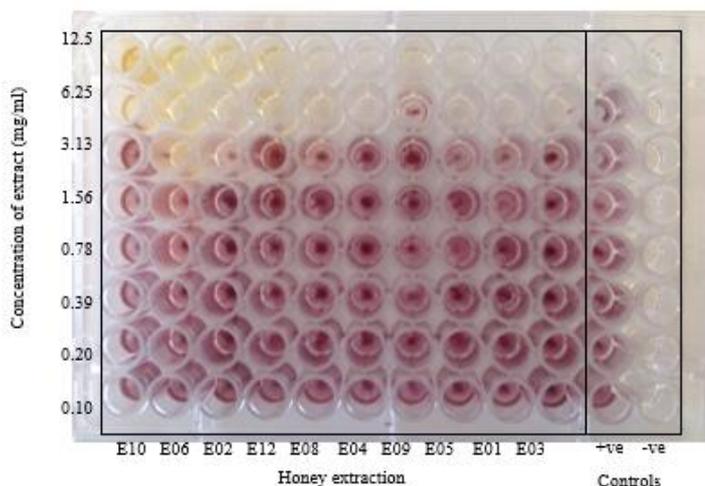


Figure 5. 5: MIC in a 96 well plate against *E.coli*

Plate contains different honey extractions at a range of concentrations incubated with *E. coli* for 24 h at 37 °C and sprayed with INT (2 mg/ml) (Positive control = vancomycin 1 mg/ml, Negative control = broth only).

#### Activity against *P. aeruginosa*

When the same extracts (E01-E12) were screened against *P. aeruginosa* a similar pattern was observed (Figure 5.6). Ethyl acetate extracts from H24 (E05), H54 (E08) and H201 (E11) all had the highest MIC of 3.13mg/ml, a MIC also observed by the hexane extract of H201 (E12). The least antibacterial effect was observed with the methanol extracts E01, E04 and E07, as demonstrated by an MIC of 12.5 mg/ml or more. Again the methanol extracts of H201 (E10) performed better than the other methanol extracts with an MIC and MBC of 6.25 mg/ml. In general, the ethyl acetate and hexane extracts showed higher levels of antibacterial activity than the methanol extracts.

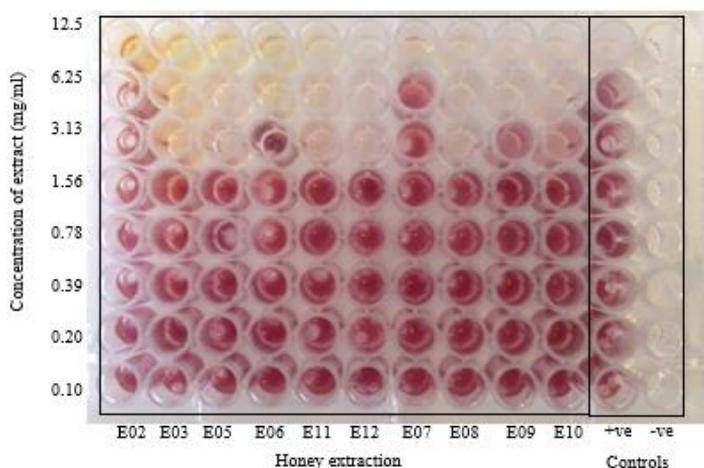


Figure 5. 6: MIC in a 96 well plate against *P. aeruginosa*

Plate contains different honey extractions at a range of concentrations incubated with *P. aeruginosa* for 24 h at 37 °C and sprayed with INT (2 mg/ml) (Positive control = tetracycline 0.01 mg/ml, Negative control = broth only).

#### 5.4.2.2 Antibacterial Analysis of Plant Extracts

The plant extracts were screened using the same MIC and MBC broth dilution assay and were assessed for activity against MRSA, *E. coli* and *P. aeruginosa*. The MIC and MBC was calculated for all plant extracts (Table 5.3) and expressed in mg/ml. All experiments were performed in triplicate and a geometric mean MIC was calculated.

As can be seen from table 5.3 all three plants contained antimicrobial compounds which inhibited bacterial growth to some extent. The level of inhibition varied between plants and was affected by the solvent used to extract the compounds and the bacterial species used to determine sensitivity. The ascending order of susceptibility was MRSA > *E. coli* > *P. aeruginosa* as was the case for the honey samples. The geometric mean MIC values for the plant extracts ranged from 0.78 mg/ml to >12.5 mg/ml (Table 5.3), highlighting the greater potency of plant extracts compared to honey extracts which ranged from 1.96 mg/ml to >12.5 mg/ml. Vancomycin had an MIC of 1.9 µg/ml against MRSA, 0.5 mg/ml against *E. coli* and an MIC of 2.5 µg/ml for *P. aeruginosa* was obtained with tetracycline.

Table 5. 3: Antibacterial activity of plant extracts

The MIC and MBC (mg/ml) of plant extracts determined by broth microdilution (n=3).

Sample	MRSA		<i>E. coli</i>		<i>P. aeruginosa</i>	
	MIC	MBC	MIC	MBC	MIC	MBC
E13	12.5	12.5	6.25	12.5	12.5	12.5
E14	3.13	6.25	6.25	12.5	12.5	12.5
E15	1.56	1.56	6.25	6.25	3.13	3.13
E16	6.25	6.25	6.25	6.25	6.25	12.5
E17	0.78	1.56	3.13	6.25	3.13	3.13
E18	3.13	6.25	6.25	12.5	6.25	12.5
E19	6.25	>12.5	6.25	6.25	12.5	12.5
E20	1.56	3.13	6.25	6.25	3.13	6.25
E21	3.13	6.25	6.25	12.5	3.13	12.5

#### Activity against MRSA

The highest level of inhibitory activity for MRSA was demonstrated by the ethyl acetate extracts of bluebell (E17), as shown by an MIC of 0.78 mg/ml. The hexane extract of woodruff (E15) and ethyl acetate extract of dandelion (E20) both had high MIC values of 1.56 mg/ml. The lowest level of inhibitory activity was demonstrated by the methanol extract of woodruff (E13) with an

MIC of 12.5 mg/ml. As you would expect, the MBC values followed a similar trend with E17 and E15 showing the highest bactericidal level of both with an MBC of 1.56 mg/ml. E19, the dandelion methanol extract had no bactericidal activity, despite an MIC of 6.25 mg/ml. These findings highlight the low level of antibacterial activity in the methanol extract compared to the ethyl acetate and hexane extracts.

#### **Activity against *E. coli***

When the plant extracts were run against *E. coli* the MIC values obtained for all samples, with the exception of E17, was 6.25 mg/ml. E17, the ethyl acetate sample from bluebell demonstrated the highest level of inhibitory activity, with an MIC value of 3.13 mg/ml. All samples killed the *E. coli* with a minimum of 12.5 mg/ml, the MBCs of E16, E17, E19, and E20 was maintained with a value of 6.25 mg/ml.

#### **Activity against *P. aeruginosa***

Against *P. aeruginosa* ethyl acetate extracts from bluebell (E17) and dandelion (E20) had the highest MIC of 3.13mg/ml, a MIC also observed by the hexane extract of woodruff (E15) and dandelion (E21). E17 and E15 had high MBCs of 3.13 mg/ml. The least antibacterial effect was observed with the methanol and ethyl acetate extracts of woodruff (E13 and E14) and the methanol dandelion extract (E19) with an MIC of 12.5 mg/ml, again the methanol extracts had the lowest level of activity.

#### **5.4.2.3 Comparison of Plant and Honey Antibacterial Activity**

The honey samples were originally chosen on the basis of the antibacterial activity detected in chapter 3 (Table 3.8). The MIC values were combined and compared using a Kruskal Wallis test and there was no significant difference ( $X^2 = 4.704$ ;  $n = 36$ ;  $z = 0.483$ ;  $p = 0.195$ ) between the four honey samples against MRSA. Against *E. coli* there was a significant difference ( $X^2 = 10.810$ ;  $n = 36$ ;  $z = -2.210$ ;  $p = 0.013$ ;  $r = 0.37$ ) across the four honey samples. A Bonferroni-Dunn's multiple comparisons test confirmed that against *E. coli* H20, the heather honey which owed its antibacterial activity to peroxide, was significantly ( $p < 0.05$ ) less active than the two honeys H24 and H201 which demonstrated non-peroxide activity in chapter 3. For *P. aeruginosa* there was a significant difference ( $X^2 = 11.667$ ;  $n = 36$ ;  $z = -3.238$ ;  $p = 0.009$ ;  $r = 0.55$ ) H20 was significantly ( $p < 0.05$ ) less active than H54 the Manuka sample.

With regards to the plant extracts there was no significant difference in the MIC of the woodruff, bluebell and dandelion extracts against MRSA ( $X^2 = 0.684$ ;  $n = 27$ ;  $z = -0.413$ ;  $p = 0.710$ ;  $r = 0.03$ ) or *E. coli* ( $X^2 = 3.054$ ;  $n = 27$ ;  $z = -1.723$ ;  $p = 0.217$ ;  $r = 0.33$ ). The woodruff extracts were

significantly ( $p < 0.05$ ) less active than the other plant extracts against *P. aeruginosa* ( $X^2 = 8.667$ ;  $n = 27$ ;  $z = -2.760$ ;  $p = 0.013$ ;  $r = 0.53$ ).

From the MIC and MBC data it can be concluded that MRSA was the most sensitive of the three bacteria tested against the plant and honey extracts. A Kruskal Wallis test revealed a significant difference ( $X^2 = 4.749$ ;  $n = 189$ ;  $z = -2.224$ ;  $p = 0.093$ ;  $r = 0.16$ ) in the log MIC values between MRSA, *E. coli* and *P. aeruginosa*. The Bonferroni-Dunn's multiple comparisons test confirmed MRSA was significantly more sensitive to the 21 extracts. There was no significant difference between the MIC results obtained for *E. coli* and *P. aeruginosa* across the 21 extracts.

The methanol extracts from the honey and plant extracts were significantly less active against MRSA compared to the ethyl acetate and hexane extracts. The Kruskal Wallis and the Bonferroni-Dunn's multiple comparisons test was also used to determine if there was a significant difference between the log MIC of methanol ( $Md = 1.10$ ,  $n = 12$ ), ethyl acetate ( $Md = 0.65$ ,  $n = 12$ ) and hexane ( $Md = 0.65$ ,  $n = 12$ ) honey extracts against MRSA. It was found that there was a significant difference ( $X^2 = 25.195$ ;  $n = 36$ ;  $z = -4.603$ ;  $p < 0.001$ ;  $r = 0.77$ ) in inhibitory activity of different solvent extractions. Bonferroni-Dunn's multiple comparisons test revealed ethyl acetate and hexane extracts were significantly ( $p < 0.01$ ) more active than methanol extracts against MRSA. A Kruskal Wallis test revealed that there was a significant difference between ( $X^2 = 19.69$ ;  $n = 27$ ;  $z = -2.892$ ;  $p = 0.04$ ;  $r = 0.44$ ) the log MIC of methanol ( $Md = 0.80$ ,  $n = 9$ ), ethyl acetate ( $Md = 0.19$ ,  $n = 9$ ) and hexane ( $Md = 0.50$ ,  $n = 9$ ) plant extracts against MRSA. Groups were compared using the Bonferroni-Dunn's multiple comparisons test confirming ethyl acetate and hexane extracts were significantly ( $p < 0.01$ ) more active than methanol extracts against MRSA.

There was a significant difference honey extracts against *E. coli* ( $X^2 = 8.446$ ;  $n = 36$ ;  $z = 0.175$ ;  $p = 0.015$ ;  $r = 0.03$ ) and *P. aeruginosa* ( $X^2 = 15.313$ ;  $n = 36$ ;  $z = -2.633$ ;  $p = 0.008$ ;  $r = 0.42$ ) using a Kruskal Wallis test. A Bonferroni-Dunn's multiple comparison test confirmed ethyl acetate extracts were significantly ( $p < 0.05$ ) more active than the hexane and methanol extracts.

In summary, the ethyl acetate extracts from the honeys showed significantly better activity against MRSA, *E. coli* and *P. aeruginosa* than the hexane and methanol extracts. From the plant extracts there was no significant difference between the different solvent extracts against *E. coli* and *P. aeruginosa*. These findings suggest compounds with high levels of antimicrobial activity are present in the ethyl acetate extracts of the honey samples, however there are also active compounds in the hexane and methanol extracts which should be considered.

In conclusion, the highest level of antimicrobial activity demonstrated across the extracts ( $n = 21$ ) was produced by the ethyl acetate extract of bluebell (E17). An MIC of 0.78 mg/ml and an MBC

of 1.56 mg/ml was seen against MRSA. The ethyl acetate extract from dandelion and hexane extract from woodruff (E15) produced MICs of 1.56 mg/ml against MRSA, highlighting their high level of antibacterial activity. The hexane extract of H24 produced an MIC of 1.96 mg/ml and MBC of 3.13 mg/ml, which was the highest level of activity seen for a honey extract. Across the four honeys, H20 was less active than the other honey samples tested. There was little difference in the activity of the three plants samples. Ethyl acetate showed the highest level of antimicrobial activity and MRSA was the most sensitive organism tested.

### 5.4.3 Thin Layer Chromatography (TLC) Assay

Different solvent systems were tested and the assay was optimised to obtain the best separation of compounds based on their polarity (Table 5.4). TLC was performed on all honey (E01-E12) and plant (E13-E21) extracts.

*Table 5. 4: Organic solvent system used for the preparation of TLC plates for compound separation.*

<b>Hexane extraction</b>	<b>Ethyl acetate extraction</b>	<b>Methanol extraction</b>
Hexane: Acetone = 6:4 (v/v)	Hexane: Acetone = 6:4 (v/v)	Acetonitrile: Methanol = 9:1 (v/v)

#### Visualisation of TLC plate

Following chromatography separation the individual compounds were visualised on plate I (section 5.3.7) using UV, iodine and vanillin to detect and isolate individual compounds. Each TLC plate was run in triplicate to confirm the presence of bands and retention factor (Rf) values were obtained for each individual band. An example of TLC visualisation of bands can be seen in figure 5.7.

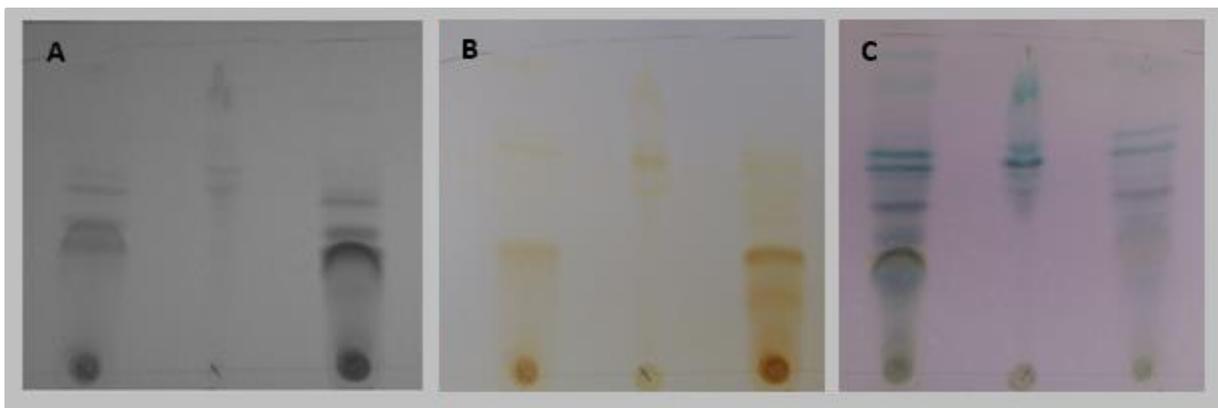
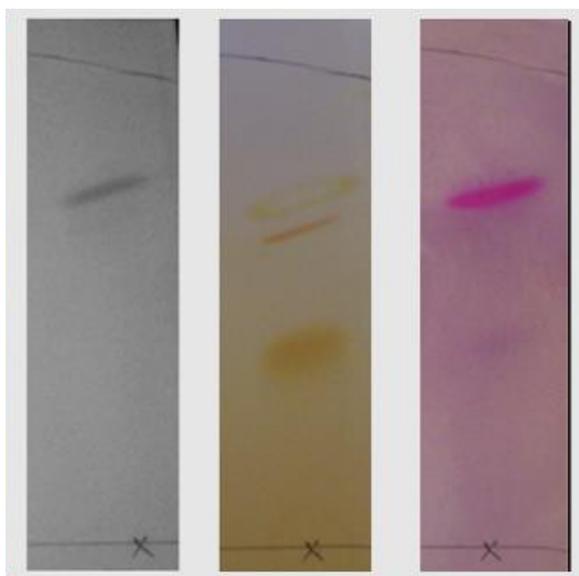


Figure 5. 7: Visualisation of TLC plate of a honey extraction

Honey extracts (E05, E08 and E11), solvent system hexane 6:4 acetone. A) UV light (grey scale) B) Iodine vapour C) Vanillin spray

### Standardisation of Thymol positive control

Thymol was used as a positive control on all TLCs run. Thymol is clearly visible under UV light, iodine vapour and vanillin spray (Figure 5.8). A characteristic pink band is visible with the vanillin spray. By using thymol as a control with standardised Rf values any experimental error or fluctuation between plates and runs can be observed. If the Rf values of 0.72 for the solvent system hexane 6:4 acetone and 0.94 acetonitrile 9:1 methanol deviated by more than  $\pm 0.1$  the experiment was repeated.



Solvent system:

Hexane 6:4 Acetone Rf = 0.72 (SE $\pm$ 0.003)

Acetonitrile 9:1 Methanol Rf = 0.94 (SE $\pm$ 0.003)

Figure 5. 8: The visualisation of positive control

Visualisation thymol control under UV light (grey scale), iodine spray and vanillin spray using the hexane 6:4 Acetone solvent system

## 5.4.4 Bioautographic Method (Chromatogram Overlay) and TLC/MS Analysis

### 5.4.4.1 Standardisation of Thymol Positive Control

A range of thymol concentrations were run in duplicate on a TLC plate and overlaid with a culture of MRSA to determine the sensitivity of the assay (Figure 5.9). Thymol was used as a positive control as it is known to possess antimicrobial activity, it is used as a pesticide and has been previously been detected in honey (Piasenzotto *et al.*, 2002). The solvent system hexane 6:4 acetone was used and an  $R_f$  of 0.72 was obtained. A minimum inhibitory concentration of 2.5  $\mu\text{g/ml}$  was determined using these assay conditions and subsequently 10  $\mu\text{g/ml}$  of thymol was included on every TLC plate to act as a positive control.

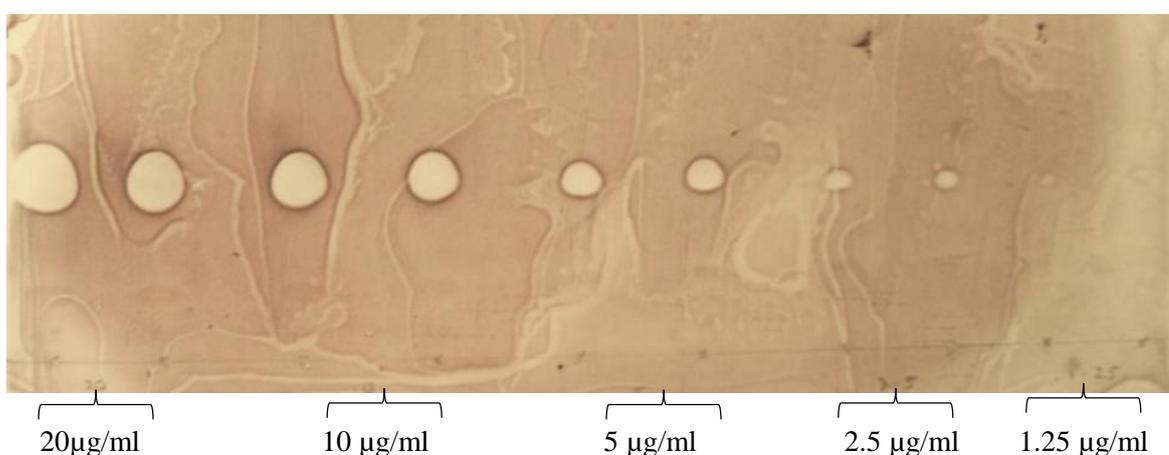


Figure 5. 9: The overlay assay of MRSA and thymol visualised using 2 mg/ml INT chloride.

### 5.4.4.2 Screening Crude Extracts for Antibacterial Activity – Honey

#### Activity against MRSA

Bioautographic assays were performed on honey organic extracts E01-E12 to identify individual compounds with antibacterial activity. TLC plate II (section 5.3.7) was overlaid and inhibitory activity was recorded as clear spots against the purple background. Each extract was tested in triplicate; any zones of inhibition with  $R_f$  values which deviated from the values obtained on the original overlay assay by  $\pm 0.2$  were also recorded.

A representative example of the overlay results obtained from ethyl acetate and hexane extracts from H24, H201, H20 and H54 are shown in figure 5.10. Bands with antibacterial activity against MRSA have been highlighted.

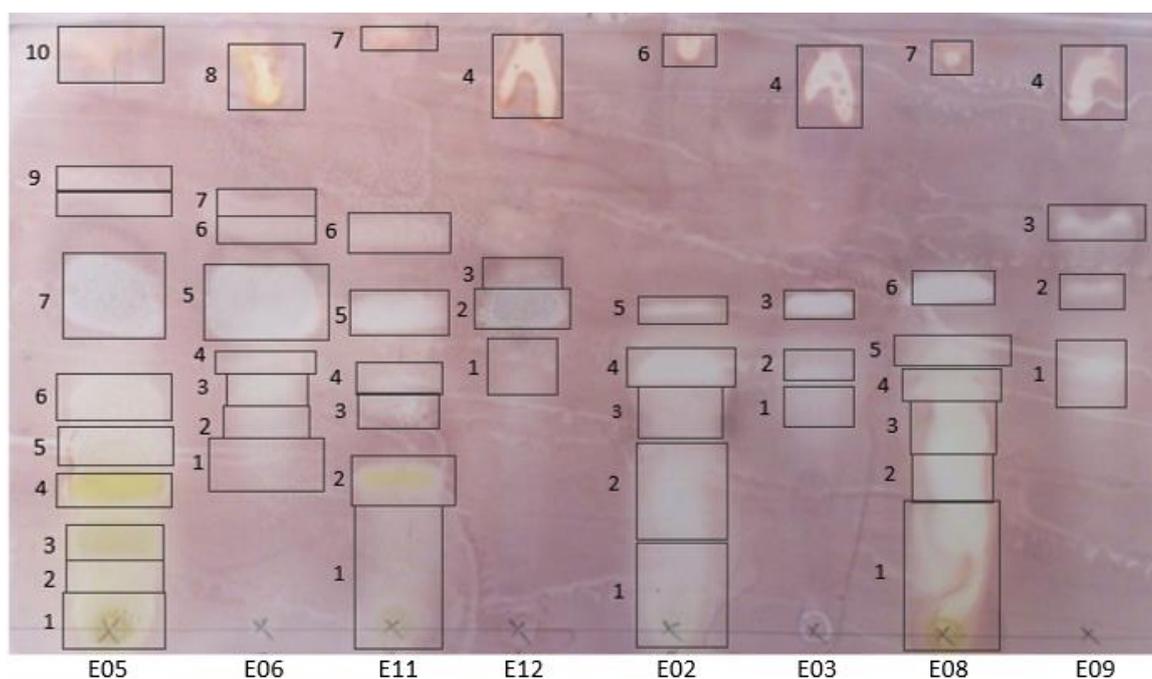


Figure 5.10: Overlay assay of honey extracts against MRSA.

Zones of clearing have been highlighted and numbered for further investigations.

The antibacterial activity of each extract was recorded for MRSA; the number of zones of inhibition, retention factor (Rf) and size of the zone was determined for each extract (Table 5.5). The areas of activity were selected based on visual data obtained from the UV, iodine, vanillin and overlay assays. Antibacterial activity against MRSA was seen in all twelve extracts generating a total of 69 antibacterial spots. These separated spots were represented by 44 different Rf values (Table 5.5). However, each antimicrobial spot was not present in each honey extract.

TLC plate III (section 5.3.7) was analysed with the TLC/MS interface. All the active bands detected on the overlay assay were assigned an  $m/z$  value of relative intensity from the MS chromatogram. The TLC assay was used for primary separation of extracts, and the TLC/MS provides further information for characterisation. Mass spectrometry is a commonly used tool for the identification of natural products and has been used for the characterisation of honey and propolis extracts (Falcao *et al.*, 2010, Gardana *et al.*, 2007, Arráez-Román *et al.*, 2006, Volpi, 2004).

Adduct formation is frequently observed in mass spectrometry analysis (Keller *et al.*, 2008). Compounds often form  $[M+H]^+$ ,  $[M+Na]^+$ ,  $[2M+H]^+$  or  $[2M+Na]^+$  adducts due to common background ions; either solvent molecules, alkali or other metal ions, or other contaminating components (Keller *et al.*, 2008). These adducts form as the MS is run in positive ESI mode and must therefore be taken into consideration when characterising peaks.

Table 5. 5: Inhibition of growth (zones of clearing) on bioautographic TLC plates by 12 extracts of 4 honey samples against MRSA.

Rf values and the area of the clear zone were recorded (+ area <10 mm, ++ area 10-20 mm, +++ >20 mm).The m/z value was also determined using the TLC/MS interface.

Extraction No.	Total No. of zones	Zone No.	Retention factor (Rf)	Antibacterial zone of clearing	Mass spec Value m/z
E01	4	1	0.05	++	379.1
		2	0.24	+++	379.1, 445.2
		3	0.45	+++	314.1, 304.3, 342.1
		4	0.92	+	251.1, 281.1
E02	7	1	0.15	+++	387.1
		2	0.41	+++	387.1, 430.9
		3	0.55	++	245.1, 430.9
		4	0.66	+	430.9, 362.9, 498.9
		5	0.79	+	430.9, 362.9, 498.9
		6	0.96	+	377.3, 491.3
E02 – Repeats		7	0.05	++	379.1, 325.1
E03	5	1	0.59	+	245.1, 295.2
		2	0.71	+	329.2, 259.1, 395.4
		3	0.80	+	279.2, 227.0, 647.6
		4	0.96	+	377.3
E03 – Repeats		5	0.90	+	377.3
E04	2	1	0.05	++	379.1, 365.1, 409.1
		2	0.94	+	253.1, 251.1, 239.1
E05	10	1	0.09	++	399.2, 403.2
		2	0.19	+	281.1, 332.3
		3	0.26	+	237.1, 281.1
		4	0.34	+	287.1
		5	0.41	+	263.1, 331.2, 365.2
		6	0.47	+	237.1
		7	0.62	++	329.2, 387.3
		8	0.74	+	253.1
		9	0.82	+	301.2, 331.2
		10	0.96	++	331.2, 329.2, 685.5
E06	10	1	0.32	++	287.1
		2	0.40	+	331.2, 581.4
		3	0.47	+	237.1
		4	0.53	+	277.1, 349.2, 302.3, 365.2, 429.3
		5	0.67	+	329.2, 387.3

		6	0.78	+	253.1
		7	0.79	+	331.2
		8	0.89	+	469.3, 329.3
E06 – Repeat		9	0.62	+	329.2, 387.3
		10	1.00	+	331.2, 365.1
E07	4	1	0.05	++	379.1
		2	0.23	+++	371.2, 231.1, 409.2
		3	0.45	+++	257.1, 413.2, 321.1
		4	0.91	+++	251.1
E08	8	1	0.20	+++	473.1, 287.1, 429.1
		2	0.35	++	315.1, 229.1, 387.1
		3	0.44	++	315.1, 285.1
		4	0.53	+	433.2, 463.1
		5	0.62	+	327.1, 297.1, 329.1
		6	0.72	+	247.1
		7	0.95	+	329.3, 685.5
E08 – Repeats		8	0.60	+	327.1
E09	5	1	0.63	+	235.1, 447.1
		2	0.71	+	335.1, 277.2
		3	0.81	+	329.2
		4	0.94	++	491.3
E09 – Repeats		5	0.30	++	227.1, 229.1
E10	2	1	0.05	++	365.1, 383.1
		2	0.91	+	235.1, 251.1
E11	7	1	0.11	+++	413.2, 347.0, 467.1
		2	0.24	++	245.1, 267.1, 223.1
		3	0.32	+	247.1, 263.1
		4	0.40	+	331.2, 281.1, 389.3
		5	0.46	++	245.1
		6	0.68	++	329.2, 251.1
		7	0.90	+	413.3, 491.3, 469.3
E12	5	1	0.35	++	389.2, 447.3, 247.1
		2	0.45	+	247.1
		3	0.53	+	317.2, 329.2, 367.3
		4	0.82	++	413.3, 491.3
E12 – Repeat		5	0.95	++	413.3
Thymol Hex 6:4 Ace	1	1	0.72	++	-
Thymol ACN 9:1 Meth	1	1	0.94	+	-

The largest number of antibacterial spots ( $n=10$ ) were seen in the ethyl acetate (E05) and hexane (E06) extracts of H24. Ethyl acetate appears to be the most effective solvent at recovering antibacterial compounds from honey as the next highest level of activity was seen in extracts E02, E08 and E11. This corresponds to the MIC and MBC data with ethyl acetate honey extracts showing the highest levels of antibacterial activity against MRSA. Using the bacterial overlay assay, 44 Rf values which are representative of different compounds proved to be inhibitory towards the growth of MRSA.

Often a compound with the same  $m/z$  value can be detected at numerous Rf values. For example,  $[M+H]^+ 329$  which was detected on 11 separate occasions was detected from spots with 9 different Rf values. Compounds were often grouped but can spread across the plate. Compounds are separated based on polarity and compounds of mid-polarity can spread across the plate. Due to the nature of TLC approach Rf values for the same compound can fluctuate, as some streaking can occur.

The aim of this research is to detect plant-derived antimicrobial compounds. The  $m/z$  values which occurred the highest number of times across the honey extracts were calculated by pooling the TLC/MS data (Table 5.6); these compounds are more likely to contribute to the overall antibacterial activity.  $[M+H]^+ 329$  was the highest occurring compound across the twelve extracts. It was detected in all four hexane extracts (E03, E05, E08 and E11) and was present in the ethyl acetate extracts of all the honeys except H20, the honey which only had peroxide antimicrobial activity (chapter 3, table 3.8). In total, this compound was detected on 5 separate occasions in H24 in E05 and E06. The second most common compound  $[M+H]^+ 331$  was found in the ethyl acetate (E05) and hexane (E06) extracts of H24 and the ethyl acetate extract of H201 (E11). Both honeys showed good levels of activity in chapter 3 (Table 3.8) and the MIC and MBC analysis (section 5.4.2).

By comparing the results obtained in this study to those published in the scientific literature it was possible to predict the likely identity of the compounds detected in the honey extracts (Table 5.6). Further investigations can be performed using databases such as SciFinder® to try and match the compound with those previously identified.

Table 5. 6: Characterisation of mass spectra values identified

Compounds which were identified more than once, and samples that were identified once but could be characterised in honey extracts. The extracts the mass values were detected in ( ‘-’ = not detected in literature).

Honey extracts			
[M+H] <sup>+</sup>	Detected in Extracts	Number of MS hits	Tentatively identified compound
329	E03, E05, E06, E08, E09, E11, E12	11	Pinobanksin-3-O-propionate or Pinobanksin-5-methyl-ether-3-acetate
331	E05, E06, E11	7	Quercetin-dimethyl ether
251	E02, E04, E07, E10	5	–
365	E04, E05, E06, E09	5	–
379	E01, E02, E04, E07	5	Detected but unknown
387	E02, E05, E06, E08	5	–
413	E07, E11, E12	5	–
245	E02, E03, E11	4	–
247	E08, E11, E12	4	–
281	E01, E05, E11	4	P-coumaric cinnamyl ester
430	E02	4	–
491	E02, E09, E11, E12	4	–
237	E05, E06	3	–
253	E04, E05, E06	3	–
287	E05, E06, E08	3	Pinobanksin-5-methyl-ether, Kaempferol or Luteolin
377	E02, E03	3	–
227	E03, E09	2	–
229	E08, E08	2	Resveratrol
235	E09, E10	2	–
263	E05, E11	2	–
277	E06, E09	2	–
315	E08	2	Pinobanksin-3-O-acetate
327	E08	2	Detected but unknown
389	E11, E12	2	–
409	E04, E07	2	–
429	E06, E08	2	–
447	E09, E12	2	–
469	E06, E11	2	–
301	E05	2	Kaempferid, Pinobanksin-5,7-dimethylether or Luteolin-methyl-ether
257	E07	1	Pinocembrin
304	E01	1	Quercetin
302	E06	1	Luteolin-5-methyl-ether or Pinobanksin-5,7-dimethylether
332	E05	1	Quercetin derivative
297	E08	1	Caffeic acid cinnamyl ester
285	E08	1	Caffeic acid phenylethyl ester Chrysin-6-methyl-ether Acacetin
295	E02	1	Fatty acid
371	E07	1	Pinobanksin-3-O-hexanoate

(Gardana et al., 2007, Arráez-Román et al., 2006, Falcao et al., 2010, Volpi, 2004, Dias et al., 2014, Pellati et al., 2011)

### Activity against *E.coli*

Bioautographic overlay was performed against *E. coli* (Figure 5.11) (Appendix G) and antimicrobial activity was detected within all of the ethyl acetate and hexane extracts.

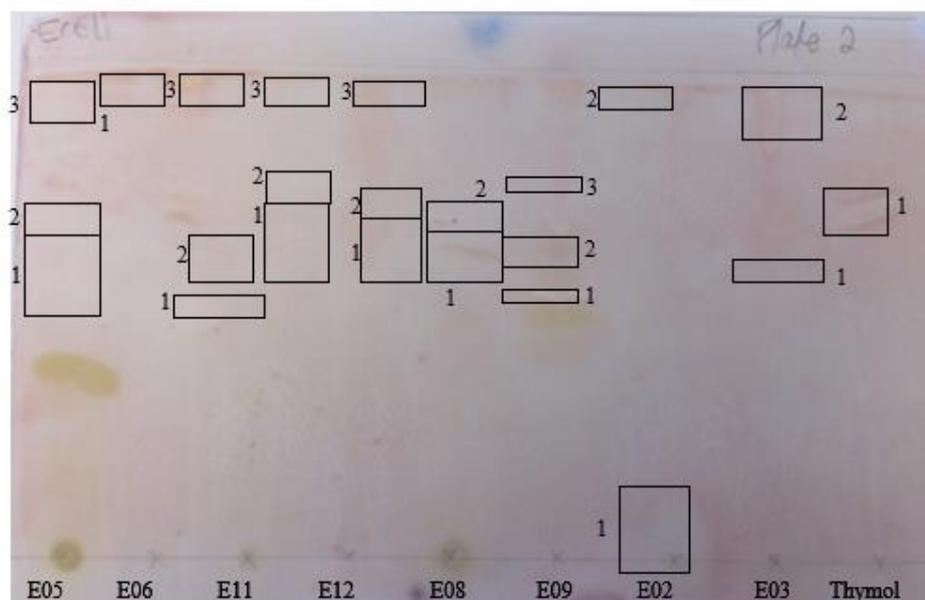


Figure 5. 11: Overlay assay of honey extracts against *E. coli*.

Zones of clearing have been highlighted and numbered for further investigations.

In total 28 clear zones were detected representing 18 different Rf values. No zones of clearing were detected from the methanol extracts. The highest number of antibacterial spots was seen in E05, the ethyl acetate extract from H24. This is consistent with the MIC/MBC data (section 5.4.2) as E05 showed good levels of activity against *E.coli* (3.13/6.25 mg/ml).

### Activity against *P. aeruginosa*

Bioautographic overlay was also performed against *P. aeruginosa* (Figure 5.12) (Appendix H) and good levels of antimicrobial activity were detected. In total 28 clear zones were detected representing 14 different Rf values.

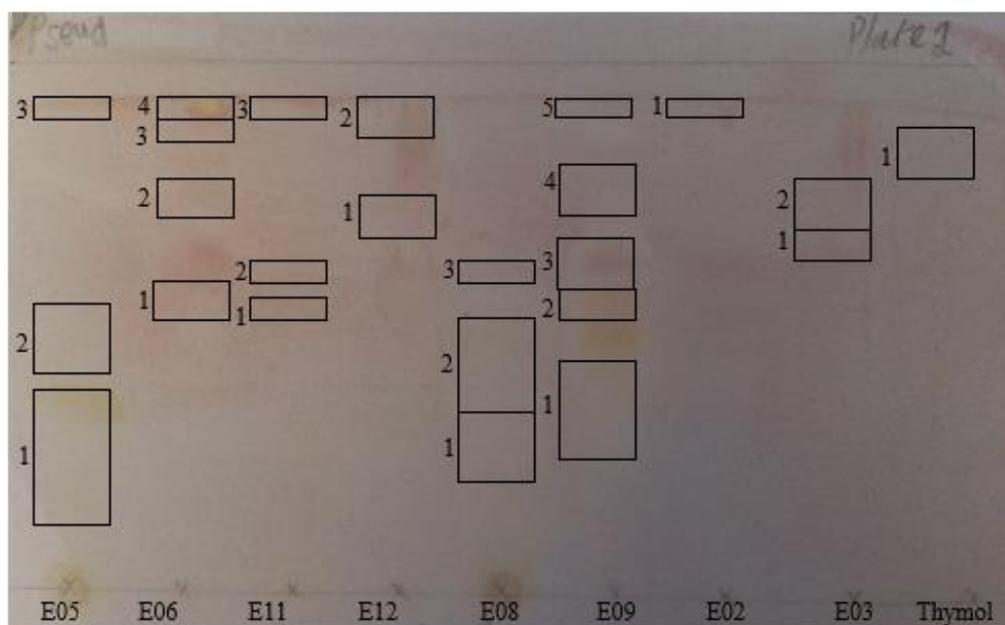


Figure 5.12: Overlay assay of honey extracts against *P. aeruginosa*.

Zones of clearing have been highlighted and numbered for further investigations.

As with the *E. coli* analysis, no zones of clearing were detected from the methanol extracts. A high number of zones was detected with ethyl acetate and hexane extracts from H24 (E05), H54 (E08) and H201 (E11), were as only three zones of clearing were produced by the ethyl acetate and hexane extracts of H20 (E03). The highest number of antibacterial spots was seen in E08, the ethyl acetate extract from H54. This is consistent with the MIC/MBC data (section 5.4.2) as E08 showed the highest levels of bactericidal against *P. aeruginosa* (3.13 mg/ml). Overall these results suggest that MRSA is more susceptible to the antibacterial compound recovered from honey than *E. coli* and *P. aeruginosa*.

#### 5.4.4.3 Screening Crude Extracts For Antibacterial Activity – Plants

##### Activity against MRSA

Bioautographic assays were also performed on woodruff, bluebell and dandelion organic extracts (E13-E21) to identify individual compounds with antibacterial activity against MRSA. The number of zones of inhibition, retention factor (Rf) and size of the zone was calculated for each extract (Figure 5.13) (Table 5.7).

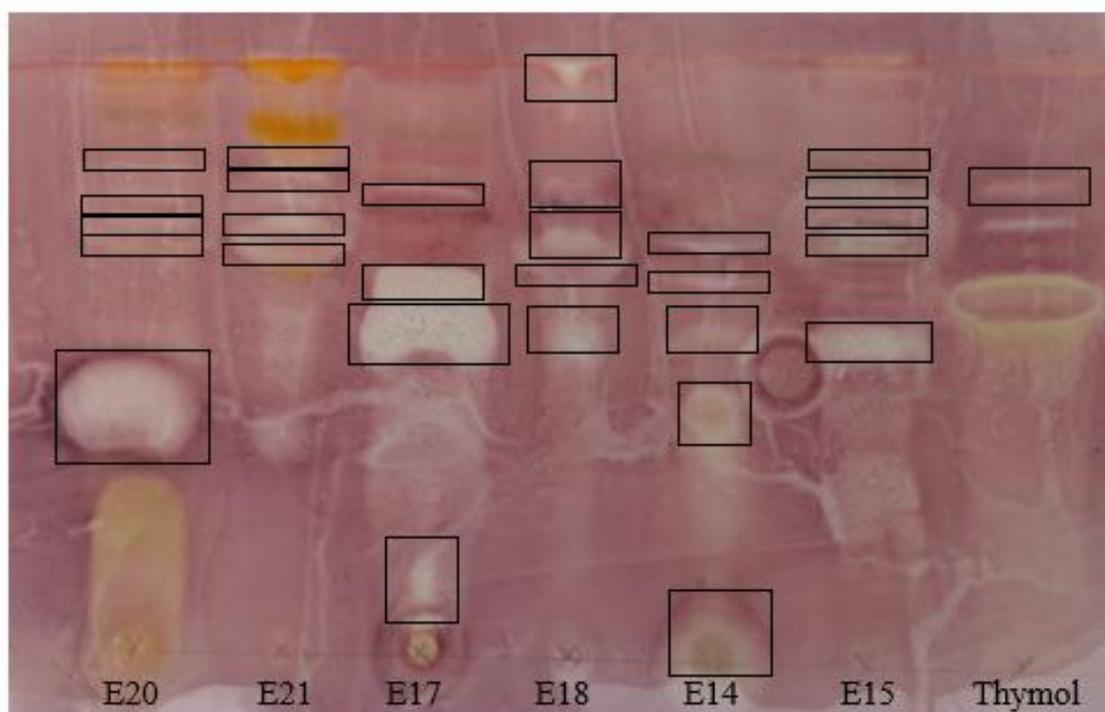


Figure 5. 13: Overlay assay of plant extracts against MRSA.

Zones of clearing have been highlighted and numbered for further investigations.

TLC-MS analysis was performed to assign an  $m/z$  value to each active spot. Antibacterial activity against MRSA was seen in all nine extracts and a total of 41 antibacterial spots were identified. These separated spots represented 32 different  $R_f$  values (Table 5.7). Different levels of activity were detected in the different extracts. Each methanol extract yielded only one antibacterial spot with low level antibacterial activity against MRSA. The largest number of antibacterial spots ( $n=7$ ) was seen in the hexane extract from dandelion (E21) and the ethyl acetate extracts from bluebell (E17) and dandelion (E20). Hexane extracts from woodruff (E15) and bluebell (E18) both yielded six antibacterial. These results again corresponded to the MIC and MBC data (section 5.4.2) in that the methanol extracts demonstrated the lowest levels of antibacterial activity against MRSA when compared to the ethyl acetate and hexane extracts. TLC/MS was performed to assign an  $m/z$  value to each spot providing further information for characterisation.

Table 5. 7: Inhibition of growth on bioautographic TLC plates by nine extracts of three plant samples against MRSA.

Extraction No.	Total no of zones	Zone no.	Retention factor (Rf) (mm)	Zone of inhibition	Mass spec Value <i>m/z</i>
E13	1	1	0.04	++	287. 1
E14	5	1	0.07	+++	287.1
		2	0.27	++	527.3, 287.0
		3	0.37	+	511.3, 430.9
		4	0.49	+	645.4, 647.4
		5	0.57	+	329.2
E15	6	1	0.06	+++	329.2, 430.9
		2	0.39	++	333.2
		3	0.46	+	360.3, 317.2, 353.3
		4	0.52	+	479.3, 315.1
		5	0.58	+	329.2, 387.2
E15 - Repeats		6	0.25	++	527.3
E16	1	1	0.37	+	307.1, 383.2, 367.2
E17	7	1	0.13	+	353.2
		2	0.33	++	335.2, 333.2, 735.2
		3	0.39	++	703.4, 335.2
		4	0.51	+	329.2, 387.2, 647.5
E17 – Repeats		5	0.04	+	307.1, 367.2
		6	0.48	++	335.2
		7	0.53	+	329.2
E18	6	1	0.33	+	430.9, 226.9, 362.9
		2	0.47	+	329.2, 430.9
		3	0.52	+	329.2, 629.5, 941.7
		4	0.61	+	615.4
		5	0.94	+	315.2
E18 - Repeats		6	0.56	+	317.2
E19	1	1	0.24	++	797.5
E20	7	1	0.26	+++	329.2, 445.2
		2	0.39	+	317.2, 353.3
		3	0.44	+	319.2
		4	0.48	+	387.2, 445.3
		5	0.52	+	329.2, 387.2
E20 -Repeats		6	0.36	++	317.2
		7	0.61	+	329.2, 387.2
E21	7	1	0.26	+	285.1
		2	0.38	+	377.2, 375.3
		3	0.44	+	671.5, 479.3, 647.5
		4	0.5	+	329.2, 387.3,
		5	0.76	+	615.5
		6	0.32	+	799.6, 329.2
E21 - Repeats		7	0.54	+	478.3, 777.6
Thymol Hex 6:4 Ace	1	1	0.73	++	
Thymol ACN 9:1 Meth	1	1	0.95	+	

(+ area <10 mm, ++ area 10-20 mm, +++ >20 mm)

The  $m/z$  values which occurred the highest number of times across the honey extracts were calculated by pooling the TLC/MS data (Table 5.8). These compounds contribute to the antibacterial activity of all nine extracts. As was the case for the honey extracts,  $[M+H]^+$  329 was the commonest compound recovered from all nine plant extracts being detected on twelve occasions. It was present in all three ethyl acetate extracts (E14, E17 and E20) and all three hexane extracts (E15, E18 and E21). As with the honey extracts it was not detected in any methanol extract.  $[M+H]^+$  387 was detected on six occasions and was present in extracts from all three plant extracts (E15, E17, E20 and E21). To determine the identity of these compounds the  $m/z$  values were compared to those published in scientific literature (Table 5.8), taking adduct formation into consideration.

*Table 5. 8: Characterisation of mass spectra values identified*

*Compounds which were identified more than once, and samples that were identified once but could be characterised in plant extracts. The extracts the mass values were detected in ('-' = Not detected in literature).*

Plant extracts			
$[M+H]^+$	Detected in Extracts	Number of MS hits	Tentatively identified compound
329	E14, E15, E17, E18, E20, E21	12	Pinobanksin-3-O-propionate or Pinobanksin-5-methyl-ether-3-acetate
387	E15, E17, E20, E21	6	Feruloyl-galactaric or glucaric acids
317	E15, E18 E20	4	Many possible plant natural products
335	E17	3	–
353	E15, E17, E20	3	–
287	E13, E14	3	Pinobanksin-5-methyl-ether, Kaempferol or Luteolin
430	E14, E15, E18	3	–
647	E14, E17, E21	3	–
307	E16, E17	2	–
315	E15, E18	2	Pinobanksin-3-O-acetate
333	E15, E17	2	Monogalloyl
367	E16, E17	2	–
445	E20	2	–
479	E15, E21	2	Isorhamnetin glucoside
527	E14, E15	2	–
615	E18, E21	2	–
319	E20	1	Myricetin
285	E20	1	Caffeic acid phenylethyl ester Chrysin-6-methyl-ether Acacetin

*(Gardana et al., 2007, Arráez-Román et al., 2006, Falcao et al., 2010)*

### **Activity against *E.coli***

A bioautographic overlay was also performed against *E. coli* (Appendix I) and nine plant extracts were screened for antimicrobial activity. In total 25 clear zones were detected representing 23 different Rf values.

Overall the size of the zones of antibacterial activity were smaller than those seen against MRSA. The level of antibacterial activity varied between the different solvent extracts. For woodruff and dandelion there was a clear trend (methanol<hexane<ethyl acetate) in the antibacterial activity using the different solvents. No antibacterial activity was seen for the methanol extracts. The largest number of antibacterial spots ( $n=7$ ) was seen in the ethyl acetate extract from bluebell (E17), followed by the ethyl acetate extract ( $n=6$ ) of woodruff (E14). E17 had the lowest MIC value recorded against *E. coli* (3.13 mg/ml).

### **Activity against *P. aeruginosa***

Bioautographic overlay was also performed against *P. aeruginosa* (Appendix J). Antibacterial activity was seen in all nine extracts and a total of 27 antibacterial spots were detected. These separated spots yielded compounds with 21 different Rf values (Appendix J). A variable range of antibacterial activity was detected across the nine different plant extracts. Methanol extracts from bluebell (E16) produced a zone of clearing on the baseline of the TLC plate, suggesting that antibacterial compounds of high polarity that failed to elute in the mobile phase. E16 had an MIC value of 6.25 mg/ml whereas E13 and E19 both had an MIC of 12.5 mg/ml.

The largest number of antibacterial spots ( $n=6$ ) was seen in the ethyl acetate extract from bluebell (E17) which had an MIC of 3.13 mg/ml. Ethyl acetate extracts from woodruff (E14) and dandelion hexane extract (E21) both had 5 spots. These results again corresponded to the MIC and MBC data (section 5.4.2); methanol extracts showed the lowest levels of antibacterial activity against *P. aeruginosa*, lower than both ethyl acetate and hexane extracts.

#### **5.4.4.4 Comparing the Antibacterial Activity of the Extracts**

The number of zones of clearing produced by the honey and plant extracts were compared (Figure 5.14). The ethyl acetate extract of bluebell (E17) had the highest level of combined antibacterial activity against the bacteria tested, followed by the ethyl acetate extract (E05) and hexane extract (E06) of the H24 honey and the ethyl acetate extract (E08) of the H54 honey.

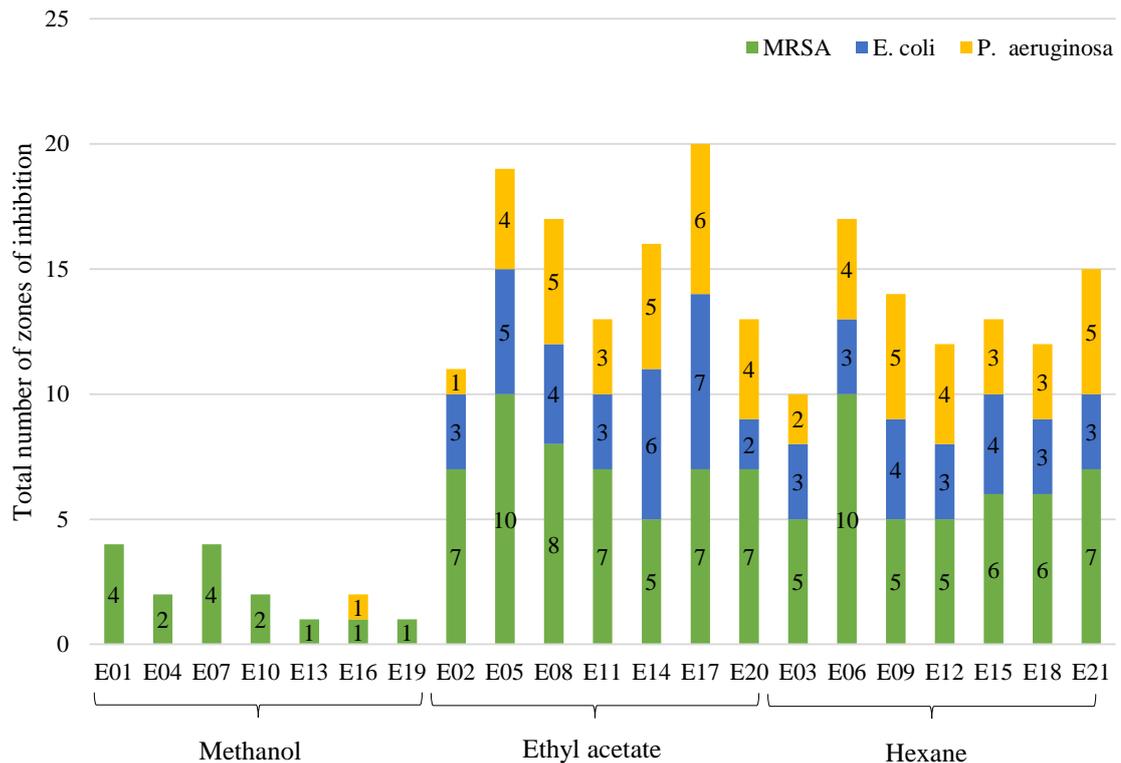


Figure 5. 14: Summary of the number of zones of clearing detected for the honey and plant extracts on the overlay assay

Samples have been grouped by solvent – methanol, ethyl acetate and hexane

There was no statistical difference ( $X^2 = 0.252$ ;  $n = 33$ ;  $z = 0.114$ ;  $p = 0.969$ ;  $r = 0.02$ ) when comparing the number of zones of inhibition produced by the four different honey extracts across the three bacteria, following a Kruskal Wallis comparison. Similarly there was no significant difference ( $X^2 = 0.327$ ;  $n = 27$ ;  $z = 0.045$ ;  $p = 0.849$ ) in the number of zones obtained from the different plant extracts.

MRSA was the most sensitive organism when tested against the twelve honey extracts and when comparing the three bacteria against the nine plant extracts it was clear MRSA was also more susceptible when compared to the two other microorganisms. A higher or equal number of zones of clearing were produced against MRSA against every extract, except E14 (Figure 5.14). The significant difference ( $X^2 = 14.020$ ;  $n = 63$ ;  $z = -3.028$ ;  $p = 0.001$ ;  $r = 0.38$ ) was determined using a Kruskal Wallis test. The number of zones of inhibition produced against MRSA was significantly higher ( $p < 0.01$ ) than the number of zones produced by the 21 extracts against *E. coli* and *P. aeruginosa* with a Bonferroni comparison test.

The methanol extracts from all four honey samples (E01, E04, E07 and E10) and plants (E13, E16 and E19) extracts showed limited activity, MRSA was the only organism sensitive to these

methanol extracts using the overlay assay (Figure 5.14). When assessing the overall activity of all the different solvent extractions against all three bacteria, the methanol extracts only produced 16 zones of clearing. Ethyl acetate extracts were responsible for 109 zones and hexane for 93 zones. This follows the trend seen with the MIC and MBC data in section 5.4.2 where methanol extracts were significantly less active than hexane extracts which were in turn less active than ethyl acetate extracts. This suggests the majority of the antibacterial compounds are isolated in the ethyl acetate extraction when using these extraction techniques.

A comparison between the MIC data (section 5.4.2) with the number of zones of inhibition produced on the bioautographic overlay assay was performed to determine if the results of the two antimicrobial techniques correlated (Figure 5.14). After reviewing the data, it was found that all the assumptions were not met to perform Pearson's correlation as data was not normally distributed therefore a Spearman's correlation test was used. The correlation analysis across all extracts for all three bacteria showed a strong inverse correlation ( $\rho = -0.612^{**}$   $p < 0.01$ ;  $n = 63$ ). The MBC data (section 5.4.2) was also analysed using a Spearman's correlation and a significant result was also obtained ( $\rho = -0.503^{**}$   $p < 0.01$ ;  $n = 63$ ). This confirms the extracts with high levels of antibacterial activity in the MIC and MBC assays produced the highest number of zones of inhibition of the bioautographic assay.

#### **5.4.4.5 Comparison of the Antibacterial Phenolic Compounds Detected in Honey and Plants**

The identity of the majority of compounds was determined by comparing their  $m/z$  values with that of standards and the scientific literature (Table 5.9). Compounds often form  $[M+H]^+$ ,  $[M+Na]^+$ ,  $[2M+H]^+$  or  $[2M+Na]^+$  adducts due to common background ions (Keller *et al.*, 2008). Adducts must therefore be taken into consideration when attempting to identify unknowns.

Both  $[M+H]^+$  species 329 and 387 were detected at high levels in the honey and plant extracts. The  $[M+H]^+$  species 329 is commonly seen in literature based on the analysis of honey or propolis and has been attributed to pinobanksin derivatives (Gardana *et al.*, 2007, Chua *et al.*, 2013).  $[M+H]^+$  329 appeared in the extracts with high levels of antimicrobial activity in the screening assays. With only one exception (E02)  $m/z$  329 was detected in all ethyl acetate extracts and hexane extracts.  $M/z$  329 was not found in any methanol extracts which correlates to the antibacterial activity described in section 5.4.4. This was also seen for the  $[M+H]^+$  387 which was detected in many of the ethyl acetate extracts (E02, E05, E08, E17, E20) and hexane extracts (E06, E15 and E21) but not in any methanol extracts. These species were both detected in all four honey samples analysed and all three plant extracts to some extent.

$[M+H]^+$  317 is an example of an  $m/z$  value which was seen 4 times from the plant extracts (in E15, E18 and E20) but only seen once in the honey extracts. Some compounds were unique to

honey extracts, pinocembrin and pinobanksin-3-O-hexanoate were detected in honey but not in the plant extracts.

$M/z$  387, 430 and 362 could not be detected in the literature search. The mass spectrum values which could not be characterised based on literature searches offer promising leads. These findings suggest there are novel unknown compounds with antibacterial activity which warrant further investigation.

Table 5. 9: Mass spectra values identified more than once in both honey and plant extracts

$[M+H]^+$	Detected in Extracts	Number of MS hits honey	Detected in Extracts	Number of MS hits plants	Tentatively identified compound
329	E03, E05, E06, E08, E09, E11, E12	11	E14, E15, E17, E18, E20, E21	12	Pinobanksin-3-O-propionate Pinobanksin-5-methyl-ether-3-acetate
387	E02, E05, E06, E08	5	E15, E17, E20, E21	6	–
430	E02	4	E14, E15, E18	3	–
287	E05, E06, E08	4	E13, E14	2	Kaempferol Luteolin Pinobanksin-5-methyl-ether
315	E08	2	E15, E18	1	Pinobanksin-3-O-acetate
362	E02	2	E18	1	–
317	E12	1	E15, E18 E20	4	Quercetin-3-methylether Isorhamnetin Quercetin-7-methyl-ether 3-Prenyl-4-(2-methylpropionyl-oxy)-cinnamic acid
285	E08	1	E21	1	Caffeic acid phenylethyl ester Chrysin-6-methyl-ether Acacetin
445	E01	1	E20	2	Quercetin O pentoside

(Gardana et al., 2007, Arráez-Román et al., 2006, Falcao et al., 2010, Volpi, 2004, Dias et al., 2014, Pellati et al., 2011) (‘–’ = Not detected in literature).

### 5.4.5 Isolation of Antibacterial Compounds

To prepare samples for extraction and subsequent analysis, extracts were run on glass backed 200  $\mu\text{m}$  analytical silica gel plates. Antibacterial bands were targeted based on overlay assay and TLC/MS data, compounds were visualised under UV light and Rf values were marked. The areas corresponding to the areas of activity were scraped off the plate, re-suspended in the original solvent for extraction. Samples were weighed and placed in individual vials. The samples were then re-suspended in HPLC grade methanol (0.01 mg/ml) before being characterised by MS.

The species  $m/z$  329 from E17 the sample which showed the highest level of antibacterial activity, was targeted for further characterisation using chromatography and the component was isolated from the band which gave the cleanest peak following TLC/MS analysis. The mass spectrum, which was performed following the isolation of this component is displayed in figure 5.15. It is noticeable that there are multiple secondary peaks within the fraction ( $m/z$  775.6 and 941.7).

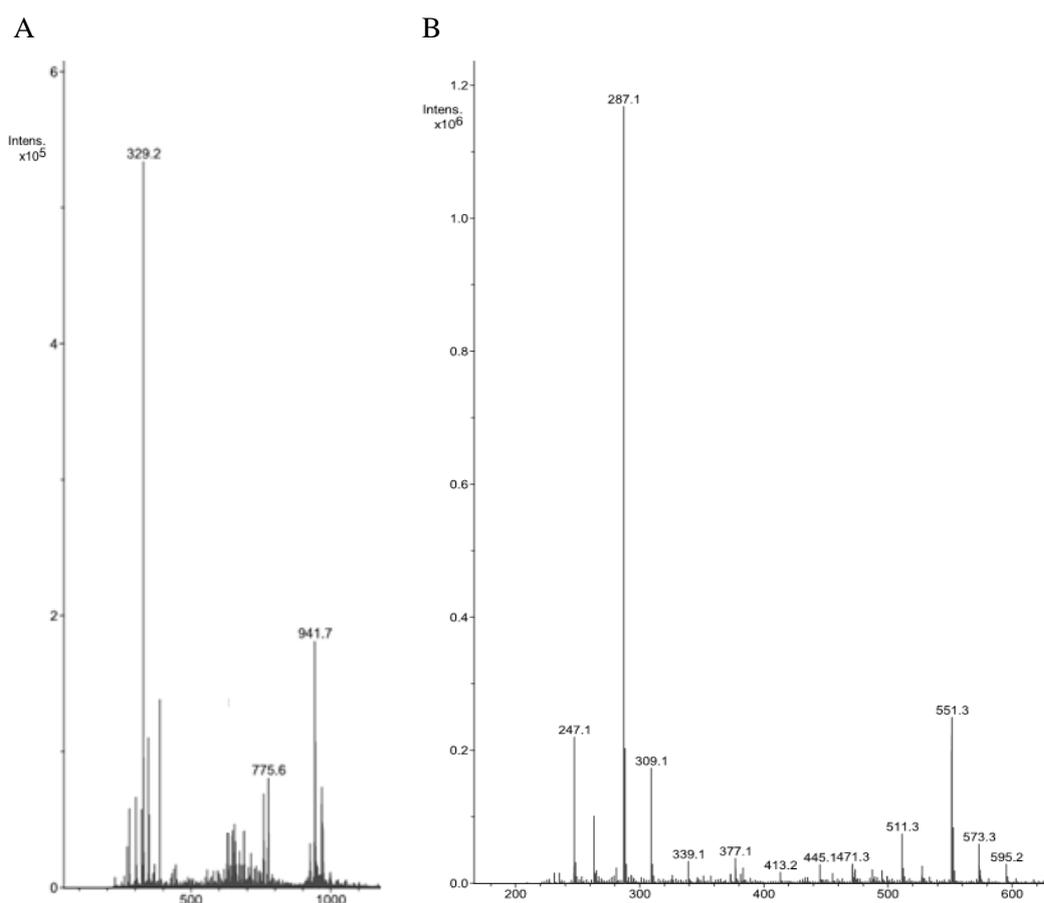


Figure 5. 15: Mass spectrum of compounds extracted from E17 and E05/E06 after separation.

(A) clear peak at  $[M+H]^+$  329 and (B) clear peak at  $[M+H]^+$  287, supported by peaks at  $[2M+H]^+$  573 and the  $[2M+Na]^+$  595.

$m/z$  387 was also targeted and extracted from the ethyl acetate extract E02 as this was the purest peak detected. Another commonly occurring compound was extracted from E05 and E06 (H24), the  $m/z$  287 was targeted and the resulting MS spectra can be seen in Figure 5.15. On the mass spectrum of E05/E06 a small peak can be seen at  $m/z$  595 for  $[2M + Na]^+$ .

By targeting these compounds the aim was to isolate enough of the individual compounds for further characterisation. Due to the low extraction yield and the crude nature of the extracts characterisation using methods such as NMR was not possible. To perform this type of analysis the solvent extraction process would need to be repeated and further optimised. Unfortunately there was insufficient amounts of the four characterised honeys to support further analysis.

## **5.4.6 High Performance Liquid Chromatography (HPLC)**

### **5.4.6.1 HPLC Analysis**

HPLC-MS is a sensitive analytical technique which was employed in an attempt to characterise the antibacterial compounds isolated from the samples. Standard compounds previously reported to occur in honey were purchased and analysed by HPLC-MS to obtain baseline data (Table 5.10). The aim was to subsequently compare the 21 honey and plant extracts to these standards and determine whether these compounds are present in the honey, and potentially detect unknown compounds. Analysis was performed at Bath University employing a variety of solvents systems and MS detection was performed in both positive and negative modes.

Not all standard compounds were detected in either or both of the ionisation modes, or by any of the UV wavelengths. In order to detect as many compounds as possible all samples were run in both polarities, and even then several compounds could not be detected. No further dilutions of the standards were analysed as the detection limits were generally poor.

Table 5. 10: HPLC of standards

The retention times, molecular weights, stock concentration and MS data of the twelve commercial standard compounds run on HPLC to compare to the honey and plant extracts ('-' = not detected)

Commercial Standard	M.W	Conc. in standard stock (µg/mL)	ESI polarity detection	[M+H] +	tR (mins)
Ellagic acid	302.19	16.6	-	303.0135	-
Naringenin	272.25	16.6	Positive and Negative	273.0757	6.9
P-coumaric acid	164.16	16.6	Positive	165.0546	7.9,11.1
Kaempferol	286.24	16.6	Negative	287.0550	7.0,9.3
Chrysin	254.24	16.6	Positive and Negative	255.0652	8.2
Syringic acid	198.17	16.6	Positive	199.0601	7.6
Trans-ferulic acid	194.18	14.2	Positive	195.0652	11.1
Rutin trihydrate	664.58	14.2	-	451.2115	-
Caffeic acid	180.16	14.2	Positive	181.0495	11
Galangin	270.24	14.2	Negative	271.0601	8.5
Gallic acid	170.12	14.2	-	171.0288	-
Hesperidin	610.56	14.2	Positive and Negative	611.1970	5.6
Thymol	150.22	14.2	-	151.1117	-

tR: Retention time, M.W: molecular weight

Colour coordinated chromatograms were obtained from the HPLC-MS analysis, the standards that gave definitive peaks can be seen in figure 5.16. These standard chromatograms were used to detect whether any of these compounds were present in the ethyl acetate and methanol fractions of the 14 honey and plant extracts. Ideally pinobanksin would have been included as a control, but the HPLC was performed prior to the discovery of this MS value and was therefore not part of the assay. The hexane extracts were omitted as due to their high polarity they were not suitable for HPLC analysis without further optimisation.

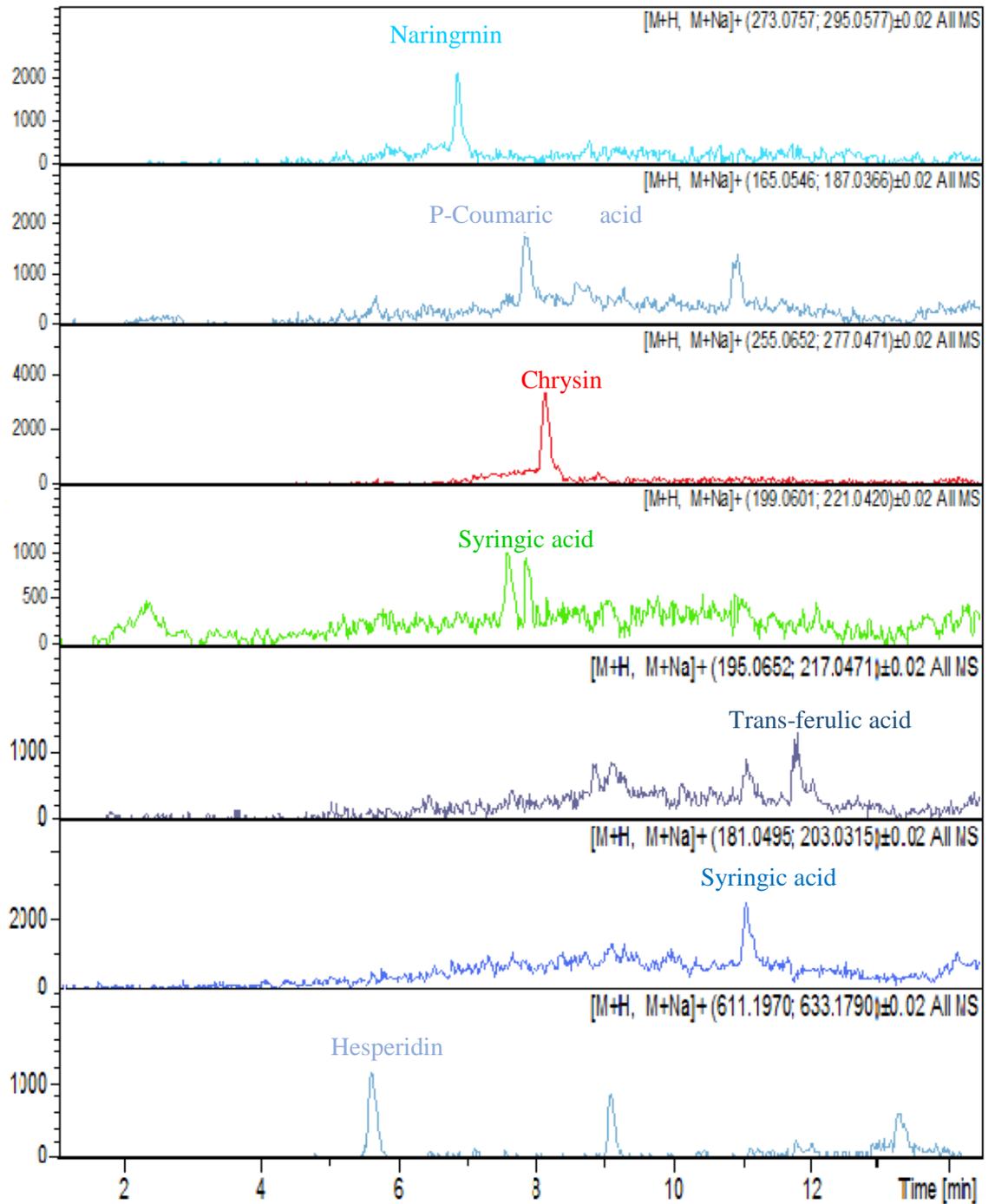


Figure 5. 16: HPLC-MS chromatogram of standard solutions, the best peak obtained from either positive or negative mode was included: continues overleaf

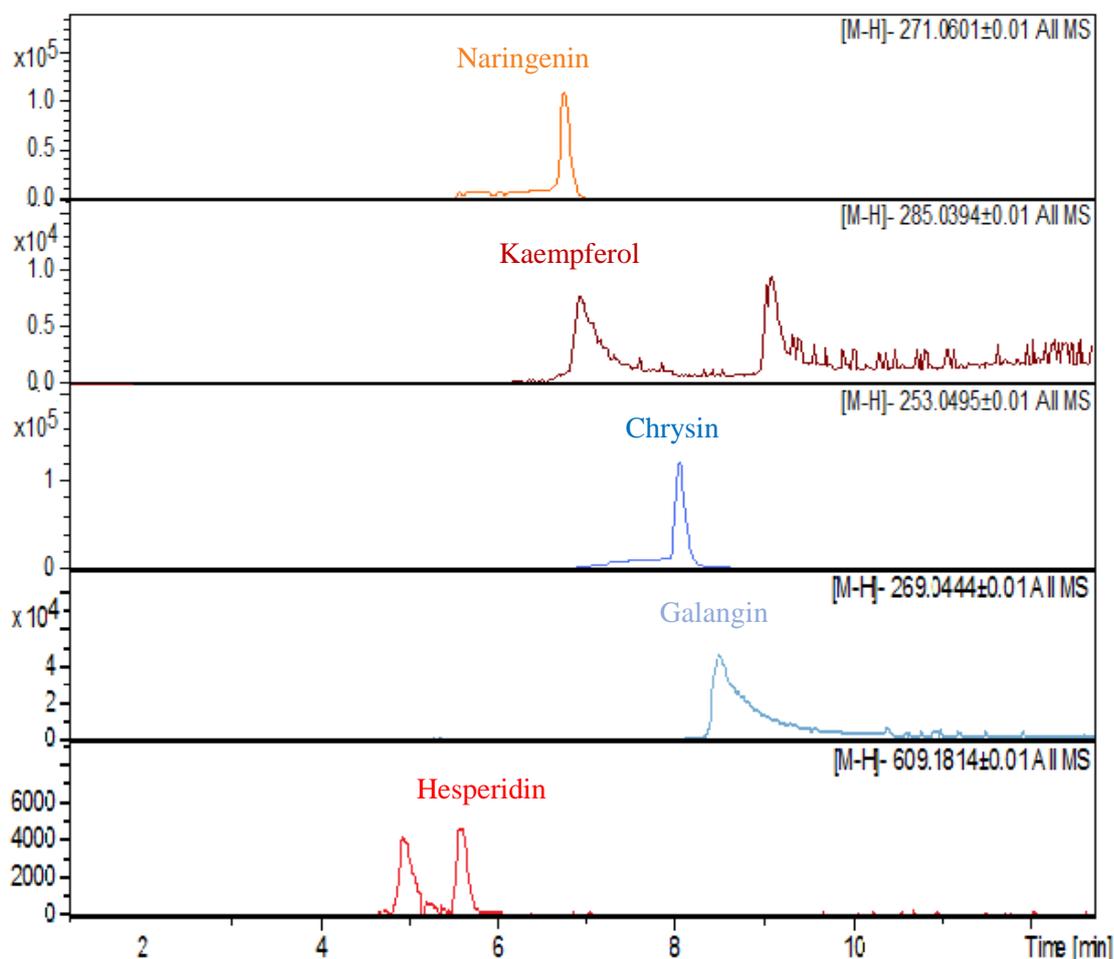


Figure 5. 16 continued: HPLC chromatogram of standard solutions

#### 5.4.6.2 HPLC Analysis –Honey and Plant Extracts

Phenolic compounds were identified in the 14 organic honey and plant extracts by comparing their retention times and mass similarities to that of the reference compounds. Chromatograms were obtained from the HPLC analysis (Figure 5.17 – 5.19), these were all analysed and data was tabulated (Table 5.11). The honey extracts had similar, but different, flavonoid profiles (Table 5.11). Due to the nature of the assay performed and time restrictions it was not possible to obtain fully quantitative data. Ideally the concentration of each compound would have been calculated. Despite this, the colour coordinated chromatograms were used to characterise the standards in each sample. Strong detection was symbolised at ‘++’ which represents an intensity of >500 on the chromatogram. Weak detection was represented by ‘+’ and this was assigned when the intensity ranged between 0 and 500. When there was no peak present ‘-’ was assigned in table 5.11.

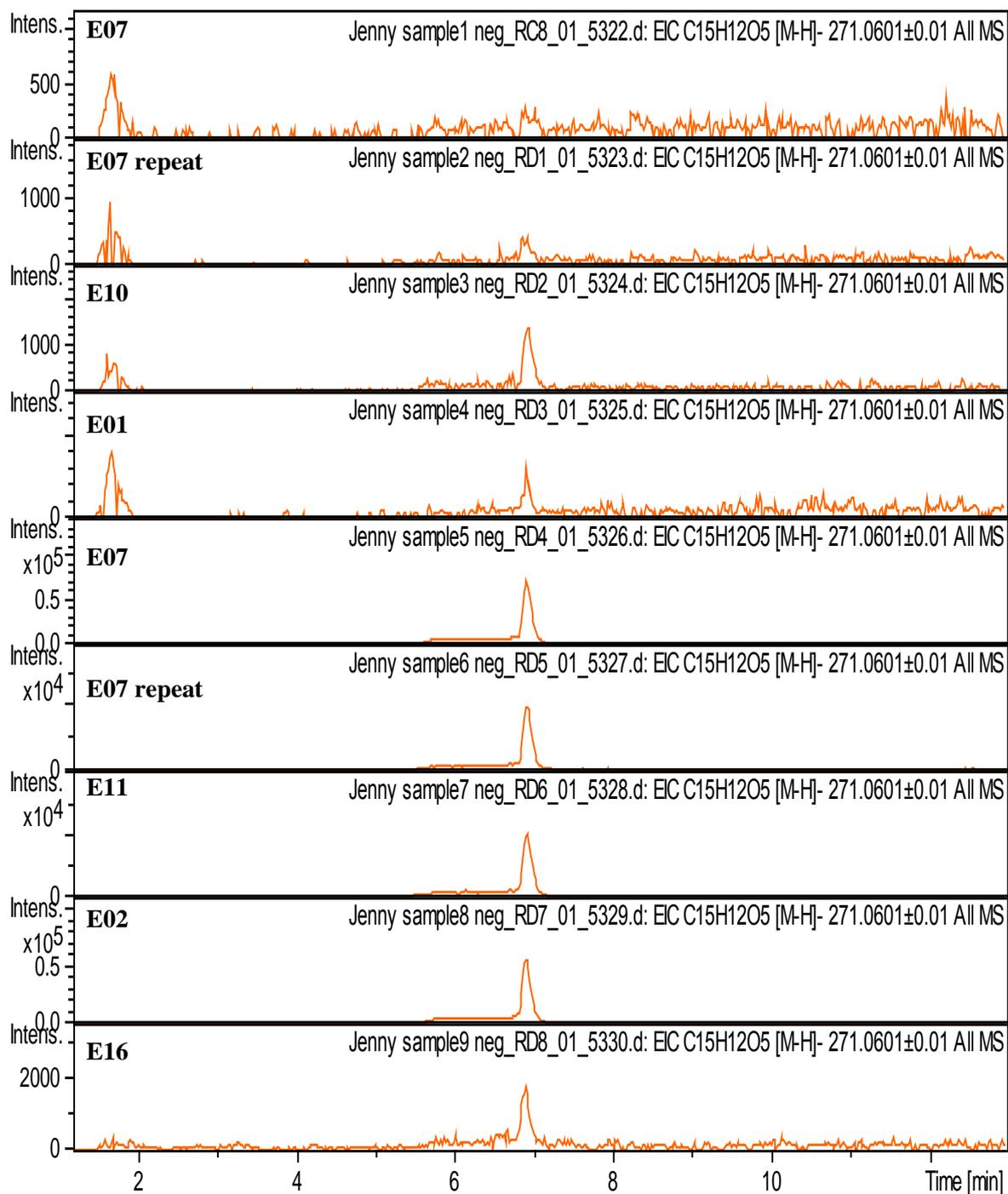


Figure 5. 17: Identification of Naringenin using HPLC in honey and plant extracts (negative mode)

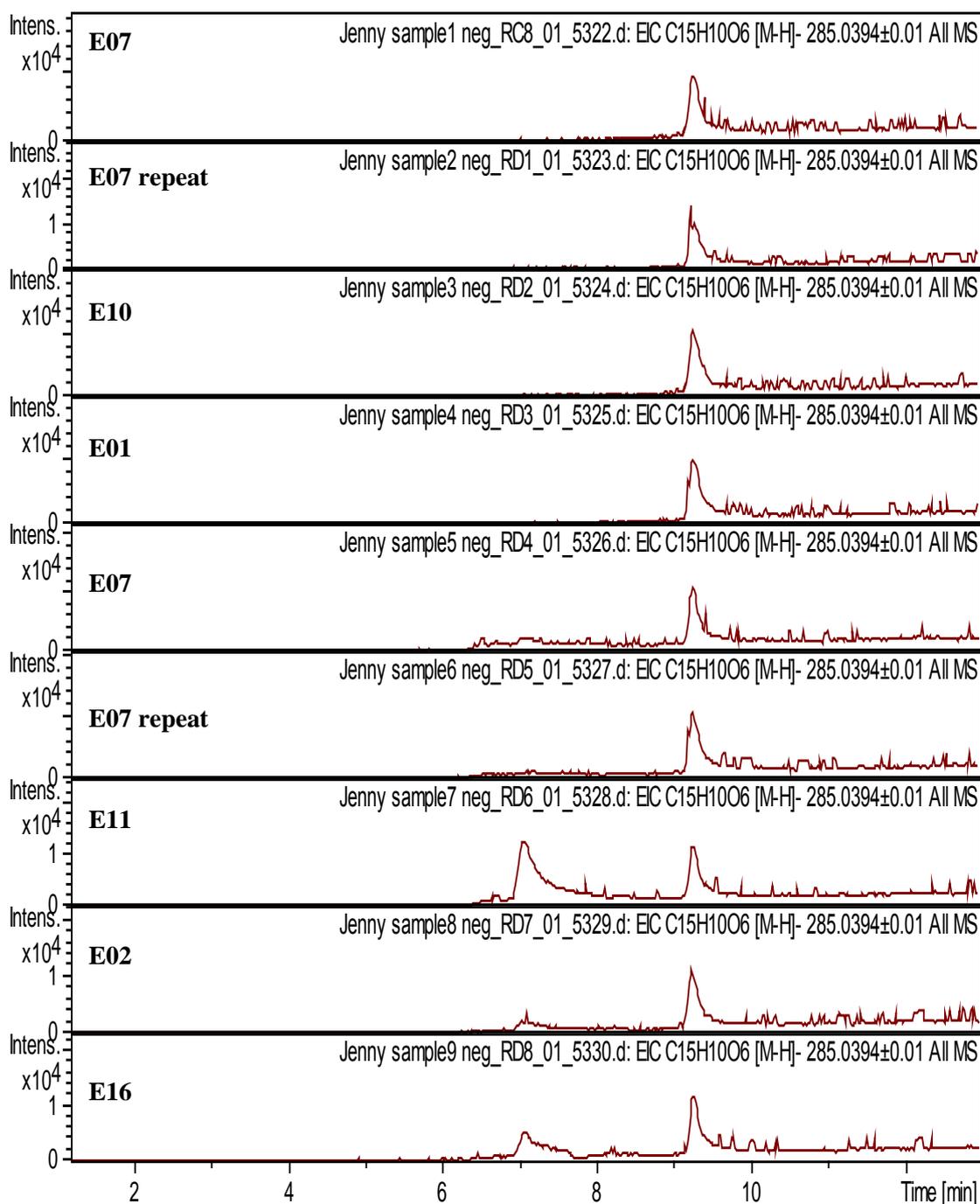


Figure 5. 18: Identification of Kaempferol using HPLC in honey and plant extracts (negative mode)

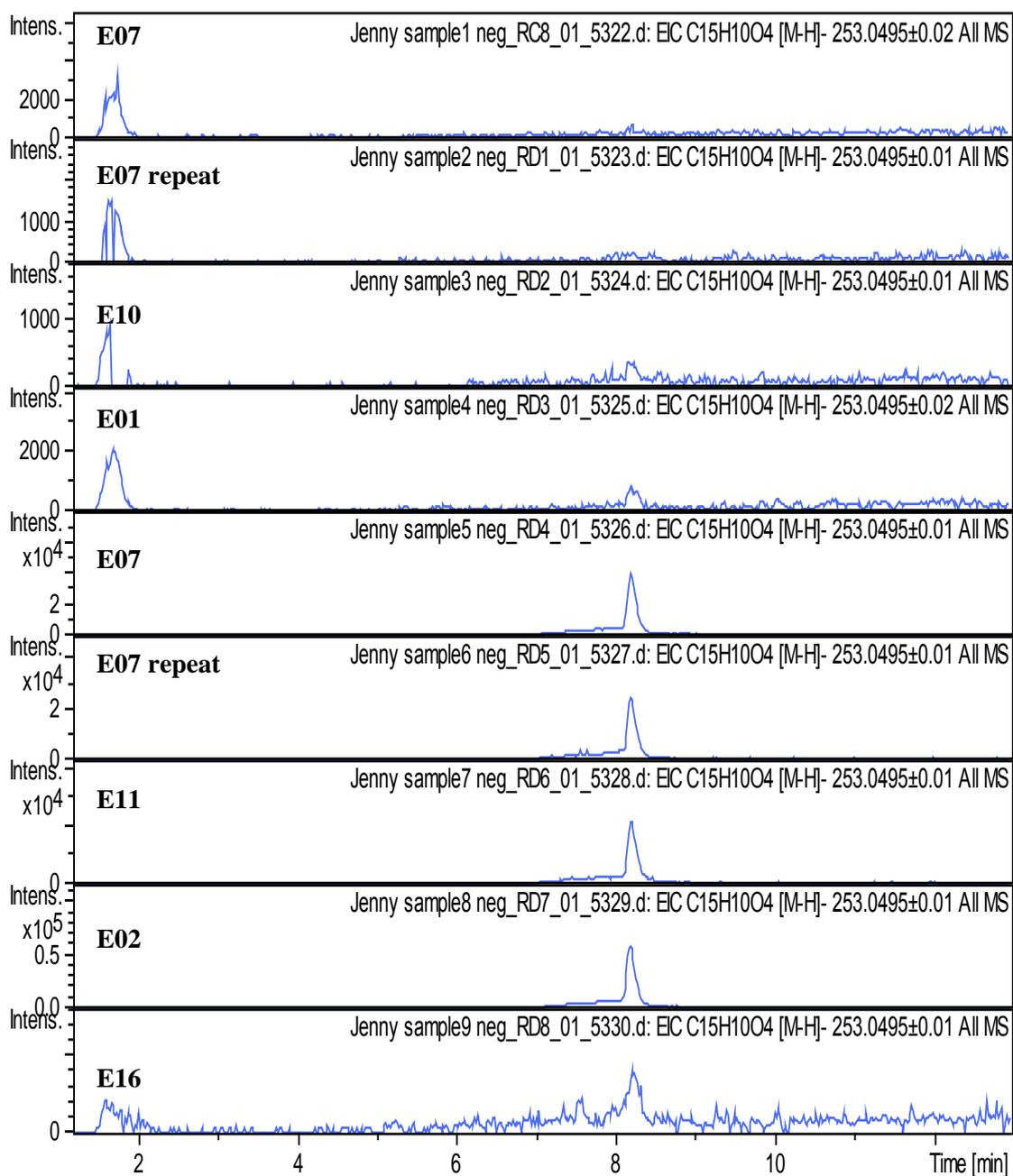


Figure 5. 19: Identification of Chrysin using HPLC in honey and plant extracts (negative mode)

Table 5. 11: The standards detected via HPLC analysis

Detection in the eight different honey extracts and six different plant extracts based on the peak intensity obtained from chromatograms (methanol and ethyl acetate) (>500 = ‘++’ strong detection, between 0 and 500 = ‘+’ weak detection, ‘-’ = not detected).

Extraction number	Sample Description	Naringenin POS	Naringenin NEG	Coumaric acid POS	Coumaric acid NEG	Kaempferol POS	Kaempferol NEG 9.3	Kaempferol NEG Both	Kaempferol NEG 7.9	Chrysin POS	Chrysin NEG	Syringic acid POS	Syringic acid NEG	Ferulic acid POS	Ferulic acid NEG	Caffeic acid POS	Caffeic acid NEG	Galangin POS	Galangin NEG	Hesperidin POS	Hesperidin NEG
E01	H20 Meth	-	++	++	-	++	++	-	-	-	+	++	-	++	-	++	-	+	-	-	-
E02	H20 E.A	++	++	++	-	++	-	++	-	++	++	-	-	++	-	++	-	++	-	-	-
E04	H24 Meth	-	++	++	-	++	++	-	-	-	++	++	-	++	-	++	-	-	-	-	-
E05	H24 E.A	++	++	++	-	++	-	++	-	++	++	++	-	++	-	++	-	++	-	-	-
E07	H54 Meth	-	++	++	-	++	++	-	-	-	+	++	-	++	-	++	-	+	-	-	-
E07	H54 Meth Repeat	-	++	++	-	++	++	-	-	-	+	++	-	++	-	++	-	+	-	-	-
E08	H54 E.A	++	++	++	-	++	++	-	-	++	++	++	-	++	-	++	-	++	-	-	-
E08	H54 E.A Repeat	+	++	++	-	++	+	-	-	++	++	++	-	++	-	++	-	++	-	-	-
E10	H201 Meth	-	++	++	-	++	++	-	-	-	+	+	-	++	-	++	-	-	-	-	-
E10	H201Meth Repeat	-	+	++	-	++	-	-	-	-	-	++	-	++	-	++	-	-	-	-	-
E11	H201 E.A	+	++	++	-	++	-	++	-	++	++	++	-	++	-	++	-	++	-	-	-
E13	Woodruff Meth	-	++	++	-	++	-	-	++	-	++	+	-	++	-	++	-	-	-	+	++
E14	Woodruff E.A	-	++	+	-	-	-	-	++	-	+	-	-	++	-	++	-	+	-	+	++
E16	Bluebell Meth	-	++	++	-	++	-	++	-	-	++	-	-	++	-	++	-	-	-	-	++
E17	Bluebell E.A	-	++	++	-	++	-	++	-	-	++	++	-	++	-	++	-	-	-	-	++
E19	Dandelion Meth	-	++	++	-	++	++	-	-	-	+	++	-	++	-	++	-	-	-	-	-
E20	Dandelion E.A	-	++	++	-	++	-	-	++	-	++	++	-	++	-	++	-	-	-	-	-

Naringenin, coumaric acid, caffeic acid, ferulic acid were detected in all fourteen extracts, highlighting their consistent presence within all the samples. Interestingly hesperidin was only detected in the woodruff extractions and bluebell extractions. Two repeats of E07, E08 and E10 were performed to ensure experimental consistency. The repeats highlighted similar results. Different reference compounds were detected and the concentration was semi-quantitatively assessed. Further work would be needed to fully clarify the concentration of the standards present in the different samples.

To compare the presence of the standards detected to the antibacterial activity a Pearson's correlation was performed as data followed a normal distribution. The level of detection (++ , + and -) was converted to numeric values for comparison (2, 1 and 0 respectively). A sum was subsequently calculated to determine the level of standards in each extract. A significant correlation was found between antimicrobial activity (zones of inhibition against MRSA) and the level of standards detected for the extracts ( $r = 0.735$ ;  $p < 0.01$ ;  $n = 21$ ). When examining the correlation across the 12 honey samples the correlation increased ( $r = 0.931$ ;  $p < 0.01$ ;  $n = 12$ ). There was no correlation when the MIC log values of all 21 extracts were analysed but there was a significant correlation when the MIC log values of the 12 honey extracts were compared to the level of standards detected ( $r = -0.726$ ;  $p < 0.05$ ;  $n = 12$ ). Suggesting the level of standard detected in the HPLC analysis related to the antibacterial activity of the extracts.

## 5.5 Discussion

The main aim of this chapter was to build on the information gained in chapter 3 and chapter 4 and to determine the identity of the antimicrobial compounds isolated from the active honey and characterised plant samples. Four honey samples were selected for analysis based on the results of the antibacterial screening assays described in chapter 3 (Table 3.8). H24 and H210 demonstrated antimicrobial activity which could not be attributed to any antimicrobial compounds previously described in literature. The H54 sample of Manuka honey also demonstrated non-peroxide antibacterial activity but this could be accredited to the presence of methylglyoxal (MGO). H20 was a Welsh heather honey with peroxide activity which was selected for comparison.

The plants of interest characterised in H24 in chapter 4 (Figure 4.5) by the 454 next generation sequencing analysis included woodruff (*Galium odoratum*), bluebell (*Hyacinthoides non-scripta*) and dandelion (*Taraxacum officinale*). While all three plants have a history of use as medicinal herbs their pharmaceutical applications and antibacterial activity have been investigated the individual compounds responsible for this activity has yet to be fully characterised (Schütz *et al.*, 2006, Izzo *et al.*, 1995, Mulholland *et al.*, 2013, Vlase *et al.*, 2014).

Three solvent extractions (methanol, ethyl acetate and hexane) were performed over a 72 hr period to ensure that no compounds were missed. By using all three solvent systems with a range of polarities, all compounds should be extracted and not overlooked. Amberlite XAD-2 separation was performed on the methanol extract to remove residual sugars and to concentrate the phenolic compounds, a method which had been previously performed by Kačániová *et al.*, (2011). A comparative study performed by Tomas-Barberan *et al.*, (1992) concluded that Amberlite XAD-2 was most appropriate for the solid phase extraction (SPE) of phenolic compounds from mixtures with significant levels of polar compounds (Tomás-Barberán *et al.*, 1992). While this approach has been shown to be an effective approach with which to recover kaempferol, p-coumaric acid and syringic acid its efficiency for quercetin was found to be only 54% suggesting that some compounds may be underrepresented using this approach (Michalkiewicz *et al.*, 2008).

The total yield of material from honey ( $n=12$ ) and plant ( $n=9$ ) extracts following methanol extraction and Amberlite separation was investigated. When comparing the solvent systems the methanol extracts produced significantly higher ( $p<0.05$ ) yields for both the honey and plant material. When comparing the overall yield obtained from honey extracts compared to plant extracts there was no significant difference.

The honey and plant extracts were subjected to broth dilution MIC and MBC analysis against Gram positive bacterium (MRSA) and Gram negative bacteria (*E. coli* and *P. aeruginosa*). Broth

analysis was chosen as non-polar compounds will diffuse more slowly than polar compounds in an agar diffusion assay thus giving the impression of reduced activity (Sánchez and Kouznetsov, 2010). Therefore a fair comparison of the hexane extract with concentrated levels of non-polar compounds could be analysed. From the MIC and MBC data (section 5.4.2) the Gram positive MRSA strain had higher susceptibility to the extracts than Gram negative bacteria (*S. aureus* > *E. coli* > *P. aeruginosa*). The same finding was described by Feás *et al.*, (2013) who investigated the antimicrobial activity of heather honey samples against eight clinically relevant strains of yeasts and bacteria (Feás *et al.*, 2013). Gram negative bacteria possess a unique hydrophilic outer membrane which is rich in lipopolysaccharides, presenting a barrier which can reduce the efficacy of certain antimicrobials (Delcour, 2009).

The potency of the plant extracts was higher than that of the honey extracts, suggesting the presence of more bioactive compounds or the presence of compounds with a higher level of antibacterial activity. By comparing the MIC and MBC values of H20, H24, H54 and H201, which have different mechanisms of action, their antibacterial activity could be assessed. There was no significant difference in the level of the antibacterial activity of the four honey samples against MRSA, possibly a reflection of the relative sensitivity of this bacteria to honey. In contrast statistical comparisons highlighted the potency of H24 and H201 against *E. coli*, and H54 against *P. aeruginosa* as they were all significantly ( $p < 0.05$ ) more antibacterial than H20. This could be due to the fact that H24, H201 and H54 owe their antibacterial activity to the presence of phytochemicals while the activity associated with H20 is due to the production of hydrogen peroxide.

It was also evident that ethyl acetate and hexane extracts had higher antibacterial activity than the methanol extracts. Plant-derived compounds have a broad range of polarities. Hexane is suitable for the extraction of non-polar compounds including waxes, oils, sterols and hydrocarbons (Manyi-Loh *et al.*, 2012). Previous studies have employed hexane extracts of honey for the detection of antibacterial compounds (Manyi-Loh *et al.*, 2012). Ethyl acetate solvent extraction is suitable for relatively polar compounds containing oxygen including polyphenols and flavonoids. Ethyl acetate extracts have been used to extract phenolic compounds (Cakir *et al.*, 2003). These compounds may be responsible for the high levels of antibacterial activity in the ethyl acetate extracts. The polarity of flavonoids varies, polar flavonoids have many hydroxyl groups C-OH in their structure, methanol was used for the extraction of highly polar compounds, Amberlite separation was performed to remove polar sugars from the methanol extracts but residual sugar may be responsible for the low level of antibacterial activity.

To identify the compounds that might be responsible for the antibacterial activity observed in the various extracts we employed a modified TLC/ bioautographic overlay based approach coupled with TLC/MS. Metabolic dyes, in the form of tetrazolium salts, were used to determine the

location of antibacterial bands on the TLC plates. The direct bioautographic approach allows target-directed isolation of biologically active molecules within a chromatogram. TLC bioautography has been known since 1946 but there has been a renewal of the application of this technique improving the results obtained (Guerrini and Sacchetti, 2014).

Previous studies have used bioautographic methods to detect antimicrobial compounds in propolis and plants (Suleimana *et al.*, 2010, Rahalison *et al.*, 1991, Farnesi *et al.*, 2009). Isla *et al.*, (2011) identified the antibacterial pinocembrin in honeys from North-western Argentina using the bioautographic method and comparing Rf values relative to standards (Isla *et al.*, 2011). TLC-bioautographic and GC-MS analysis have been used to characterise the bioactive compounds found in essential oils (Nickavar *et al.*, 2014) and leaf extracts (Annegowda *et al.*, 2013). These studies involved the physical removal of active bands from the TLC plate. The additional isolation and suspension step reduces compound yield and can potentially introduce contaminants.

In total 110 zones of inhibition were observed when all of the results from the honey and plant extracts ( $n=21$ ) against MRSA were combined, highlighting the sensitivity of this bacterium to antibacterial compounds. With the exception of one zone against *P. aeruginosa* (E16), none of the methanol extracts produced zones of inhibition against the Gram negative bacteria. In comparison with MRSA, the low permeability of the outer membrane of Gram negative bacteria restricts penetration of antimicrobials into the bacterium (Denyer and Maillard, 2002). Interestingly against *P. aeruginosa* only three zones of clearing were produced by the ethyl acetate and hexane extracts of H20. Whereas the H24, H54 and H201 extracts produced 8, 10 and 7 zones of inhibition respectively. This highlights the higher level of active compounds in H24, H54 and H201 compared to H20.

The results of the MIC and MBC data showed a significant correlation ( $p<0.01$ ) to the number of zones of inhibition observed using the bioautographic assays, suggesting active compounds were successfully separated and identified using the TLC bioautographic assay. The antibacterial activity of the extracts against clinically relevant isolates was concluded using both antimicrobial screening assays. On the bioautographic assay the methanol extracts produced the lowest number of antibacterial bands and MRSA was the most sensitive microorganism tested highlighting the similarities between the results obtained from the two techniques.

The Rf value and the area of the zone of inhibition calculated for each band using the bioautographic assays can also assist in the characterisation of bioactive compounds detected against *E. coli* and *P. aeruginosa*. In the results tables (section 5.4.4) numerous  $m/z$  values were detected at equal Rf values highlighting the need for further analysis to fully characterise these compounds which are active against *E. coli* and *P. aeruginosa*. Due to the nature of the TLC

analysis streaking or clumping of different compounds can occur, however several differences could be noticed and TLC-MS characterised the individual bands of activity against MRSA.

In this study TLC, the bioautographic MRSA assay and the TLC-MS interface were combined and it was possible to directly allocate  $m/z$  values to active bands without the need for further separation. Individual spots were directly assign  $m/z$  values using the TLC-MS interface. The  $m/z$  values obtained were compared to literature describing the previous characterisation of honey, propolis and plant-derived compounds (Gašić *et al.*, 2014, Gardana *et al.*, 2007, Falcao *et al.*, 2010). The species  $m/z$  329 was detected eleven times across the four honeys and twelve times in the three plants. It was detected in all four hexane extracts (E03, E05, E08 and E11), and it was also detected in the ethyl acetate extracts of all honeys except H20, the honey which only had peroxide activity in chapter 3 (Table 3.8).

The  $[M+H]^+$  species 329 is reported to occur in honey and propolis and has been identified as pinobanksin derivatives ( $M=328$ ), pinobanksin-3-O-propionate and pinobanksin-5-methyl-ether-3-acetate (Gardana *et al.*, 2007, Chua *et al.*, 2013, Falcao *et al.*, 2010, Gašić *et al.*, 2014). Pinobanksin (3,5,7-trihydroxyflavanol) and its derivatives are antioxidant flavonoids which possess antibacterial activity (Chua *et al.*, 2013, Gardana *et al.*, 2007) and thus may be responsible for the large number of the antibacterial bands detected in the screening assay.

Pinobanksin is commonly found in propolis which is a resinous substance produced by the bees from certain trees, traces of propolis is also found in honey. Pinobanksin has been characterised in Poplar *spp.*, Pinus *spp.* and *B. dracunculifolia* resin, pinobanksin demonstrated antibacterial activity (Park *et al.*, 2002, English *et al.*, 1992, Vardar-Ünlü *et al.*, 2008). Pinobanksin is produced from pinocembrin, by hydroxylation adjacent to the ketone. Pinocembrin (5,7-dihydroxyflavanone) is one of the primary flavonoids isolated from the variety of plants including Lauraceae and Asteraceae families. The  $[M+H]^+$  species 329 was common in all three plant extracts, based on a search of the relevant literature pinobanksin has not previously been extracted from the flowers of woodruff, bluebell or dandelion, however methodologies vary significantly and their phytochemical profiles have not been fully characterised. As this  $m/z$  value was detected in all three samples further plant species would need to be analysed to determine if these results were unique to the three plants analysed in this study.

To fully characterise the flavonoid, ideally the TLC-MS interface would be linked to high resolution mass spectrometry (HRMS) to obtain highly accurate  $m/z$  values and fragmentation analysis. More detailed structural information has been reported by application of tandem mass spectrometry (MS-MS) (Pyrzynska and Biesaga, 2009). MS-MS could be used to determine if the 287  $m/z$  value was Kaempferol, luteolin or pinobanksin-5-methyl-ether. Gardana *et al.*, (2007) characterised the two pinobanksin derivatives, extracted from propolis, using liquid

chromatography–tandem mass spectrometry with MS-MS analysis as shown in figure 5.18 (Gardana *et al.*, 2007).

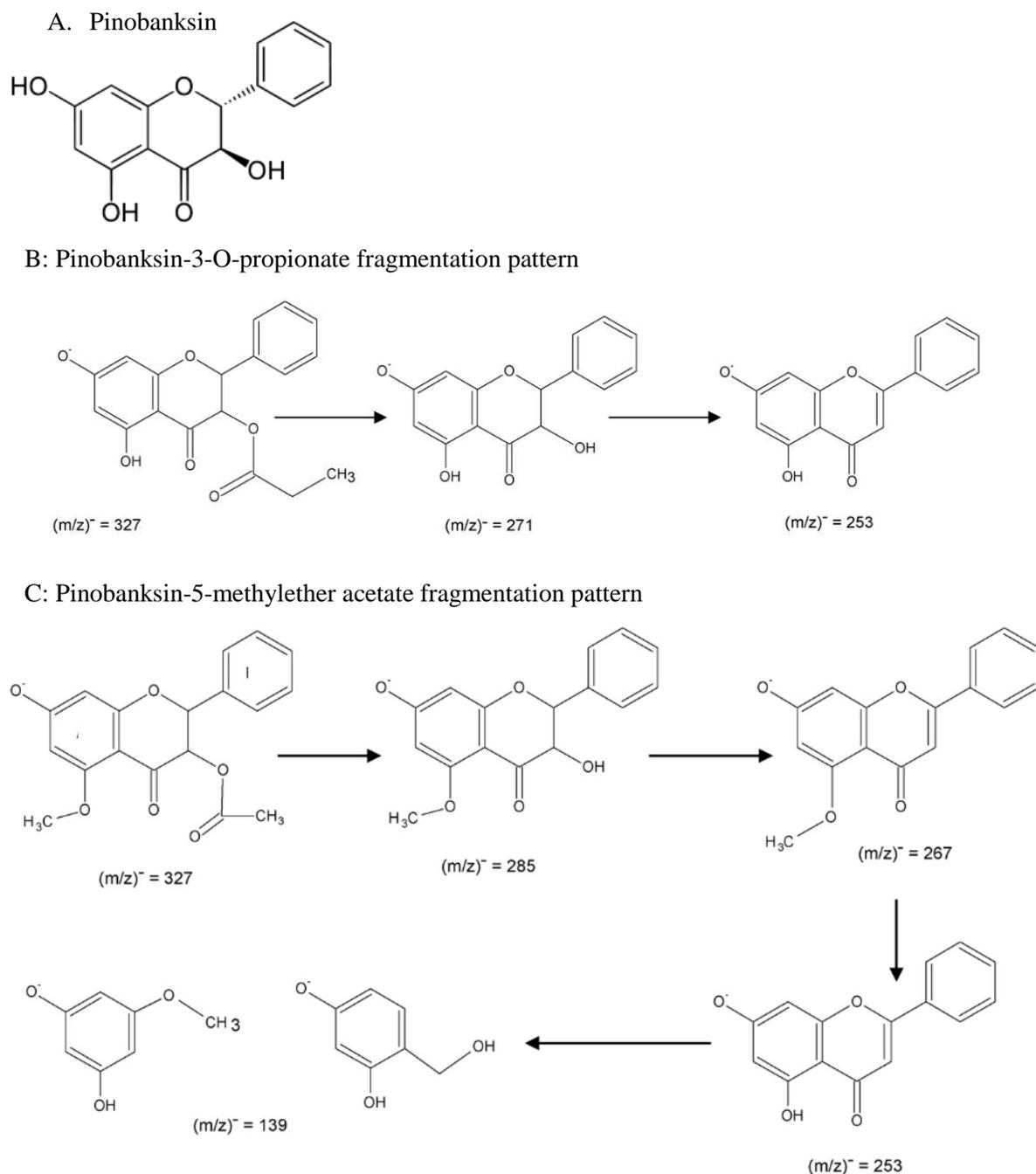


Figure 5. 20: Chemical structure of pinobanksin ( $m/z$ 329) and the fragmentation pattern of the two pinobanksin derivatives attributed to an  $m/z$  of 328.

Other  $m/z$  values observed could not be characterised based on a literature search. Presuming  $[M+H]^+$  ionisation,  $m/z$  387 and 430 which were detected in the extracts offer promising leads and warrant further investigation. With the aim of characterising these unknown compounds bands were extracted from TLC plates and resuspended in organic solvent to elute the desired compounds. The aim was to recover sufficient material to perform nuclear magnetic resonance spectroscopy (NMR), unfortunately insufficient material was obtained and at the necessary level

of purity to support this analysis. A scaled-up extraction would be required to obtain sufficient yield from the TLC separation method.

The primary method for analyte identification is often a chromatographic method coupled with high or ultra-pressure liquid chromatography (HPLC/UPLC). These techniques can also be coupled to MS or NMR for detection of new compounds. Many studies have used HPLC or capillary electrophoretic (CE) for the analysis of phenolics and flavonoids in honey and plants, as summarised by Pyrzynska and Biesaga (2009). HPLC analysis revealed the presence of the 13 known compounds across all of the extracts examined in this study. These compounds have been commonly identified in both honey and also plant extracts (Dias *et al.*, 2014, Schütz *et al.*, 2006). When the antibacterial activity of the overlay assay was compared to the number of known phenolic compounds which were detected, a statistically significant match was obtained, suggesting phenolic compounds may be contributing to the antibacterial activity of the honey samples. For a reliable clarification of a novel natural product compound it would be necessary to combine MS with other spectroscopic techniques (e.g. UV and NMR) (Pyrzynska and Biesaga, 2009). Before this could happen the extraction and purification protocols employed in this study would require further optimisation.

In conclusion this chapter highlights the main difficulties of natural product separation and identification. Successful isolation, separation and characterisation of antibacterial compounds in honey and plant extracts has been achieved. By combining traditional and more recently developed chemical techniques, this chapter reports differences in the growth inhibitory potency of various solvent extracts, their antimicrobial effect on different clinically relevant bacteria and differences in the potency between different honeys and plant species. The antibacterial activity of H<sub>2</sub>O<sub>2</sub>, which only displayed peroxide activity in chapter 3, was not as potent as the three other honeys tested; confirming the high levels of non-peroxide activity in the three other samples. The direct analysis using a TLC-MS interface accelerates characterisation and improves the quality of analysis. In this study, these rediscovered research methods were used as an activity guided screening strategy to determine whether novel compounds could be attributed to any biological activity detected. To date, few studies have utilised these techniques for the identification of natural products within honey.

Finally, the principle of the methods used has been proven by isolating and tentatively characterising antimicrobial compounds. Plant-originated bioactive components were transferred to honey by foraging bees. Several unidentified compounds which were directly associated with the antibacterial fraction of honey and plant extract may provide leads for novel compounds or derivatives. By combining bioautography and TLC-MS analysis, activity-guided separation could be performed an efficient method in terms of both time and resources. These are promising leads but would need future investigation to purify in greater yields to allow full characterisation.

# Chapter 6

## **6. GENERAL DISCUSSION**

## 6.1 General Discussion

Honey and medicinal plants have long been utilised for their healing powers and it is therefore unsurprising that in the present day nearly 50% of all pharmaceuticals worldwide have originated from plant derived sources (Beutler, 2009, Cragg and Newman, 2013). The composition of honey is strongly associated with its botanical and geographical origin and understanding the floral composition of honey has a wide variety of applications (Kaškonienė and Venskutonis, 2010). While the antimicrobial activity of individual honey polyphenols has been studied (Pyrzynska and Biesaga, 2009, Aljadi and Yusoff, 2002, Estevinho *et al.*, 2008, Silici *et al.*, 2010, Isla *et al.*, 2011) a comparable and comprehensive characterisation of the activity of these compounds has yet to be undertaken.

Published studies investigating the antimicrobial activity of honey have employed a range of different methods which makes comparisons of the results generated by these different studies almost impossible (Carina *et al.*, 2014, Kwakman and Zaat, 2012). The aim of this study was to develop optimised antimicrobial screening assays which could be used to screen individual honey samples for the presence of antibacterial compounds. The identification of the specific compounds responsible for this activity was subsequently performed through chemical analysis. A DNA sequencing approach was also developed in order to identify those plants which were the original source of antimicrobial compounds. During the optimisation of the antimicrobial screening assay it was discovered that different culture media components interfered with antibacterial activity. These findings highlighted the need to consider the role of culture media when seeking to assess the antibacterial activity of honey and its derivatives. For this reason an LB agar diffusion assay was developed which was used to screen 220 UK honey samples for antibacterial activity. MRSA was used as a model organism to assess the antibacterial activity of the natural honeys. Of this total, 194 (88%) demonstrated some level of antibacterial activity.

Honey contains two distinct mechanisms of antimicrobial activity, peroxide and non-peroxide based (Brudzynski *et al.*, 2011, Kwakman *et al.*, 2011b). The peroxide based activity is thought to be due to the action of glucose oxidase which is an enzyme produced by bees. (White *et al.*, 1963), this mechanism has now been fully described (Bucekova *et al.*, 2014). The level of hydrogen peroxide present in honey is also effected by the level of natural catalase in pollen (Weston, 2000). The non-peroxide activity has been linked to the presence of phytochemicals such as MGO which is derived from the Manuka plant. MGO is responsible for the pronounced antibacterial activity of Manuka honey (Mavric *et al.*, 2008, Adams *et al.*, 2009, Stephens *et al.*, 2010).

To determine the basis of the antibacterial activity seen in the honey samples a series of methods developed by Kwakman *et al.*, (2010) were employed. These methods allowed antibacterial

factors (hydrogen peroxide, MGO, Defensin-1 and pH) to be successively neutralised. Often the difficulty with fully characterising activity is the numerous antibacterial factors in honey but by successively neutralising each component and reassessing the residual activity all factors were taken into consideration. This ensured a thorough and comprehensive study was performed. Unlike the findings described by Cooper and Wheat (2008) four of the honey samples screened (H24, H139, H180 and H201) owed their antibacterial activity, in part, to non-peroxide compounds other than MGO. The fact that the activity of these compounds was effected by pH suggests that they may be acidic in nature.

The chemical composition of a particular honey will vary depending on the floral sources of the nectar and pollen collected by honey bees (Dong *et al.*, 2013, Kaškonienė and Venskutonis, 2010, Tomás-Barberán *et al.*, 2001). By characterising the plants which contribute to the making of a honey sample further information can be obtained about the phytochemicals which may be responsible for the antibacterial activity. A number of chemical methods have been used to investigate the floral composition of honey; polyphenolic profiles, physico-chemical properties (e.g., pH, conductivity and sugar), amino acid and protein content, volatile compounds and trace elements have all been studied (Bogdanov *et al.*, 2004). Reliable characterisation of honey requires the classification of more than one class of compounds, preferably in combination (Kaškonienė and Venskutonis, 2010).

Melissopalynology has been routinely employed since it was first introduced by Louveaux *et al.*, (1978) for pollen analysis and floral characterisation (Louveaux *et al.*, 1978). Microscopic analysis is unsuitable for rapid, high throughput analysis of pollen, the approach is time-consuming, requires specialist knowledge and expertise and has a laborious counting procedure. DNA metabarcoding and NGS technologies have the potential to revolutionise the capacity of traditional pollen analysis. NGS analysis of pollen does not require a high level of taxonomic expertise, a greater sample size can be screened and processing time is significantly reduced.

Researchers have utilised molecular techniques to analyse pollen and the floral composition of honey using plant specific primers and probes (Laube *et al.*, 2010) and DNA barcoding (Valentini *et al.*, 2010, Olivieri *et al.*, 2012, Bruni *et al.*, 2015, Galimberti *et al.*, 2014). To date few studies have fully utilised these DNA based approaches and as a consequence protocols have yet to be fully optimised (Soares *et al.*, 2015, Guertler *et al.*, 2014, Lalmangaihi *et al.*, 2014). To our knowledge this is the first study which has attempted to combine traditional melissopalynology with NGS DNA barcoding technologies (Roche 454 and Illumina) to characterise DNA from honey.

In this study, melissopalynology and DNA based methods were used to characterise the floral profiles of honey samples which demonstrated antibacterial activity. The universal primer *rbcL*

was utilised for the DNA barcoding of UK honey samples using Roche 454 and Illumina technology. Comprehensive DNA plant profiles were produced for ten of the honeys using each of the NGS techniques. This represents the largest study to date in which DNA based methods were used to characterise the pollen content of honey. In previous studies Bruni *et al.*, (2014) characterised four samples while Valentini *et al.*, (2010) only looked at two samples (Bruni *et al.*, 2015, Valentini *et al.*, 2010).

Microscopy and DNA based methods were effective methods with which to characterise the pollen content of honey. A comparison was made between the Melissopalynology, 454 and Illumina results. There were significant differences in the results obtained with each technique. The possible reasons for these differences are numerous and are likely to reflect problems in sampling strategy, sample diversity, methodological bias and data processing. This difference may reflect the relative differences in the number of pollen grains which were examined using the two approaches. Melissopalynology analysis is based on the characterisation of ~300 pollen grains from 2 g of honey. The DNA approach is not limited by laborious counting procedures and 40 g of each honey was analysed. These differences are also effected by the high level of discrimination obtained with NGS compared to microscopy, abundant plant taxa were successfully detected using all three techniques.

Different levels of plant taxa were detected across all three techniques. Many of the plant taxa that were detected using microscope analysis were not detected by DNA barcoding. Many of these taxa were represented by single pollen grains. With DNA barcoding lower abundance species may not yield amplicons, therefore the NGS sequencing approach is not suitable for the identification of species with <1% abundance within a sample (Hajibabaei *et al.*, 2011). While melissopalynology provides a tried and tested method, DNA metabarcoding provides a promising new tool for pollen identification. The level of discrimination to family level was vastly improved using metabarcoding. By performing repeat sampling it could be concluded that 454 DNA metabarcoding also has a much higher reproducibility when compared to melissopalynology with 63% compared to 28% similarity respectively.

Given that one of the main aims of this project was to identify the plants which were the source of the antimicrobial phytochemicals detected in the active honeys the characterisation was focused on the pollen from the two UK honeys (H24 and H201). These two honeys were shown to contain non-peroxide based antibacterial activity (chapter 3, table 3.8). The dominant plant taxa identified in H24 were bluebell (*Hyacinthoides non-scripta*), dandelion (*Taraxacum officinale*) and woodruff (*Galium odoratum*). Dandelion represented the dominant taxa in H201. Further investigations of dandelion, bluebell and woodruff were subsequently performed to determine if these plants were contributing to the antibacterial activity of these honeys.

The extraction of phenolic compounds from honey involves sampling a representative sample, homogenisation and extraction (organic solvents or SPE) for the removal of sugars and preconcentration of compounds (Pyrzynska and Biesaga, 2009). To identify individual antibacterial compounds in the honey and plants identified in this study a solvent based extraction strategy coupled with TLC, MS and HPLC was employed. Honey and plant extracts were rich in phenolic compounds, the extracts had a broad spectrum of antimicrobial activity. Antibacterial activity was detected in the ethyl acetate and hexane extracts against MRSA, *E. coli* and *P. aeruginosa* using a broth microdilution assay and the TLC/bioautography overlay methods. This suggests non-polar compounds extracted in non-polar solvents have the highest level of antibacterial activity.

A range of analytical techniques have been used to determine the phenolic profile of honey and plant extracts. Separation techniques commonly include HPLC and EC, coupled with a diode-array detector (DAD) and advanced MS techniques (Pyrzynska and Biesaga, 2009). The HPLC analysis revealed a correlation between the number of phenolic compounds identified and the antibacterial activity of the extracts, but further investigations would be required to fully characterise the source of the activity.

In a recent study of honey samples from Northwest Argentina, Isla *et al.*, (2011) employed a similar approach to that used in this study. TLC/bioautography overlay was used to detect and identify the presence of antibacterial compounds, they were able to tentatively identify individual compounds such as pinocembrin (Isla *et al.*, 2011). In this study TLC/bioautography was combined with MS, an approach which has previously been used to characterise phenolic compounds as markers with which to determine the botanical origin of propolis (Bertrams *et al.*, 2013, Kasote *et al.*, 2015). Using an approach very similar to that used in this study Kasote *et al.*, (2015) identified pinocembrin, pinobanksin and possibly pinobanksin-3-O-pentanoate as antibacterial compounds within a sample of South African propolis (Kasote *et al.*, 2015). The TLC/MS interface offers an efficient, rapid approach to the separation and characterisation of bioactive compounds.

While it was not possible to confirm the identity of all the antibacterial compounds in the honey and plant samples an indication as to the possible identity of at least some of these compounds was obtained. These included pinobanksin-3-propionate and pinobanksin-5-methylether acetate. These findings support those of Kasote and Isla and provide further evidence as to the antibacterial activity of this class of compounds in honey (Isla *et al.*, 2011, Kasote *et al.*, 2015).

It has been suggested that while pinobanksin contributes to the antibacterial activity of honey and propolis its only represents a minor component (Bogdanov, 1989). While this may be the case it does not negate the fact that this compound represents a potential lead for drug development. In

addition to pinobanksin other antibacterial compounds were detected in the samples for which a homolog could not be detected in the literature suggesting that they may represent novel compounds. Further work will be required to fully characterise these promising leads.

The detection of some compounds in the honey extracts that were also seen in the plant extracts suggests DNA based pollen analysis succeed in its aim to identify the plants which were the possible source of the antibacterial compounds. It is unknown whether these compounds may be abundant in all flowering plants and compounds would need to be fully characterised to confirm this hypothesis.

Ultimately, combining this bioautographic approach for multiple honey and plant samples provides a powerful tool for rapid activity-targeted characterisation for phenolic analysis. There is significant evidence that a pinobanksin derivative may be contributing to the antibacterial activity of the honey samples. Due to the large number of antibacterial bands detected on the overlay assay, some of which could not be characterised, it would be fair to suggest different compounds are also contributing to the overall antibacterial activity.

## **6.2 Limitations and Future Work**

The composition of honey is dependent on storage and time of collection and the chemical composition of every honey sample is unique, making it difficult to fully characterise its activity. Despite optimising the antimicrobial screening assays (agar diffusion, broth dilution and TLC bioautography) there may be compounds which are too dilute to detect due to the sensitivity of the methods. Further investigations could also be preformed of different strains of bacteria or more strains of MRSA to broaden the depth of these findings.

The DNA barcoding method is a robust, effective method for the characterisation of plant species. However none of the DNA marker regions work for every plant species and the level of discrimination varies (Li *et al.*, 2015). The Plant Working Group (PWG) of the Consortium for the Barcoding of Life (CBOL) pioneered the use of universally agreed DNA regions *matK* and *rbcL* for the identification of plants (CBOL Plant Working Group *et al.*, 2009). This study utilised the *rbcL* region which exhibits some limitation in discrimination power (Hollingsworth *et al.*, 2011) however this marker provides a useful backbone for barcoding as *rbcL* is relatively easy to amplify. Many *rbcL* sequences are also available in the Genbank database compared to the other markers (Li *et al.*, 2015).

Valentini *et al.* (2010) originally used the *trnL* approach to characterise plants isolated from honey (Valentini *et al.*, 2010). More recently Bruni *et al.*, (2014) and Galimberti *et al.*, (2014) concluded that the *rbcL* and *trnH-psbA* plastid regions were most effective in achieving maximum universality and highest discrimination of pollen collected from honey and bees (Bruni *et al.*,

2015, Galimberti *et al.*, 2014). For future analysis the use of *rbcL* in combination with other plastid or nuclear loci would improve the discrimination and quality of the results.

The main limitation of the barcoding approach is the semi-quantitative characterisation of plant flora, the exact number of detectable biological units is effected by PCR target-associated biases (Bruni *et al.*, 2015, Berry *et al.*, 2011). Species of higher abundance or higher affinity for primer binding sites, capture more primer (Hajibabaei *et al.*, 2011), it is therefore unfeasible to quantify the number of sequences in terms of biological units. In future work, additional molecular techniques, such as real-time PCR based on sequence characterised amplified regions (SCARs) could be used to obtain relative abundances for plant species (Bruni *et al.*, 2015). To account for sequencing or PCR error, all unique sequences represented by <10 reads were removed from the data set ensuring only data of good quality is used. Pyrosequencing-specific errors, sometimes termed “sequencing noise” (Větrovský and Baldrian, 2013) are minimised by rigorous data processing, by removing sequences of low quality and below 250bp. The Illumina sequencing also lessened this issue due to the higher number of reads and increased sensitivity.

To improve extraction, amplification and sequencing success further optimisation of the DNA extraction step could be performed. A recent study by Soares *et al.*, (2015) demonstrated the superior efficacy of the Wizard extraction method and automated DNA extraction proposed by Guertler *et al.*, (2014) was faster and resulted in higher DNA yield and sufficient DNA purity for successful amplification (Guertler *et al.*, 2014, Soares *et al.*, 2015). By improving the quality and yield of the DNA the number of PCR cycles could subsequently be reduced; minimizing PCR errors and biases which become more pronounced as more cycles are performed (Wu *et al.*, 2010). These methodologies could be explored in future studies.

Further investigations would need to be performed to determine the relationship and the overall antibacterial effect of the compounds in this study. There may be a synergistic effect occurring and this would require further analysis to draw comprehensive conclusions. High resolution MS (HSMS) and Tandem MS (MS-MS) with fragmentation analysis would improve the chances of characterising novel compounds. MS analysis can also be combined with additional spectroscopic techniques including UV, fluorescence and NMR for phenolic characterisation. In a complex mixture such as honey the array of compounds can make separation difficult even when separation is based on polarity or molecular size. If separation is achieved, another problem that can arise is achieving a sufficient compound weight for identification purposes, as seen in this study. Ideally, optimised extraction techniques would be performed; further validating methodologies and ensuring enhanced levels of recovery and repeatability. By combining HPLC with advanced MS and NMR full characterisation of novel natural products from honey or plant extracts would be possible (Pyrzynska and Biesaga, 2009).

### 6.3 Concluding Remarks

Whilst the antibacterial properties of honey have been known for years, a comprehensive analysis of honey derived phenolic compounds and their antimicrobial activity has yet to be undertaken. The aim of this thesis was to identify, isolate and characterise plant-derived antibacterial compounds that have the potential to be developed as therapeutics for the treatment of clinically relevant pathogens.

Using the screening assay developed during this study (chapter 3) four UK honey samples (H24, H139, H180 and H201) out of 194 owed their antibacterial activity, in part, to activity of unknown compounds. These samples were obtained from non-commercial hives, three from a hive in Aberdovey and one from a hive in Southampton. These active compounds are likely to be plant-derived chemical components, however synergy, environmental factors or the processing performed by the honey bee may be contributing to their potency. The fact that the activity of these compounds was affected by a decrease in acidity suggests that the activity of the compounds are affected by pH. The discovery of this novel activity warranted further investigation.

The phenolic profile is variable and primarily dependent on the botanical and geographical origin of the flora which contributed to the making of the honey. A combination of microscopy and novel DNA based sequencing approaches were employed to identify the pollen grains which had been deposited in the honey. From the range of plants identified dandelion, bluebell and woodruff were selected for further characterisation as they were the plants in high abundance in the active sample H24 and H201. Chemical characterisation of honey and plants samples identified the presence of a number of antibacterial compounds which were common to both, these included known pinobanksin derivatives and unknown compounds suggesting that these plants may be the original source of these compounds.

From the results it appears the antibacterial activity of these samples is multifactorial, hydrogen peroxide, low pH, high sugar content and phenolic compounds are all contributing to the activity. This warrants further investigation to fully characterise the unknown compounds. By combining microbiological screening with analytical chemistry and DNA sequencing an approach which is capable of identifying potentially novel plant derived antibacterial compounds has been developed. These unknown compounds or combinations of phenolic compounds may prove to be useful natural antimicrobials with therapeutic applications in an era where antibiotic resistance is becoming ever more prevalent. Further work is required to refine the sensitivity and specificity of the system and to fully characterise the compounds which have been identified in this study.

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# 8. APPENDIX

## Appendix A

Paper accepted and due to be published on the 26<sup>th</sup> of August 2015.

### PLOS ONE

#### Using DNA metabarcoding to identify the floral composition of honey: A new tool for investigating honey bee foraging preferences —Manuscript Draft—

<b>Manuscript Number:</b>	PONE-D-15-12624R1
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<b>Full Title:</b>	Using DNA metabarcoding to identify the floral composition of honey: A new tool for investigating honey bee foraging preferences
<b>Short Title:</b>	Floral composition of honey.
<b>Corresponding Author:</b>	Natasha de Vere National Botanic Garden of Wales Carmarthenshire, UNITED KINGDOM
<b>Keywords:</b>	honey bee; <i>Apis mellifera</i> ; DNA metabarcoding; rbcL; honey; plant diversity; pollen; 454 pyrosequencing; floral composition; high throughput sequencing; melissopalynology; foraging preferences; pollinators; insects; pollination
<b>Abstract:</b>	<p>Identifying the floral composition of honey provides a method for investigating the plants that honey bees visit. We compared melissopalynology, where pollen grains retrieved from honey are identified morphologically, with a DNA metabarcoding approach using the rbcL DNA barcode marker and 454-pyrosequencing. We compared nine honeys supplied by beekeepers in the UK.</p> <p>DNA metabarcoding and melissopalynology were able to detect the most abundant floral components of honey. There was 92% correspondence for the plant taxa that had an abundance of over 20%. However, the level of similarity when all taxa were compared was lower, ranging from 22-45%, and there was little correspondence between the relative abundance of taxa found using the two techniques. DNA metabarcoding provided much greater repeatability, with a 64% taxa match compared to 28% with melissopalynology.</p> <p>DNA metabarcoding has the advantage over melissopalynology in that it does not require a high level of taxonomic expertise, a greater sample size can be screened and it provides greater resolution for some plant families. However, it does not provide a quantitative approach and pollen present in low levels are less likely to be detected.</p> <p>We investigated the plants that were frequently used by honey bees by examining the results obtained from both techniques. Plants with a broad taxonomic range were detected, covering 46 families and 25 orders, but a relatively small number of plants were consistently seen across multiple honey samples. Frequently found herbaceous species were <i>Rubus fruticosus</i>, <i>Filipendula ulmaria</i>, <i>Taraxacum officinale</i>, <i>Tritolium</i> spp., <i>Brassica</i> spp. and the non-native, invasive, <i>Impatiens glandulifera</i>. Tree pollen was frequently seen belonging to <i>Castanea sativa</i>, <i>Crataegus monogyna</i> and species of <i>Malus</i>, <i>Salix</i> and <i>Quercus</i>.</p> <p>We conclude that although honey bees are considered to be supergeneralists in their foraging choices, there are certain key species or plant groups that are particularly important in the honey bees environment. The reasons for this require further investigation in order to better understand honey bee nutritional requirements.</p> <p>DNA metabarcoding can be easily and widely used to investigate floral visitation in honey bees and can be adapted for use with other insects. It provides a starting point for investigating how we can better provide for the insects that we rely upon for pollination.</p>
<b>Order of Authors:</b>	Jennifer Hawkins Natasha de Vere Adelaide Griffith

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## Appendix B

Refractive index conversion table for the calculation of honey water content proposed by the International Honey Commission (68).

<b>Water Content,</b>	<b>Refractive Index</b>	<b>Water Content</b>	<b>Refractive Index</b>
g/100 g	20°C	g/100 g	20°C
13.0	1.5044	19.0	1.4890
13.2	1.5038	19.2	1.4885
13.4	1.5033	19.4	1.4880
13.6	1.5028	19.6	1.4875
13.8	1.5023	19.8	1.4870
14.0	1.5018	20.0	1.4865
14.2	1.5012	20.2	1.4860
14.4	1.5007	20.4	1.4855
14.6	1.5002	20.6	1.4850
14.8	1.4997	20.8	1.4845
15.0	1.4992	21.0	1.4840
15.2	1.4987	21.2	1.4835
15.4	1.4982	21.4	1.4830
15.6	1.4976	21.6	1.4825
15.8	1.4971	21.8	1.4820
16.0	1.4966	22.0	1.4815
16.2	1.4961	22.2	1.4810
16.4	1.4956	22.4	1.4805
16.6	1.4951	22.6	1.4800
16.8	1.4946	22.8	1.4795
17.0	1.4940	23.0	1.4790
17.2	1.4935	23.2	1.4785
17.4	1.4930	23.4	1.4780
17.6	1.4925	23.6	1.4775
17.8	1.4920	23.8	1.4770
18.0	1.4915	24.0	1.4765
18.2	1.4910	24.2	1.4760
18.4	1.4905	24.4	1.4755
18.6	1.4900	24.6	1.4750
18.8	1.4895	24.8	1.4745

## Appendix C

Screening results of 220 UK honey sample; Water content and pH reading of honeys, Average zone of inhibition produced against MRSA on LB agar diffusion assay (minus the 6mm well). The phenol equivalent (%) calculated using the phenol standard curve and broth based assay results at honey concentrations 50% and 25%. ‘+’ = bacterial growth, ‘-’ = no bacterial growth). Manuka samples shown in red text.

Honey sample ID	Water content (g/100g)	pH reading	Agar diffusion assay			Broth dilution assay	
			Zone of clearing (mm)	Standard error	Phenol equivalent	Honey 50%	Honey 25%
H1	17.0	3.89	0.00	0.00	2.96	+	+
H2	19.8	3.75	13.50	0.26	10.48	-	-
H3	18.2	3.67	16.67	0.28	12.25	-	-
H4	22.8	3.42	0.00	0.00	2.96	+	+
H5	19.4	4.21	0.00	0.00	2.96	+	+
H6	17.0	4.33	10.83	0.21	9.00	-	-
H7	19.6	3.71	10.92	0.31	9.04	+	+
H8	20.2	3.69	0.00	0.00	2.96	+	+
H9	21.4	3.62	0.00	0.00	2.96	+	+
H10	20.2	3.33	0.00	0.00	2.96	+	+
H11	21.6	4.21	5.25	0.39	5.89	+	+
H12	17.6	3.75	13.08	0.26	10.25	-	-
H13	18.0	3.89	0.00	0.00	2.96	+	+
H14	19.2	3.90	0.00	0.00	2.96	+	+
H15	23.0	3.92	6.00	0.46	6.30	+	+
H16	16.6	3.68	6.42	0.34	6.54	+	+
H17	19.0	4.12	10.33	0.14	8.72	+	+
H18	21.6	4.44	0.00	0.00	2.96	+	+
H19	15.6	4.23	9.50	0.23	8.25	+	+
H20	21.2	4.42	5.25	0.22	5.89	-	+
H21	21.8	3.90	10.33	0.22	8.72	+	+
H22	16.2	3.78	17.33	0.28	12.62	+	-
H23	17.0	4.02	14.92	0.26	11.27	-	+
H24	17.2	4.13	8.17	0.32	7.51	+	+
H25	18.0	3.99	10.75	0.28	8.95	-	+
H26	22.8	3.41	6.58	0.23	6.63	+	+
H27	22.0	4.22	6.17	0.42	6.40	-	+
H28	16.2	3.90	10.08	0.34	8.58	-	+
H29	22.0	3.33	7.08	0.34	6.91	+	+
H30	21.0	3.76	12.08	0.42	9.69	-	-
H31	19.6	3.45	9.33	0.26	8.16	+	+
H32	22.0	3.97	10.58	0.23	8.86	-	+
H33	20.2	4.10	10.17	0.24	8.63	-	-
H34	18.2	3.58	7.08	0.31	6.91	+	+
H35	17.0	3.87	14.83	0.24	11.23	+	+
H36	20.6	3.75	16.50	0.31	12.15	+	+
H37	22.8	3.67	5.25	0.35	5.89	+	+
H38	17.6	3.41	10.67	0.28	8.90	+	+
H39	21.2	3.43	15.92	0.34	11.83	+	+
H40	19.6	3.73	4.67	0.33	5.56	+	+
H41	18.0	3.81	16.00	0.25	11.88	+	+
H42	20.0	3.56	17.00	0.54	12.43	+	+
H43	18.8	3.55	15.17	0.39	11.41	+	+

H44	17.0	3.76	0.00	0.00	2.96	+	+
H45	19.8	3.80	2.58	0.23	4.40	+	+
H46	17.4	3.54	12.25	0.28	9.79	+	+
H47	22.6	4.09	9.83	0.46	8.44	+	+
H48	21.0	4.12	0.00	0.00	2.96	+	+
H49	17.6	3.29	13.08	0.38	10.25	+	+
H50	21.2	4.33	14.17	0.46	10.85	-	+
H51	22.0	4.54	11.50	0.40	9.37	+	+
H52	19.6	3.66	8.83	0.24	7.88	-	-
H53	17.2	3.76	7.42	0.40	7.09	-	-
H54	19.0	3.47	6.96	0.19	6.84	-	-
H55	17.6	3.63	6.00	0.14	6.30	-	-
H56	20.8	3.92	0.00	0.00	2.96	+	+
H57	21.6	3.87	9.08	0.26	8.02	+	+
H58	17.6	4.24	13.50	0.34	10.48	-	-
H59	20.6	4.23	12.75	0.45	10.06	-	-
H60	20.8	4.55	13.83	0.41	10.67	+	+
H61	19.0	3.78	13.42	0.36	10.44	-	-
H62	18.6	3.92	12.92	0.19	10.16	+	+
H63	20.6	4.23	7.00	0.35	6.86	+	+
H64	17.2	3.46	11.83	0.24	9.55	-	+
H65	20.8	3.98	6.17	0.21	6.40	+	+
H66	17.0	3.76	12.00	0.43	9.65	-	-
H67	19.8	3.45	14.00	0.25	10.76	+	+
H68	15.6	3.56	1.58	0.26	3.84	+	+
H69	16.8	3.34	7.42	0.23	7.09	+	+
H70	21.2	4.23	0.00	0.00	2.96	+	+
H71	17.0	4.25	0.00	0.00	2.96	+	+
H72	20.0	3.75	1.17	0.27	3.61	+	+
H73	19.8	3.64	8.92	0.31	7.93	+	+
H74	21.2	3.86	11.33	0.31	9.28	+	+
H75	18.8	3.46	10.58	0.23	8.86	+	+
H76	19.8	3.46	14.08	0.31	10.81	+	+
H77	16.0	4.11	10.00	0.21	8.53	+	+
H78	17.8	4.34	12.67	0.26	10.02	+	+
H79	20.0	4.24	12.42	0.42	9.88	+	+
H80	17.0	3.78	7.08	0.34	6.91	+	+
H81	21.0	4.45	14.17	0.46	10.85	+	+
H82	16.4	3.34	14.17	0.24	10.85	-	-
H83	22.8	4.65	13.42	0.26	10.44	-	+
H84	21.4	3.78	13.58	0.19	10.53	-	-
H85	16.4	3.45	14.42	0.29	10.99	-	-
H86	20.2	3.57	6.00	0.43	6.30	-	+
H87	21.6	3.46	10.67	0.33	8.90	+	+
H88	20.4	3.36	9.75	0.25	8.39	+	+
H89	22.2	3.47	2.33	0.36	4.26	+	+
H90	16.0	4.12	3.00	0.30	4.63	+	+
H91	21.0	3.35	6.42	0.38	6.54	+	+
H92	16.2	4.34	15.25	0.41	11.46	-	-
H93	22.6	4.24	15.08	0.26	11.37	+	+
H94	21.2	4.57	16.25	0.64	12.02	-	-
H95	17.6	3.47	19.25	0.43	13.69	+	+
H96	21.6	4.42	12.25	0.60	9.79	+	+
H97	20.2	3.56	17.75	0.28	12.85	+	+
H98	21.2	4.24	10.33	0.28	8.72	-	+
H99	21.6	4.25	19.08	0.65	13.59	-	-
H100	20.2	3.52	18.50	0.78	13.27	+	-
H101	17.6	4.42	1.50	0.19	3.80	+	+
H102	17.8	4.61	13.92	0.47	10.72	+	+
H103	21.6	3.74	16.00	0.33	11.88	-	-

H104	22.8	3.52	17.50	0.78	12.71	+	+
H105	17.0	4.13	15.67	0.36	11.69	-	+
H106	21.0	3.64	0.00	0.00	2.96	+	+
H107	19.6	4.13	7.92	0.15	7.37	-	-
H108	17.0	3.73	11.75	0.48	9.51	+	+
H109	19.6	4.13	2.33	0.19	4.26	+	+
H110	21.2	3.47	15.42	0.47	11.55	-	+
H111	17.8	4.22	5.42	0.31	5.98	+	+
H112	22.0	3.33	9.33	0.22	8.16	+	+
H113	15.6	4.42	10.08	0.36	8.58	+	+
H114	21.4	3.75	6.42	0.31	6.54	+	+
H115	20.4	3.36	17.17	0.41	12.53	+	+
H116	20.2	3.74	7.58	0.29	7.19	+	+
H117	21.6	3.63	6.75	0.39	6.72	+	+
H118	19.8	3.64	1.67	0.14	3.89	+	+
H119	16.6	3.64	12.75	0.33	10.06	-	+
H120	18.2	4.12	9.83	0.32	8.44	-	+
H121	20.8	3.75	0.00	0.00	2.96	+	+
H122	20.6	4.22	12.25	0.49	9.79	+	+
H123	18.0	3.64	7.25	0.49	7.00	+	+
H124	20.8	3.37	4.58	0.29	5.51	+	+
H125	20.2	3.85	2.17	0.17	4.17	+	+
H126	17.4	4.51	11.33	0.26	9.28	+	+
H127	20.8	3.47	13.08	0.29	10.25	+	+
H128	21.0	3.74	5.92	0.23	6.26	+	+
H129	17.6	3.53	15.42	0.47	11.55	-	+
H130	15.6	3.64	2.50	0.19	4.35	+	+
H131	22.0	4.69	0.00	0.00	2.96	+	+
H132	18.0	4.96	10.25	0.33	8.67	+	+
H133	23.0	3.90	13.33	0.28	10.39	+	+
H134	21.2	3.68	2.67	0.19	4.45	+	+
H135	17.2	4.04	12.83	0.34	10.11	+	+
H136	22.0	3.84	13.75	0.39	10.62	+	+
H137	20.4	4.06	11.50	0.34	9.37	+	+
H138	21.0	4.42	1.50	0.26	3.80	+	+
H139	18.0	3.99	8.75	0.22	7.84	-	-
H140	21.2	3.86	8.92	0.31	7.93	+	+
H141	15.4	4.46	13.00	0.25	10.20	-	-
H142	23.0	3.86	8.00	0.28	7.42	+	+
H143	21.0	3.86	9.75	0.30	8.39	+	+
H144	17.2	3.92	10.67	0.22	8.90	-	-
H145	21.6	3.61	10.92	0.23	9.04	-	-
H146	21.6	3.53	0.00	0.00	2.96	+	+
H147	21.2	3.25	12.92	0.26	10.16	+	+
H148	19.6	3.84	13.67	0.36	10.58	-	-
H149	22.2	3.78	10.17	0.24	8.63	+	+
H150	16.8	4.41	12.42	0.23	9.88	-	-
H151	16.4	3.70	8.67	0.26	7.79	+	+
H152	20.6	4.35	0.00	0.00	2.96	+	+
H153	22.8	4.23	8.50	0.29	7.70	+	+
H154	17.6	3.86	11.67	0.50	9.46	-	-
H155	18.2	3.86	3.50	0.23	4.91	+	+
H156	21.0	3.46	0.00	0.00	2.96	+	+
H157	17.6	3.72	10.00	0.25	8.53	-	-
H158	21.2	3.92	7.90	0.19	7.36	+	+
H159	20.2	3.57	0.00	0.00	2.96	+	+
H160	17.6	3.39	12.83	0.21	10.11	-	+
H161	17.8	3.63	18.92	0.26	13.50	-	-
H162	20.6	3.98	8.92	0.51	7.93	+	+

H163	16.0	3.74	11.67	0.45	9.46	+	+
H164	22.8	3.54	13.17	0.21	10.30	+	+
H165	2.18	4.23	0.83	0.11	3.42	+	+
H166	18.0	3.58	12.33	0.19	9.83	+	+
H167	21.8	3.94	2.92	0.08	4.59	+	+
H168	21.2	4.31	13.58	0.19	10.53	+	+
H169	19.8	3.48	18.50	0.57	13.27	+	+
H170	19.0	3.84	12.42	0.26	9.88	+	+
H171	18.8	4.22	18.58	0.29	13.32	-	-
H172	17.6	3.25	5.75	0.39	6.16	+	+
H173	18.6	4.13	5.58	0.36	6.07	+	+
H174	20.0	3.46	0.00	0.00	2.96	+	+
H175	21.2	3.75	5.42	0.29	5.98	-	-
H176	17.0	3.47	5.33	0.28	5.93	+	+
H177	22.0	3.75	8.17	0.39	7.51	+	+
H178	21.6	3.68	4.42	0.31	5.42	+	+
H179	16.8	3.84	13.17	0.17	10.30	-	+
H180	17.6	3.55	5.67	0.22	5.00	+	+
H181	21.6	3.69	1.92	0.15	4.03	+	+
H182	17.6	4.42	8.00	0.17	7.42	+	-
H183	22.8	4.41	3.42	0.26	4.86	+	+
H184	18.8	3.85	6.08	0.34	6.35	+	+
H185	16.8	3.35	6.50	0.26	6.58	+	+
H186	22.6	4.24	10.00	0.25	8.53	-	-
H187	22.2	3.94	13.92	0.26	10.72	-	-
H188	21.8	4.13	5.92	0.26	6.26	+	+
H189	21.8	3.68	6.83	0.21	6.77	+	+
H190	21.4	3.36	6.50	0.31	6.58	+	+
H191	15.4	3.95	11.58	0.29	9.41	-	-
H192	16.8	3.31	1.42	0.23	3.75	+	+
H193	19.4	3.64	4.83	0.17	5.65	+	+
H194	23.0	3.68	1.75	0.18	3.94	+	+
H195	17.4	4.34	0.00	0.00	2.96	+	+
H196	21.0	3.69	10.00	0.28	8.53	-	-
H197	21.8	3.64	11.50	0.19	9.37	-	+
H198	15.6	3.93	4.42	0.15	5.42	+	+
H199	21.2	4.49	2.58	0.19	4.40	+	+
H200	20.4	4.41	15.67	0.26	11.69	-	-
H201	17.0	4.22	7.83	0.17	7.33	+	+
H202	17.8	4.26	12.78	0.13	10.08	-	+
H203	19.8	4.29	12.89	0.19	10.14	-	-
H204	15.6	3.62	9.22	0.16	8.10	-	+
H205	20.6	3.87	7.67	0.18	7.23	+	+
H206	20.4	3.92	9.67	0.32	8.35	-	+
H207	16.8	3.68	11.78	0.16	9.52	-	+
H208	21.2	4.31	12.89	0.16	10.14	-	-
H209	18.8	3.64	10.22	0.16	8.66	-	+
H210	21.0	4.14	1.44	0.18	3.77	+	+
H211	20.2	3.86	7.89	0.13	7.36	+	+
H212	20.8	4.13	6.22	0.25	6.43	-	+
H213	15.4	4.34	11.56	0.19	9.40	-	-
H214	17.2	4.51	0.44	0.18	3.21	+	+
H215	21.2	3.25	8.78	0.13	7.85	+	+
H216	21.6	4.68	0.00	0.00	2.96	+	+
H217	16.2	3.34	9.89	0.30	8.47	+	+
H218	21.8	4.35	7.56	0.26	7.17	+	+
H219	19.4	3.37	8.78	0.25	7.85	+	+
H220	17.0	3.94	5.33	0.26	5.93	-	-

## Appendix D

Python script written for DNA barcoding analysis

```
#####  
BLAST sequences against a local BLAST database  
# We created a local BLAST database from GenBank and then filtered the  
# database for plant based GI's therefore cutting down our search area. We also  
# have a local BLAST database for the extra UK species that we have barcoded but  
# have yet to upload to GenBank.  
# The output of this program is used as input for "blast_summary.py"  
# Change the defines for your specific requirements.  
# We assume that the sequences have already had their primers and tags removed  
# and are stored in fasta files with each file being for one tag.  
#####  
  
# Imports  
import os  
import glob  
from Bio import SeqIO  
from Bio.Blast.Applications import NcbiblastnCommandline  
  
# Defines  
# Change these for your specific needs  
  
# Main directory for work  
workdir = os.path.dirname(os.path.realpath(__file__))  
  
# Local BLAST database and GI filter list  
blast_db = '%s/./blast-db' % workdir  
  
# FASTA directory, where to find the sequences  
fasta_dir = '%s/./fasta' % workdir  
  
# Output of our BLAST results  
outdir = '%s/./blast_results' % workdir  
  
# Given a directory this returns a list of fasta files  
# Change if 'fa' is not the extension that you want to find.  
def get_fasta_files(fasta_dir):  
    abs_path = os.path.abspath(fasta_dir)  
    print('Looking for fasta files in: ',abs_path)  
    return glob.glob('%s/*.fasta' % abs_path)  
  
# Returns the output file  
def outfile(dir,file):  
    return '%s/%s.csv' % (dir,os.path.splitext(os.path.basename(file))[0])  
  
# BLASTS the sequence file against the local database  
def ncbi_blast(in_file,out_file,dbp):  
    cur_dir = os.getcwd()  
    os.chdir(dbp)  
    # Replace the database names with your own local databases  
    # ...plus we are using 8 threads so change according to the resources available
```

```

cmd_line = NcbiblastnCommandline(query=in_file, db="nt_ncbi_plants fpuk", out=out_file,
outfmt="10 std score stitle", max_target_seqs=20,num_threads=8)
cmd_line()
os.chdir(cur_dir)

# Each fasta file contains a set of sequences that were
# matched for a given tag. They are either reverse or forward.
# We blast them to the database.
def blast_sequences( fastas, odir, db_dir ):
    for file in fastas:
        print( 'Processing: ', file )
        ncbi_blast(file,outfile(odir,file),db_dir)

# Main
print('Running')
os.chdir(workdir)
fasta_files = get_fasta_files(fasta_dir)
blast_sequences(fasta_files,outdir,blast_db)
print('Done')

#####
Given the output of "blast_with_ncbi.py" we process each tag file that
# contains all the BLAST results and output a CSV file with our best guess
# at what the BLAST result is informing us.
#
# Change the defines for your own specific requirements
#####

# Imports
import os
import re
import glob
import csv
from collections import defaultdict

# Defines

# Work dir where this script is
workdir = os.path.dirname(os.path.realpath(__file__))

# BLAST results directory that was created by "blast_with_ncbi.py"
file_dir = '%s/./blast_results' % workdir

# Where to place our summaries
res_dir = '%s/./blast_summary' % workdir

# Regular expression to allow us to "mark" results that come from
# our created UK barcodes
uk_re = re.compile('NMW|NBGW|RBGE')

# Makes a directory if does not already exist
def mkdir(dname):
    if not os.path.exists(dname):
        os.makedirs(dname)

# Given a directory this returns a list of ext files
def ls(dir,ext):

```

```

return glob.glob('%s/*.%s' % (dir,ext))

# Returns the path to the CSV results file
def mkcsv(dir,file):
    return '%s/%s.csv' % (dir,os.path.splitext(os.path.basename(file))[0])

# Gets the list of species and bit scores
def get_species(matched):
    species_list = []
    for match in matched:
        bits = match[3].strip()
        species = match[4]
        is_ncbi = species.rsplit('|')
        is_uk = species.split('-')

        # Test to see if this ncbi
        if len(is_ncbi) > 2:
            # From NCBI
            str = is_ncbi[-1]
            parts = str.split(' ') # Always a space at the start
            genus = parts[1]
            spec = parts[2]
            if uk_re.search(str):
                spec += '*'
            species_list.append('%s %s (%s)' % (genus,spec,bits))
        else:
            # From UK
            species_list.append('%s %s* (%s)' % (is_uk[1],is_uk[2],bits))

    return species_list

# Extracts the species from the matched list
def extract_species(matched):
    species_list = []
    for match in matched:
        species = match[4]
        is_ncbi = species.rsplit('|')
        is_uk = species.split('-')

        # Test to see if this ncbi
        if len(is_ncbi) > 2:
            # From NCBI
            str = is_ncbi[-1]
            parts = str.split(' ') # Always a space at the start
            genus = parts[1]
            spec = parts[2]
            if uk_re.search(str):
                spec += '*'
            species_list.append('%s %s' % (genus,spec))
        else:
            # From UK we mark this with a *
            species_list.append('%s %s*' % (is_uk[1],is_uk[2]))

    unique = list(set(species_list))
    species_list = []

```

```

for spp in unique:
    tmp = spp.split(' ')
    species_list.append([tmp[0],tmp[1]])

return species_list

# Look through the genus in the list and if one of them
# has a greater influence of 60% then pick that one
def genus_percentage(spp):
    total = len(spp)
    genus = defaultdict(list)

    for sp in spp:
        gen = sp[0]
        if not gen in genus:
            genus[gen] = 1
        else:
            genus[gen] += 1

    for gen in genus:
        if (float(genus[gen])/float(total))*100.0 >= 60:
            return '%s %%' % gen

    return 'Various'

# Returns the match type for the species it can be:
# Zero
# Species
# Genus
# Various
def get_match_type(species):
    if len(species) == 0:
        return '----'
    if len(species) == 1:
        return '%s %s' % (species[0][0],species[0][1])

    genus = species[0][0]
    for item in species:
        if genus != item[0]:
            return genus_percentage(species)

    if species[0][1][-1] == '*':
        genus = '%s*' % genus

    return genus

# Outputs the header
def header(fd):
    fd.write('SID,Number-Of-Sequences,Score,Match,Top Species\n')

# Output a row in the file
def row(fd,sid,numberof,score,type,species,blast_results):
    try:
        # ID
        fd.write('%s,' % sid)
        # Number of sequences for this sequence

```

```

fd.write('%d,' % int(numberof))
# Top score
fd.write('%f,' % float(score))
# Type of match ... speices, genus, various
fd.write('%s' % type)
except:
    print(sid)
    bang

# Top bit score taxa matches
for item in species:
    fd.write('%s %s' % (item[0],item[1]))

# We also output all the BLAST results on the same line with their bit score
# So if something does not look correct we can take a look at the full results
top_10 = get_species(blast_results)
fd.write(',')

for spp in top_10:
    fd.write('%s' % spp)

fd.write('\n')

# Process one set of blast results for each ID
def process_set(fd,blast_results):
    top_bit_score = 0.0
    top_set = []

    # Work out the top bit score set
    for blast_id in blast_results:

        # Sort the entries by top bit score
        blast_results[blast_id].sort(key = lambda row: row[3],reverse=True)

        # Go through the entries and pick out all the top bit scored ones
        for entry in blast_results[blast_id]:
            if float(entry[3]) >= float(top_bit_score):
                top_set.append(entry)
                top_bit_score = entry[3]
            else:
                break

        # Extract the species and the matched type
        # i.e. does the top match to a specific species, genus or various?
        species_list = extract_species(top_set)
        matched = get_match_type(species_list)

        # When our sequences where processed they where given the tags
        # id-number, where number was the number of sequences within the
        # huge data-set that matched exactly with this sequence. i.e. the
        # sequences where merged. So we extract the number for the summaries
        numberof = 1
        if "-" in blast_id:
            parts = blast_id.split('-')
            if parts[0].isdigit():
                numberof = parts[1]

```

```

        # Output a row in the summary file for this ID set
        row(fd,blast_id,numberof,top_bit_score,matched,species_list,blast_results[blast_id])

# Goes through a list of BLAST CSV results and summaries each file as a CSV file
def blast_summary(dir,files):
    # Create the summary folder
    mkdir(dir)

    # Process the BLAST results file by file
    for file in files:
        print('Processing: %s' % file)
        in_fd = open(file,'r')
        out_fd = open(mkcsv(dir,file),'w')
        records = csv.reader(in_fd,delimiter=",")

        header(out_fd)

        working_set = defaultdict(list)
        last_id = ""

        # Go through each line in the file
        for row in records:
            # Check to see if we have got to the end of a set for
            # a particular ID. There will be a set of results per ID
            if len(working_set) > 0 and last_id != row[0]:
                # Process this ID's set
                process_set(out_fd, working_set)
                working_set.clear()

                # Grab the data from the line
                sid = row[0]
                percent_score = row[2]
                bit_score = row[11]
                description = row[13]
                # Save off the data into the set
                last_id = sid
                working_set[row[0]].append([sid,1,percent_score,bit_score,description])

        # There might be one left to process
        if len(working_set) > 0:
            process_set(out_fd, working_set)
            working_set.clear()

        in_fd.close()
        out_fd.close()

# Main
print("")
print('Running')

# Get the BLAST results
files = ls(file_dir,'csv')

# Create the summaries for each BLAST result file
blast_summary(res_dir,files)
print('Enend')

```

## Appendix E

### Next generation sequencing - Illumina trial run methods

#### Methods

#### Honey samples analysed

For the initial Illumina sequencing run 16 honeys were analysed; some of the honeys had been previously analysed on the 454 run and melissopalynology, other samples were freshly prepared. DNA extractions were carried out as described previously for the 454 pyrosequencing run (chapter 4, section 4.3.3.2). Four repeats of each sample was run.

The honey samples analysed of the trial 454 run:

Index No.	Honey ID
1	H14
2	H23
3	H24
4	H25
5	H34
6	H36
7	H39
8	H54
9	H107
10	H110
11	H114
12	H160
13	H171
14	H207
15	H213
16	H217

#### Polymerase Chain Reaction (PCR) Amplification

A single round of PCR was used to incorporate *rbcL* universal primers and identification tags. The universal forward and reverse *rbcL* primers were attached to the unique sequence tags. These ligated adapters were purchased from Sigma and resuspended with molecular biology grade water (Sigma) to 100  $\mu$ M. To obtain sequence data from many individual samples in a pool of DNA unique tags are required. Ligating primers with unique tags were placed onto individual DNA samples before sequencing. A 1 in 10 dilution was performed and working stocks (10 $\mu$ M) were stored at -20 °C until required.

The PCR was performed using the following reagents: 12.5  $\mu$ l PCR Biomix (Bioline), 1.0  $\mu$ l BSA and 8.0  $\mu$ l molecular biology grade water (Sigma). A 21.5  $\mu$ l volume of master mix was added to each PCR tube. Due to the specificity of each individual primer and tag pair 0.5  $\mu$ l of each one was added to each PCR tube individually. Each adapter is unique for each honey sample; 0.5  $\mu$ l

of both the forward and reverse of the pair was still added. Subsequently 2.5 µl of extracted DNA was added, PCRs were again run at a total volume of 25 µl.

The PCR cycle was run using the same conditions as previously described (chapter 4, section 4.3.3.3): initial denaturing at 95 °C for 2 mins, followed by 30 cycles of 95 °C for 2 mins, 50 °C for 90 secs, 72 °C for 40 secs with a final extension at 72 °C for 5 mins and 30 °C for 10 secs. The PCR reaction products were analysed on a 1% agarose gel as previously described (chapter 4, section 4.3.3.3) with the presence of visible bands confirming that amplification had been successful. In total 16 honey samples labelled with different adaptor tags were successfully amplified ready for Illumina sequencing.

### **High throughput Illumina sequencing**

Following the TruSeq® DNA sample preparation guide the tagged honey DNA samples were prepared for next generation sequencing. A preliminary run was carried out using a 300 cycle kit (2 x 150 bp) to assess whether the proposed methodology works. In brief, an AMPure XP bead (Beckman Coulter) clean-up was performed following Illumina instructions to clean up the DNA samples. Samples were again run on a 1% agarose gel and visualised to ensure DNA remained within the samples after the wash steps.

Following purification DNA was quantified using a Qubit® 20 Fluorometer (Invitrogen). A Qubit® double stranded DNA BR Assay was followed according to the manufacturers' instructions. Standards were used to calibrate the device; fluorescence vs. concentration graph is produced by a two point calibration. DNA samples were subsequently diluted to 1 µg/ml based on the reading obtained on the fluorometer. To create a pooled sample of DNA 2 µl of each of the samples were combined.

The TruSeq® DNA sample preparation guide was followed for incorporation of a single 'A' nucleotide to the 3' end of blunt fragments. Then the DNA was then prepared for hybridization onto the flow cell by the addition of Illumina indexing adapters. A PCR step was used to enrich the DNA which have adapter molecules at both ends and increase the amount of DNA in the library. The PCR was run following the Illumina protocol. The sample was cleaned using AMPure XP Beads after the PCR step. An agarose gel was run to ensure the PCR had been successful and the clean-up step had removed any undesired DNA fragments.

### **Data analysis**

The Illumina run was carried out in Aberystwyth University (IBERS) and the DNA sequences were recorded from the mixed honey sample. Software written using Python was used to further

process the results received from the Illumina sequencing. A script was produced to assess the number of sequences which were exact matches for both tag and primers.

### Next generation sequencing - Illumina Trial run

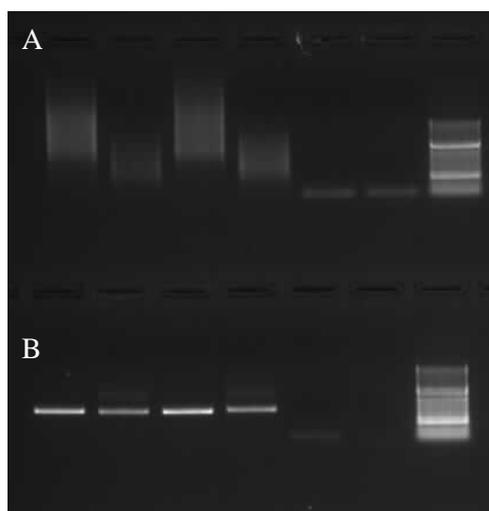
#### Results

An agarose gel was run to ensure the presence of a band and ensure there was still DNA present, a bright band was seen. Sixteen honey samples were run on the Illumina sequencer, a pooled sample with different extractions and repeats labelled with different tags were analysed. In total 11,908,466 sequence reads were obtained from the Illumina run. In total 4,722,610 (39.7%) exact matches were found for tag and primer pairs.

It was expected that the forward and reverse primers for a pair would have the same tags and that these tags could then be used to piece together the fragments of the *rbcL* gene, this was not seen. Forward and reverse reads with different tags were observed; therefore complete plant DNA sequences could not be resolved. These unexpected results are believed to be due to chimera production during the PCR of the Illumina adapters. Complete plant DNA sequences could not be resolved and further optimisation was performed. Based on these findings a new protocol was trailed (chapter 4, section 4.3.5), the Illumina Metagenomic Sequencing Library Preparation Protocol was used.

#### Next generation sequencing – Illumina further optimisation

To further optimise the Illumina protocol an adapted *rbcL* primer was used to target an amplicon region that is 84bp shorter. This was necessary as amplification success using the *rbcL590-t* primer was inconsistent. The intensity and clarity of the band on the agarose gel image greatly improved (Figure 1). The short *rbcL* amplicon primer was subsequently used.



Gel image showing the amplification success of the original *rbcLr590* (A) primer compared to the short *rbcLr506* (B).

## Appendix F

The ten other honey samples analysed using traditional pollen characterisation methods.

<b>Honey ID</b>	<b>Collection code</b>	<b>Hive location</b>	<b>Co-ordinates (Lat/Long)</b>
H110	H11	Llangynnwr, Carmarthenshire, Wales	51.851048, -4.239368
H192	H12	New Quay, Llandysul, Dyfed, Wales	52.182299, -4.343473
H14	H13	Whitland, Carmarthenshire, Wales	51.914968, -4.56642
H37	H14	Coytrahen, Bridgend, Wales	51.564926, -3.602989
H1	H15	Bancyfelin, Carmarthen, Wales	51.83602, 51.83602
H216	H16	Llanidloes, Powys, Wales	52.472334, -3.614685
H191	H17	Unknown	
H188	H18	Pontyclun, Rhondda Cynon Taff, Wales	51.561718, -3.37766
H189	H19	Sycamore Close, Bridgend, , Wales	51.525054, -3.579158
H187	H20	Bridgend, Neath Port Talbot, Wales	51.544786, -3.684888

Order	Family	Taxa	H11	H12	H13	H14	H15	H16	H17	H18	H19	H20
Apiales	Apiaceae	Ligustrum										58
Asparagales	Amaryllidaceae	Allium										
Asparagales	Asparagaceae	Hyacinthoides non-scripta										
Asparagales	Asparagaceae	Scilla										
Asparagales	Xanthorrhoeaceae	Hemerocallis		564								
Asterales	Asteraceae	Achillea millefolium		33								
Asterales	Asteraceae	Aster	3233									
Asterales	Asteraceae	Bellis	5857						961			1101
Asterales	Asteraceae	Centaurea		22								
Asterales	Asteraceae	Chrysanthemum										474
Asterales	Asteraceae	Cirsium	13	166	68							
Asterales	Asteraceae	Crepis	163			18						
Asterales	Asteraceae	Erigeron								301		
Asterales	Asteraceae	Helianthus										795
Asterales	Asteraceae	Hypochaeris								250		
Asterales	Asteraceae	Lactuca										
Asterales	Asteraceae	Leucanthemum										
Asterales	Asteraceae	Solidago	72	27	18							10
Asterales	Asteraceae	Taraxacum officinale	50237	23782	15788	15400	62904	146	37493	1759	481	2982
Brassicales	Brassicaceae	Arabis				30						
Brassicales	Brassicaceae	Brassica										
Brassicales	Brassicaceae	Brassica napus				4926					32	
Brassicales	Brassicaceae	Brassica oleracea										
Brassicales	Brassicaceae	Cardamine								342		
Brassicales	Brassicaceae	Erysimum				174						41
Caryophyllales	Caryophyllaceae	Arenaria								247		
Caryophyllales	Caryophyllaceae	Cerastium										633
Caryophyllales	Polygonaceae	Rumex						206				
Cornales	Cornaceae	Cornus		1764								13
Cornales	Hydrangeaceae	Hydrangea		150		12						
Cornales	Hydrangeaceae	Philadelphus	12			20						
Dipacales	Adoxaceae	Sambucus / Viburnum		351	1260			55				
Dipacales	Caprifoliaceae	Lonicera						883				
Ericales	Balsaminaceae	Impatiens glandulifera								433	198	13283
Ericales	Ericaceae	Calluna vulgaris		13711								
Ericales	Primulaceae	Anagallis						2086				
Escalloniales	Escalloniaceae	Escallonia		11	40							
Fabales	Fabaceae	Fabaceae										
Fabales	Fabaceae	Genista										
Fabales	Fabaceae	Lathyrus										
Fabales	Fabaceae	Ononis										
Fabales	Fabaceae	Trifolium				218		523		12		
Fabales	Fabaceae	Trifolium pratense										
Fabales	Fabaceae	Trifolium repens				14957		3394		301	84	
Fabales	Fabaceae	Ulex			61							
Fabales	Fabaceae	Vicia sativa		237								349
Fabales	Polygalaceae	Polygala				14191		1641		262		652
Fagales	Fagaceae	Castanea sativa										
Fagales	Fagaceae	Quercus										
Gentianales	Gentianaceae	Centaurium		1194						671		
Geraniales	Geraniaceae	Geranium								632	120	
Lamiales	Lentibulariaceae	Pinguicula?		1694								
Lamiales	Orobanchaceae	Euphrasia						2125				103
Lamiales	Plantaginaceae	Digitalis / Antirrhinum / Veronica		4315							57	943
Lamiales	Scrophulariaceae	Verbascum		311								
Laurales	Lauraceae	Persea	23	42		46						
Liliales	Liliaceae	Lilium										
Magnoliales	Magnoliaceae	Magnolia										
Malpighiales	Hypericoideae	Hypericum	2036									
Malpighiales	Salicaceae	Salix			2003	10						
Myrtales	Onagraceae	Chamerion angustifolium		32								
Myrtales	Onagraceae	Epilobium		570				979				499
Oxalidales	Oxalidaceae	Oxalis		588						38	33	219
Pinales	Pinaceae	Pinus						94				
Poales	Poaceae	Agrostis capillaris		724								
Poales	Poaceae	Arrhenatherum elatius		26								
Poales	Poaceae	Festuca		76								
Poales	Poaceae	Holcus lanatus		248				48		13		
Poales	Poaceae	Poa pratensis		1319				2536		877		
Poales	Poaceae	Trisetum						71				
Polypodiales	Athyriaceae	Athyrium filix-femina				157						
Ranunculales	Papaveraceae	Papaver		1072								
Ranunculales	Ranunculaceae	Helleborus foetidus								656		
Rosales	Rhamnaceae	Ceanothus			348							
Rosales	Rosaceae	Alchemilla		612								
Rosales	Rosaceae	Cotoneaster			87							
Rosales	Rosaceae	Crataegus monogyna			439						41	
Rosales	Rosaceae	Filipendula ulmaria										
Rosales	Rosaceae	Malus			1522							
Rosales	Rosaceae	Prunus			8029						162	
Rosales	Rosaceae	Rosa		74		328				66		161
Rosales	Rosaceae	Rubus fruticosus		3066		10363				2509	159	2434
Rosales	Rosaceae	Sorbus			258							
Sapindales	Sapindaceae	Acer										
Saxifragales	Grossulariaceae	Ribes		40		145		16				
Saxifragales	Saxifragaceae	Heuchera / Astilbe										
Saxifragales	Saxifragaceae	Saxifraga		1979	338	1171		760				

Figure 2 - Summary of plant taxa detected through Illumina analysis in the other honey samples

## Appendix G

Inhibition of growth on bioautographic TLC plates by 12 extracts of 4 honey samples against *E. coli* ('+' area <10mm, '++' area 10-20mm, '+++>20mm, '-' no zone of inhibition).

Extraction No.	Total No. of zones	Zone no.	Retention factor (R <sub>f</sub> )	Zone of inhibition
E01	0	–	–	–
E02	3	1	0.05	+++
E02 - Repeat		2	0.96	+
		3	0.58	+
E03	3	1	0.42	+++
		2	0.58	+
		3	0.87	++
E04	0	–	–	–
E05	5	1	0.55	+++
		2	0.67	+
		3	0.98	+
E05 - Repeat		4	0.42	+
		5	0.44	+
E06	3	1	0.98	+
E06 - Repeat		2	0.52	+
		3	0.44	+
E07	0	–	–	–
E08	4	1	0.57	++
		2	0.66	+
E08 -Repeat		3	0.44	+
		4	0.97	+
E09	4	1	0.54	+
		2	0.58	+
		3	0.67	+
		4	0.97	+
E10	0	–	–	–
E11	3	1	0.34	+
		2	0.65	+
		3	0.98	+
E12	3	1	0.57	++
		2	0.66	+
		3	0.97	++
Thymol Hex 6:4 Ace	1	1	0.72	++
Thymol ACN 9:1 Meth	1	1	0.94	+

## Appendix H

Inhibition of growth on bioautographic TLC plates by 12 extracts of 4 honey samples against *P. aeruginosa* ('+' area <10mm, '++' area 10-20mm, '+++>20mm, '-' no zone of inhibition).

Extraction No.	Total No. of zones	Zone No.	Retention factor (R <sub>f</sub> )	Zone of inhibition
E01	0	–	–	–
E02	1	1	0.93	+
E03	2	1	0.68	++
		2	0.79	++
E04	0	–	–	–
E05	4	1	0.25	+++
		2	0.45	+++
		3	0.90	+
		4	0.72	+
E05 - Repeat				
E06	4	1	0.52	+
		2	0.73	+
		3	0.84	+
		4	0.88	+
E07	0	–	–	–
E08	5	1	0.20	+++
		2	0.36	+++
		3	0.89	+
E08 - Repeat		4	0.42	+
		5	0.91	+
E09	5	1	0.35	+++
		2	0.47	+
		3	0.58	++
		4	0.72	++
		5	0.89	+
E10	0	–	–	–
E11	3	1	0.48	+
		2	0.54	+
		3	0.89	+
E12	4	1	0.64	+
		2	0.97	+
E12 - Repeat		3	0.33	+
		4	0.42	+
Thymol Hex 6:4 Ace	1	1	0.73	++
Thymol ACN 9:1 Meth	1	1	0.95	+

## Appendix I

Inhibition of growth on bioautographic TLC plates by 9 extracts of 3 plant samples against *E. coli* ('+' area <10mm, '++' area 10-20mm, '+++>20mm, '-' no zone of inhibition).

Extraction No.	Total No. of Zones	Zone no.	Retention factor (R <sub>f</sub> )	Zone of inhibition
E13	0	–	–	–
E14	6	1	0.43	+
		2	0.48	+
		3	0.55	+
		4	0.60	+
		5	0.66	+
		6	0.95	+
E15	4	1	0.62	+
		2	0.71	+
		3	0.95	+
E15 - Repeats		4	0.42	+
E16	0	–	–	–
E17	7	1	0.28	+
		2	0.45	+
		3	0.56	+
		4	0.61	+
		5	0.65	+
		6	0.68	+
E17 - Repeats		7	0.31	+
E18	3	1	0.54	+
		2	0.70	+
		3	0.94	+
E19	0	–	–	–
E20	2	1	0.77	+
		2	0.94	+
E21	3	1	0.56	+
		2	0.74	+
		3	0.88	+
Thymol Hex 6:4 Ace	1	1	0.72	++
Thymol ACN 9:1 Meth	1	1	0.94	+

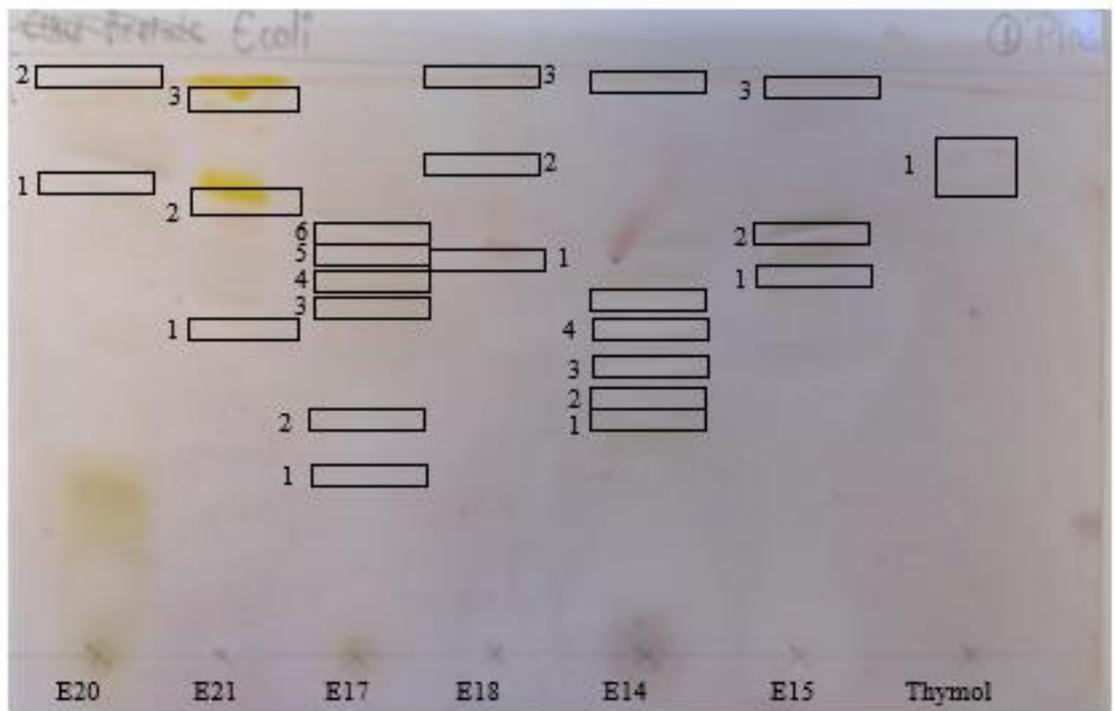
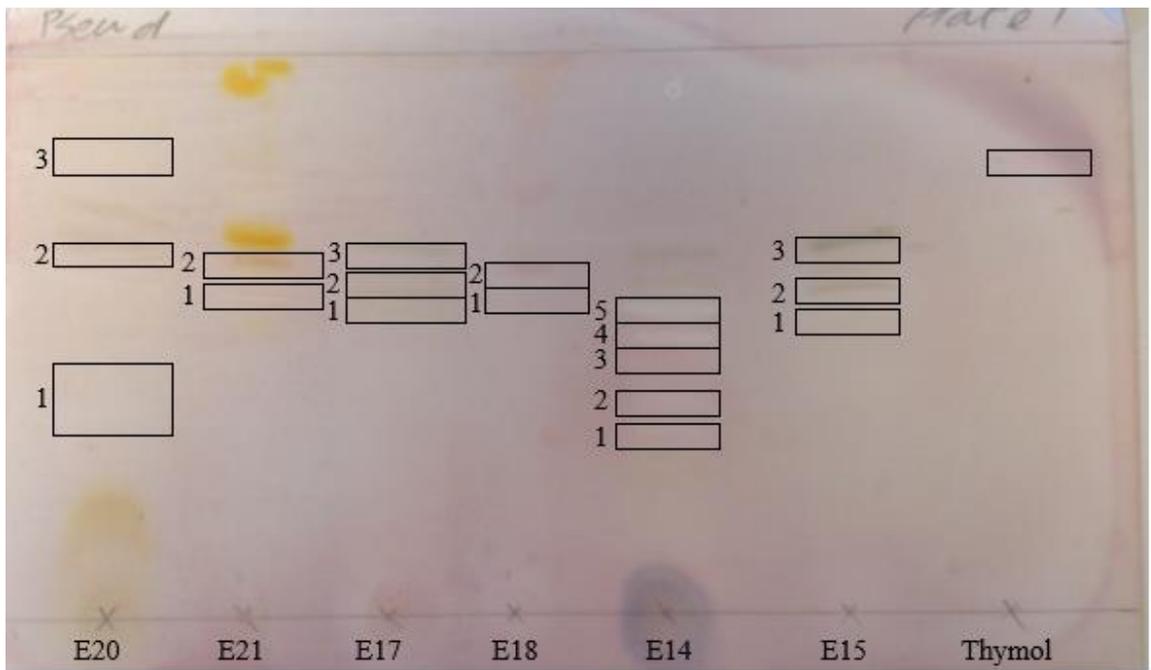


Figure 3 - Overlay assay of plant extracts against *E. coli*. Zones of clearing have been highlighted and numbered for further investigations.

## Appendix J

Inhibition of growth on bioautographic TLC plates by 9 extracts of 3 plant samples against *P. aeruginosa* ('+' area <10mm, '++' area 10-20mm, '+++>20mm, '-' no zone of inhibition).

Extraction No.	Total No. of Zones	Zone no.	Retention factor (R <sub>f</sub> )	Zone of inhibition	
E13	0	-	-	-	
E14	5	1	0.32	+	
		2	0.36	+	
		3	0.47	+	
		4	0.52	+	
		5	0.56	+	
E15	3	1	0.50	+	
		2	0.54	+	
		3	0.58	+	
E16	1	1	0.04	+	
E17	6	1	0.52	+	
		2	0.57	+	
		3	0.60	+	
		4	0.63	+	
		E17 - Repeats	5	0.32	+
			6	0.44	+
E18	3	1	0.56	+	
		2	0.6	+	
E18 - Repeats		3	0.45	+	
E19	0	-	-	-	
E20	4	1	0.30	+++	
		2	0.62	+	
		3	0.80	++	
E20 - Repeats		4	0.36	++	
E21	5	1	0.58	+	
		2	0.63	+	
E21 - Repeats		3	0.30	+	
		4	0.42	+	
		5	0.77	+	
Thymol Hex 6:4 Ace	1	1	0.73	++	
Thymol ACN 9:1 Meth	1	1	0.95	+	



*Overlay assay of plant extracts against P. aeruginosa. Zones of clearing have been highlighted and numbered for further investigations.*