Development of a novel multidisciplinary approach to evaluate honey for biomedical applications

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A thesis submitted for the award of Ph.D.

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Declaration

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

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To my family, everything I do is because of you.

Dissemination of the project outcomes

Publications

- Angioi, R.; Morrin, A.; White, B. The Rediscovery of Honey for Skin Repair: Recent Advances in Mechanisms for Honey-Mediated Wound Healing and Scaffolded Application Techniques. *Appl. Sci.* **2021**, *11*, 5192. https://doi.org/10.3390/app11115192
- Angioi, R.; Morrin, A.; White, B. Advantages of a multifaceted characterisation of honey, illustrated with Irish honey marketed as heather honey. *ACS Food Science & Technology.* https://doi.org/10.1021/acsfoodscitech.3c00475

Poster presentations

- *Unlocking honey's secrets with analytical chemistry.* Analytical Research Forum 2022 (ARF2022), an event organised by the Royal Society of Chemistry (London - June 14th, 2022).
- *Honey's Antimicrobial Properties: Is There Only Room for Manuka Honey?* E-poster with 3-min talk. EWMA2023 Conference – Antimicrobials session (Milan – May 2nd-5th, 2023),
- *The rediscovery of Irish honey to treat wound infections.* 71st International Congress of the Society for Medicinal Plant and Natural Product Research (GA) "Unlocking Nature's Pharmacy for a Sustainable Future" (Dublin – July 2nd-5th, 2023).

Oral presentations

- *Enhancing burn healing using honey hydrogel wound dressings.* 5th DCU Annual Chemistry Day 2020, organised by the School of Chemical Sciences, Dublin City University (virtual conference – June 5th, 2020)
- *Enhancing burn healing using honey hydrogel wound dressings.* Chemistry le Chéile (Chemistry Together) - Forwarding Careers of Women in Chemistry, organised by the Institute of Chemistry of Ireland (ICI), (virtual conference – December 9th, 2020)

- *Unlocking honey's secrets with analytical chemistry* - Finalist. Tell it Straight Postgraduate Research Communications Competition, organised by DCU Postgraduate Office (Dublin - March 8th, 2023).
- *Multidisciplinary approach towards a comprehensive characterisation of Irish heather honey: composition screening, antibacterial properties, and honey-loaded topical formulation design.* 74th Irish Universities Chemistry Research Colloquium, organised by the University of Galway and the Institute of Chemistry of Ireland (Galway - June 14th-15th, 2023)
- *Multidisciplinary approach towards a comprehensive characterisation of Irish heather honey: composition screening and antibacterial properties.* PSE Young Scientists' Meeting 2023 "Trends in Natural Products" organised by the Phytochemical Society of Europe (Paris – June 28th-30th, 2023).

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List of Abbreviations

10-HAD	10-Hydroxy-2-decenoic acid
AD	Atopic Dermatitis
ANOVA	One-way Analysis of Variance
CAR	Carboxen
CFU	Colony Forming Unit
CMC	Carboxymethyl Cellulose
DFU	Diabetic Foot Ulcers
DHA	Dihydroxyacetone
DVB	Divinylbenzene
GAE	Gallic Acid Equivalent
GC	Gas Chromatography
GDL	D-(+)-Gluconic acid d-lactone
GOx	Glucose Oxidase
HMF	Hydroxymethylfurfural
HPLC	High Performance Liquid Chromatography
HS-SPME	Headspace-Solid Phase Micro Extraction
LCMS/MS	Liquid Chromatography-tandem Mass Spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantification
MBC	Minimum Bactericidal Concentration
MDR	MultiDrug Resistant
MFC	Minimum Fungicidal Concentration
MGH	Medical-Grade Honey
MGO	Methylglyoxal
MH	Manuka Honey
MIC	Minimum Inhibitory Concentration
MMPs	Matrix Metalloproteinases
MPI	Ministry of Primary Industry of the New Zealand government
MS	Mass Spectrometry
MVTR	Moisture Vapour Transmission Rate
MWCO	Molecular Weight Cut Off
NIST	National Institute of Standards and Technology (US Department of Commerce)
OD	Optical Density
OD600	Optical Density at 600 nm
PAH	Polycyclic Aromatic Hydrocarbons
PBS	Phosphate Buffered Saline
PDMS	Polydimethylsiloxane
PTFE	Polytetrafluoroethylene
RI	Retention Index/Indices
RJ	Royal Jelly
ROS	Reactive Oxygen Species

RT	Retention Time/Times
SDA	Saboured Dextrose Agar
SPE	Solid Phase Extraction
spp.	Several species
STH	Strawberry Tree Honey
TIC	Total Ion Chromatogram
TPC	Total Phenolic Content
TSA	Trypticase Soy Agar
TSB	Trypticase Soy Broth
UMF™	Unique Manuka Factor
USE	Ultrasound-assisted Solvent Extraction
VOC	Volatile Organic Compounds
WVTR	Water Vapour Transmission Rate

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Abstract

Development of a novel multidisciplinary approach to evaluate of honey for biomedical applications

Roberta Angioi

A multifaceted approach is needed to fully characterise honey composition and evaluate its beneficial effects in biomedical applications. Here we investigated the composition of four samples of Irish honey, marketed as heather honey, and one sample of manuka honey, examined their inhibitory effect on bacterial growth, and developed honey-loaded scaffolds for topical application. The melissopalynological analysis confirmed only one Irish sample as unifloral heather honey. Aroma compounds, previously suggested as markers of botanical origin, were measured in the headspace of Irish heather honey (C9-norisoprenoids with 3,5,5-trimethyl-cyclohexenic structure) and manuka honey (2-methoxyacetophenone and 2'-hydroxyacetophenone) via Gas Chromatography/Mass Spectrometry (GC/MS). Honey's antibacterial activity against *S. aureus* and *E. coli* was evaluated *in vitro*, with bacterial growth inhibition of 93-100% induced by 50% (w/v) honey solutions in the broth microdilution assay, while the Minimum Bactericidal Concentration (MBC) was established at a honey concentration between >50% and 25% (w/v). An alternative strategy to evaluate honey's antibacterial properties via GC/MS was proposed and tested with *E. coli* by monitoring the variation in emission of distinctive volatile metabolites (i.e., indole and linear alcohols) when bacterial growth was inhibited by honey in liquid culture. Honey-based hydrogels were fabricated with the aim to improve honey's applicability, and their functional performance evaluated regarding swelling degree for absorption of wound exudate and honey release rate. All the hydrogels developed here, regardless of the honey presence, exerted a significant antibacterial effect against *S. aureus* (but not against *E. coli*) *in vitro*, which should therefore be attributed to the intrinsic antibacterial properties of the polymer formulation.

It has been demonstrated in this work that Irish heather honey has an antibacterial effect comparable to manuka honey, that its botanical origin can be characterised using GCMS methods, and that it can be successfully incorporated into hydrogels whereupon its release can be manipulated. Together, these elements provide a robust foundation for further investigations into the potential of Irish honey for biomedical use.

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Chapter 1 The rediscovery of honey for skin repair: recent advances in mechanisms for honey-mediated wound healing and scaffolded application techniques

Part of this chapter was published in the manuscript: Angioi, R.; Morrin, A.; White, B. The Rediscovery of Honey for Skin Repair: Recent Advances in Mechanisms for Honey-Mediated Wound Healing and Scaffolded Application Techniques. *Appl. Sci.* **2021**, *11*, 5192. https://doi.org/10.3390/app11115192

1.1 Honey and its myriad of therapeutic properties

Honey is a sugar-based food product obtained from a broad range of botanical sources (unifloral or multifloral) and different geographical origins. This diversity is reflected by the distinctive pattern of aroma, flavour, colour, and texture of different honey varieties. These organoleptic features, described by the sensory characterisation, are highly interrelated with the physicochemical composition, including soluble bioactive compounds and volatile organic compounds (VOCs), that constitutes the chemical fingerprint of that specific honey variety[1–4]. Reports of using honey and its related bee products (propolis, beeswax, pollen) in traditional medicine stretch back centuries, since the first reports from Sumer, and then in ancient Egypt, Greece, Rome and Asia[5], due to their appreciated and renowned health benefits. However, despite the many hypotheses on the mechanisms for the numerous beneficial effects, honey's full potential has started to be unlocked only recently, with more comprehensive characterisations of its physicochemical composition (as described in Section 1.1.1) and the further investigation of the medical activity of natural bioactive compounds also found in honey[6–15]. Honey has been shown to possess beneficial properties with diverse applications in otorhinolaryngology (conditions of the head and neck)[16,17], respiratory tract diseases[18,19], cardiovascular diseases[20–22], metabolic disorders[23–26], and oncology[27,28]. However, the focus of this review is on the use of honey in the management and treatment of skin disease and, in particular, wounds of various natures, exploring the possible mechanisms by which honey may enhance skin regeneration.

Wound healing is a highly articulated process involving the sophisticated cooperation of numerous cells, mediators, cytokines throughout four phases (coagulation, inflammation, proliferation and remodelling)[29]. It is influenced by various factors, whose disbalance can cause complications, with improper hypertrophic scarring and impaired tissue repair, resulting in chronic wounds[30–33]. Among wound complications, infections represent a major concern, given the alarming surge in antibiotic resistance incidence in recent years[34,35]. In this context, the use of topical agents should be regarded as a valuable alternative to systemic antibiotics when the latter is not deemed essential. Local antimicrobial administration allows a high concentration of active compounds in

the delimited affected area, with limited or no side effects on the healthy circumjacent skin[36]. Furthermore, the use of natural antimicrobial agents, for which antibacterial resistance has not been reported, is becoming more prominent as complementary or alternative options to conventional treatments[37]. Honey, with its extensive use in traditional healing and dermatology, represents a valuable candidate to promote wound healing and complete skin regeneration.

The renewed attention to traditional approaches and apitherapy applications in skin medicine[38,39] coincides with advanced strategies for wound repair gaining increasing importance. In particular, functionalised dressings are being designed not only to cover the wound and protect it from external contaminations but also to actively enhance and accelerate the healing process[40]. These sophisticated developments in wound care might represent the bridge between the history of ethnopharmacology and phytotherapy and the needs of future medicine, with the final goal of developing a cost-effective complementary addition to conventional medications. The main features pursued in an ideal wound dressing are the ability to support healing and shield the wound from further harm and tissue loss whilst incorporating satisfactory fluid control properties. These properties serve to avoid maceration (damage from the over-retention of fluids on the surrounding healthy skin). The ideal dressing should also adhere delicately to the skin, avoiding secondary damage upon removal of the dressing from the newly formed skin underneath and preventing excessive scarring[41].

Our review focuses on the latest research demonstrating the promising therapeutical virtues of different honey types, with particular attention to the articles published in the last five years. Starting with the compositional characterisation of honey, in relation to different honey varieties and particular compounds, honey's antimicrobial, antioxidant and anti-inflammatory properties and the suggested mechanisms are described. Then, the application of honey in the management of different skin diseases and wounds is presented, with case reports and clinical studies, to highlight the latest evidence that demonstrates its healing benefits and also its limitations. Finally, the state-of-the-art of honey incorporation into scaffolds and technological devices for wound healing is illustrated, with a critical analysis of the pivotal parameters that can be used to optimise and further enhance the intrinsic remarkable properties of honey.

1.1.1 Honey physicochemical composition

Honey is the product of various modifications, mainly enzymatic and operated by honey bees, of the nectar or other secretions of plants, i.e. Nectar Honey or Honeydew Honey, respectively, as defined by the Revised Codex Standard for Honey[42], which meets all the legal requirements for food products, from chemical composition to labelling, in order to guarantee a quality product that conforms to the highest standards. From a chemical point of view, honey is a super-saturated solution of sugars, mainly fructose and glucose, at a concentration of not less than 60 g/100 g. Other sugars (mono-, di, tri- and polysaccharides) are also contained, as up to 80% of the product's total composition, with high variability in the relative ratio depending on the botanical origin, although not specifically linked to it[43]. The sugar mixture itself and water content are both indicators of honey's optimal ripeness and also represent the primary factors in assessing adulteration while defining the rheological features responsible for honey texture[44]. Honey moisture content should not exceed 20%, although for heather honey (*Calluna*) a value of 23% is deemed acceptable[42].

Sugar mixture and water content are not the only physicochemical parameters by which honeys can be characterised. A maximum quantity of 0.1 g/100 g water-insoluble solid particles[42] derived from honey collection, such as pollen, are typically also present and can be used for the melissopalynological analysis as part of the botanical classification[45]. Honey also contains minerals and vitamins; proteins are mainly represented by enzymes such as invertase, diastase and glucose oxidase. Among the amino acids found in honey, proline is often quantified, with a minimum value of 180 mg/kg required as an indicator of maturation and authenticity[46]. Proline has been found to be the most abundant amino acid in Estonian honeys (257 - 1328 mg/kg), followed by phenylalanine and glutamine[47]. Another parameter that is strictly monitored is hydroxymethylfurfural (HMF), whose quantity should not exceed 40 mg/kg (80 mg/kg for honey from tropical countries)[42]. It is a by-product of the Maillard reaction between amino acids and sugars and of the acidic degradation of monosaccharides (mainly fructose) and a marker of long storage at high temperatures[48].

1.1.2 Honey phenolic fraction and bioactive compounds

Phenolic compounds are recognised as responsible for the widely investigated antioxidant properties of honey, as indicated by a positive correlation with the water- and lipid-soluble antioxidant capacity[47]. Their content is derived from its floral origin (both if it is single-origin or multifloral) and contributes to honey's colour[49]. A significant correlation ($r = 0.6$, $n = 36$, $p < 0.001$) was confirmed by Kavanagh et al., with Irish dark honeys showing higher Total Phenolic Content (TPC)[50]. Interestingly, the same study has also revealed that phenolic compounds were found to be significantly more abundant in Irish urban honeys than in rural samples. More specifically, in this study, Irish heather honey showed comparable chemical composition to manuka honey (MH) with regard to its TPC (68.16 ± 2.73 and 62.43 ± 10.03 mg GAE/100 g, respectively, where GAE stands for gallic acid equivalents). A TPC of 88.7 mg GAE/100 g and a lipid-soluble antioxidants capacity of 60.7 mg TE (Trolox equivalents)/100 g represented the highest values among the Estonian honeys analysed by Kivima et al., and they were both registered in heather honey, which was also the darkest sample, with the most red tones[47]. Noticeable levels of total phenolics were also confirmed by Salonen et al.[43] for heather honey from Nordic countries. This highlights the potential of heather honey for biomedical applications, considering that honey antioxidant activity is correlated to the beneficial effects of honey on various health issues[51] and to the antibacterial efficacy[43]. Similar TPC values for manuka honey to those observed by Kavanagh et al. have also been measured by Nguyen et al. (72.1 ± 2 and 75.4 ± 0.8 mg GAE/100 g), but in this case, they were shown to be significantly higher than other unifloral honeys from traditional Indian medicinal plants such as tulsi plant (*Ocinum tenuiflorum L.*) and alfalfa (*Medicago sativa*), respectively 50.6 ± 2.7 and 18.3 ± 0.3 mg GAE/100 g[49]. Moreover, additional factors may act independently or jointly with the honey phenolics, leading to an additive or synergistic (potentiated) effect. As such, further investigations on the medicinal effects of alternative honey varieties to manuka honey could still disclose a high therapeutic power.

The variety of phenolic and structurally related compounds observed in honey is determined mainly by the floral origin and the geographical collocation[52]. Some examples are listed here and shown in Table 1.1, merely to offer a brief (but by no means exhaustive) illustration of the diverse assortment of phenolics measured in the latest

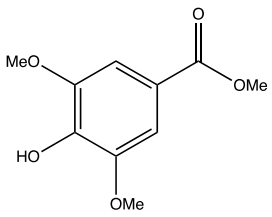
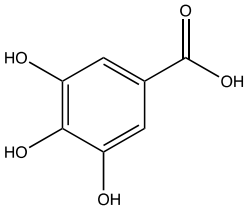
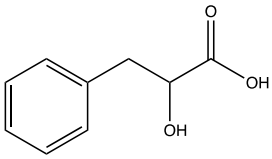
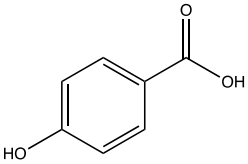
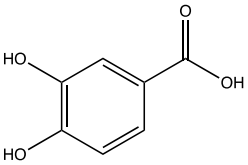
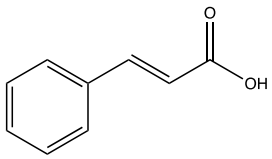
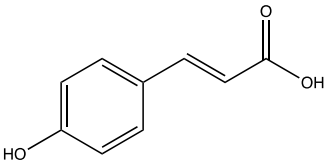
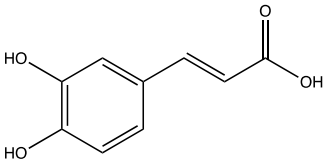
published characterisation of different honey varieties. The honey phenolic fraction can include benzoic acids such as syringic acid, its aldehyde syringaldehyde and its ester methylsyringate. The latter has been detected in Iranian unifloral honeys such as Persian rose, hawthorn and thyme[53]. Methylsyringate represented up to 43.87% of the total phenolic fraction of manuka honey (1.79 ± 0.030 mg/100 g of honey)[53,54], and it has been identified as a biomarker of asphodel honey, with a minimum concentration of 122.6 mg/kg suggested for the botanical origin confirmation[55,56]. Gallic acid was the most concentrated phenolic acid quantified in strawberry tree honey (3.92 ± 0.16 mg/100 g of honey), accounting for 54.44% of the phenolic acid content, followed by 4-hydroxybenzoic acid and caffeic acid (1.29 ± 0.02 and 1.22 ± 0.06 mg/100 g, respectively)[57]. Homogentisic acid has been identified in particularly high concentrations in strawberry tree honey (346.8 ± 17.2 mg/kg)[56], of which it represents, together with abscisic acid isomers (109.6 ± 4.2 and 116.9 ± 4.7 mg/kg), a specific marker of botanical origin[58,59]. Ellagic acid, typically found in raspberry honey, was also detected in lingonberry honey by Salonen et al. (0.39 ± 0.24 mg/kg and 0.48 mg/kg, respectively)[43]. Phenyllactic acid and para-hydroxybenzoic acid were the benzoic acids found in the highest abundance in honeys from Iran (semi-quantified based on peak area)[53]. Cinnamic acid has been detected in chestnut honey[60] and is typically found in high concentrations in heather honey, together with myricetin and abscisic acid derivatives[47]. Coumaric acid is the most represented cinnamic acid derivative in buckwheat honey (5.69 mg/kg)[43]. Among the flavonoids, pinobanksin has been found to be abundant across various Iranian honey samples of different botanical origin (semi-quantified based on peak area)[53]. It was also the most abundant flavonoid, together with pinocembrin identified in manuka honey (36% and 23% of total flavonoid content, at concentrations of 0.28 ± 0.047 and 0.18 ± 0.035 mg/100 g of honey, respectively)[54]. The flavonol kaempferol was the most represented flavonoid (1.90 ± 0.05 mg/100 g of honey, 41.2% of the total flavonols content) in strawberry tree honey, with quercetin and luteolin being also highly abundant (0.90 ± 0.02 and 0.79 ± 0.10 mg/100 g of honey)[57]. Chrysin has been found in various unifloral honeys, such as astragal, chicory, white clover, and hawthorn honey[53]. The screening and isolation of individual compounds in honey in these studies was predominantly used to identify markers of botanical origin and to characterise honey's organoleptic profile. While phenolic

compounds have been extensively researched, and the rationale for their use as ethno-medicinal products has been confirmed by numerous studies[52,61], typically research in this area has not focused on the determination of individual phenols in honeys. Instead, TPC and its correlation with biological activities of interest is investigated, and composite phenolic extracts are evaluated in biological studies for their combined effect[52,62]. These natural anti-inflammatory and antioxidant agents are widely present in various plants and natural products of traditional use in wound care and folk medicine for various skin conditions[63]. Polyphenol-rich crude extracts from Alaskan wild berries showed promising potential *in vitro* for tissue regeneration and wound closure, in particular, due to the proanthocyanidin fractions playing a significant role in the expression of extracellular matrix constituents, such as integrins and collagen, and stimulating mitochondrial vital processes[64]. Bletilla striata phenols-rich extract has been shown to effectively promote healing in a mice model of burn wounds, with notable wound reduction by the fifth day of treatment compared to control. The components of the beneficial extract were identified as protocatechuic acid, p-hydroxybenzoic acid, caffeic acid, p-hydroxy-benzaldehyde, 3-hydroxycinnamic acid, and ferulic acid[65]. These bioactive compounds and structurally related compounds have also been identified in honeys of various botanical sources[43,47,60,66,67], suggesting that honey might share the same virtues and represent a valuable agent in wound medicine.

Royal jelly (RJ) has been highly regarded in traditional medicine and widely investigated for its distinctive antimicrobial properties[68]. RJ's peculiar compound, 10-hydroxy-2-decenoic acid (10-HDA), also known as queen bee acid, has shown promising results *in vitro* on human colorectal cancer by tackling critical pathways in the pathogenesis of carcinomas, markedly reducing the levels of cytokines involved in pro-inflammatory signalling and exerting bactericidal activity against pathogens responsible for infections of the gastrointestinal tract[69]. Significant downregulation of melanin production and inhibited expression of melanogenesis-related peptides were observed in melanoma cells upon treatment with 10-HDA, showing potential to treat hyper-pigmentation skin conditions[70]. In light of their noteworthy beneficial properties, the identification of RJ compounds in honey could be of significant interest for the applications of honey in skin treatment. 10-HDA was found in high concentrations in the aliphatic acids fraction analysed in pine herbhoney and multifloral honey by Isidorow et al.[71]. It was also

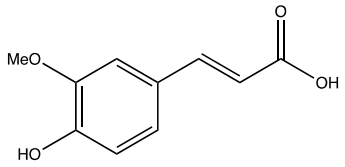
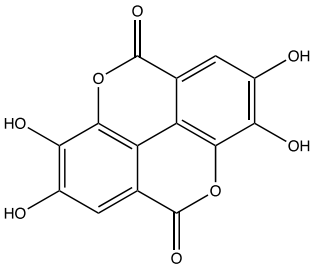
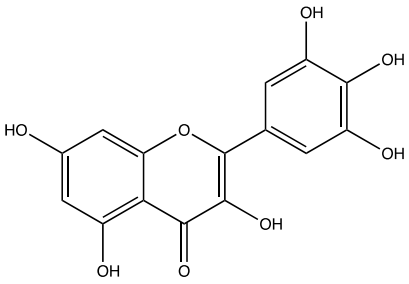
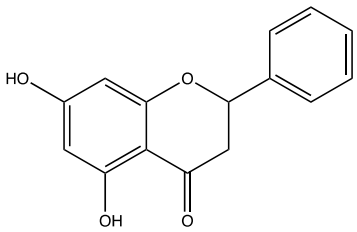
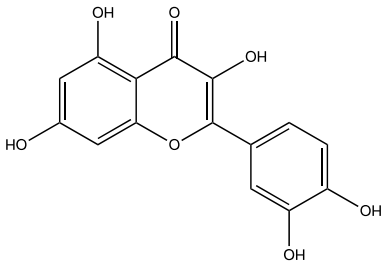
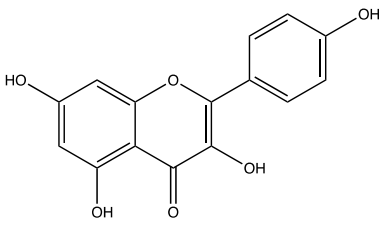
identified by Levya-Jimenez in Iranian honeys of different botanical origins, although dihydroxy-decenoic acid was the most abundant RJ-derived compound in these samples. However, this was not found to be present at significant concentrations even in the most actively antimicrobial samples[53], suggesting it might not significantly contribute to this activity. RJ-derived fatty diacids (decanedioic and decenedioic acids) have been detected in Scottish honeys, together with their glycosides, with preliminary data finding them more concentrated in antimicrobially active samples[67].

Table 1.1 Chemical structure of some of the most frequently researched phenolic compounds detected in honey and examples of their occurrence in honeys of different botanical origins. Me = methyl group.

Chemical structure	Compound name	Honey varieties
	Methylsyringate	Persian rose, Hawthorn, and Thyme[53], Asphodel[55,56], Agastache[72], Manuka[54,67]
	Gallic acid	Strawberry tree[57], Chestnut[60], Mint and Raspberry[73]
	Phenyllactic acid	Agastache and Jarrah[72], Manuka[67]
	p-Hydroxybenzoic acid	Strawberry tree [57], Chestnut [60], Raspberry, Sunflower, and Mint[73], Buckwheat [74]
	Protocatechuic acid	Chestnut[60], Raspberry, Mint, Thyme, and Honeydew[47,73], Buckwheat[74]
	Cinnamic acid	Chestnut[60], Heather[47]
	Coumaric acid	Raspberry, Sunflower, and Thyme[73], Buckwheat[43,74], Juazeiro[66], Tilia, Honeydew, and Sunflower[75]
	Caffeic acid	Strawberry Tree[57], Chestnut[60]

Continues on page 10

Table 1.1 (continued)

Chemical structure	Compound name	Honey varieties
	Ferulic acid	Buckwheat[74], Juazeiro[66]
	Ellagic acid	Raspberry and Lingonberry[43], Juazeiro[66]
	Myricetin	Strawberry Tree[57], Thyme, Rape, Mint, Raspberry, and Sunflower[73], Malicia[66], Heather[47]
	Pinocembrin	White clover, Hawthorn, Black cumin[53], Manuka[54], Tilia, Acacia, Honeydew, and Sunflower[75]
	Quercetin	Strawberry Tree[57], Juazeiro and Malicia[66], Brassica[47], Honeydew and Acacia[75]
	Kaempferol	Strawberry Tree[57], Juazeiro and Malicia[66], Brassica[47]

1.1.3 Antimicrobial properties of honey

The determinant factor for honey antimicrobial activity is yet to be unequivocally agreed on, which complicates the comparison between honey varieties described in healing applications. A conventional simplification leads to a distinction between peroxide and non-peroxide antibacterial activity: the first being hydrogen peroxide-mediated and the latter often identified with manuka honey but not limited to it. However, the hypothesis of a multifactorial system seems more verisimilar, and it is generally accepted[76] (Figure 1.1).

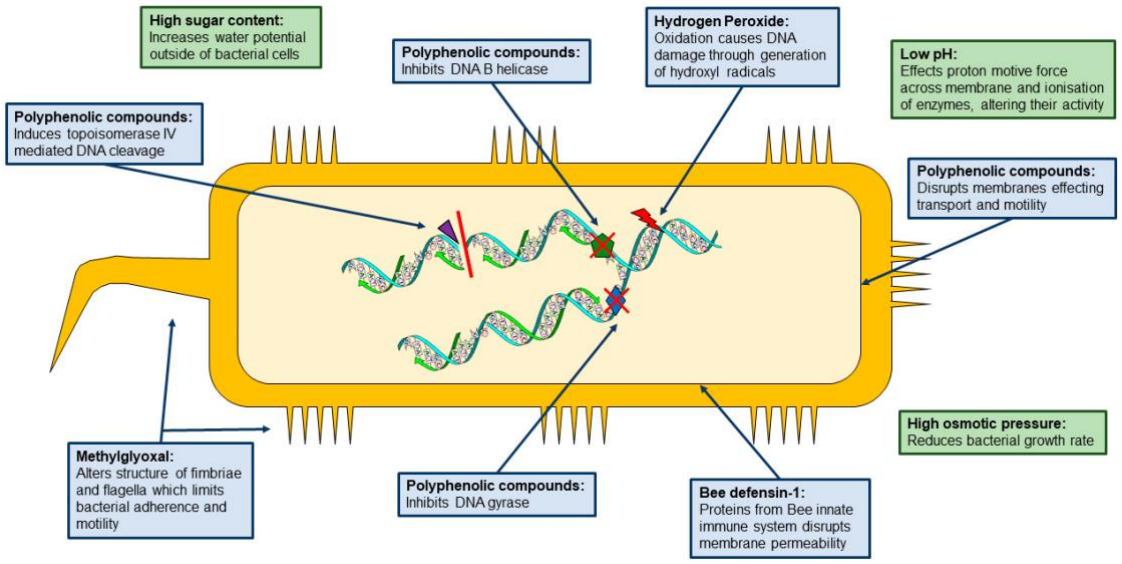


Figure 1.1 "The main constituents attributed to honey's antimicrobial activity and their mechanism of action. Direct inhibitory factors affect cellular mechanisms (blue), indirect inhibitory factors have a wider ranging effect on the bacterial cell (green)." Reproduced from Antibiotics 2019, 8, 251; Nolan et al. Dissecting the Antimicrobial Composition of Honey[77].

1.1.3.1 Hydrogen peroxide

The main mechanism for the widely clinically observed capacity of honey to fight bacterial infections and promote skin regeneration is due to the production of hydrogen peroxide (H_2O_2) during the glucose oxidase (GOx)-mediated conversion of glucose to its correspondent acid. Furthermore, honey also upregulates AQP3 aquaporin's expression, with consequently improved diffusion of H_2O_2, which results in increased calcium Ca^{2+} cytoplasmatic levels by Ca^{2+} channel activation. Different Ca^{2+} release intensities and kinetics have been linked to different honey varieties. Augmented intracellular Ca^{2+} thereupon governs numerous biochemical chains of events that

ultimately boost wound repair[78]. However, conflicting results are reported in the literature about the role played by H_2O_2 in the antibiotic efficacy of honey, and this might also be due to the time-sensitive variations of H_2O_2 levels after honey production. H_2O_2 content in Western Australian honeys did not show a correlation with the antiseptic performance, although H_2O_2 reduction operated by the enzyme catalase did affect the samples' antimicrobial power[79]. Comparable results to these reported by Roshan et al. were also described by Bucekova and co-workers about honeydew honeys, with no significantly different results in the antibacterial *in vitro* evaluations, despite divergent H_2O_2 concentrations across the samples[80]. On the other hand, opposite observations were collected by the same author in an attempt to elucidate the contribution of H_2O_2 to the antimicrobial effect of different blossom honeys - namely rapeseed, acacia and wildflower, ordered here by increasing antibacterial activity. In fact, Bucekova et al. reported a statistically significant correlation between the antibacterial effect and both total phenolic and H_2O_2. However, interestingly, no statistical correlation was registered between the measured H_2O_2-producing enzyme GOx and the levels of H_2O_2. Furthermore, while again dramatically inhibited by treatment with catalase, such antimicrobial activity was instead not affected when GOx was proteolytically digested upon the addition of proteinase-K[45]. These results suggest that another system, other than the protein-controlled one, must be co-responsible for the honey peroxide-mediated activity. Therefore, these observations could confirm the hypothesis of a phytochemical-dependent production of H_2O_2 in honey as an alternative to the widely accepted GOx biochemical pathway[80]. A synergism between H_2O_2 and phenolics has also been linked to the antiseptic activity exerted by Corsican honeys through irreversible plasmidic DNA damage on *P. aeruginosa* cultures[81].

1.1.3.2 The bee-derived peptide, defensin-1

Defensin-1, originally named royalisin due to its first isolation in royal jelly is a peptide of 51 amino acids (5523 Da of molecular mass), produced by bees with immunity and a defence function against pathogens such as *Paenibacillus larvae*[82–84]. It exerts antibacterial effects mainly against Gram-positive bacteria[83,85], although inhibition against Gram-negative and fungi has been observed in defensin-1 obtained via

recombinant technique[86,87]. The mechanism of action has been hypothesised to involve ionic interaction with the bacterial membrane[85]. Multiple disulfide bonds in its structure have been reported to confer stability to it at high temperatures and low pH[83]. However, other studies suggested that heat exposure at temperatures above 55 °C and microwave treatment may induce the formation of aggregated structures, resulting in diminished and fully inactivated antibacterial effect of honey, respectively[85,88].

Defensin-1 has been suggested as a crucial element to the antibacterial activity of non-manuka honeys, but its concentrations are highly variable depending on the botanical origin and geographical location and moreover are typically low, suggesting that its role in the antibacterial activity of honey may be negligible[80,89,90]. For example, defensin-1 has been identified as one of the bactericidal factors of Revamil™ honey, together with MGO and H_2O_2[91]. On the other hand, in honeydew honey, defensin-1 levels did not seem to influence the antibacterial effect against *S. aureus*, confirmed by the uncompromised antibacterial activity despite protein inactivation by peptidase[80]. In contrast, proteinase K-treated Greek honeys had their microbiological properties negatively impacted, as demonstrated by higher honey concentrations needed to achieve the same bacterial inhibition (i.e., higher MIC)[92]. In addition to its antibacterial activity, defensin-1 has been shown to exert a pivotal contribution to the skin regenerative effect traditionally shown by honey and royal jelly. The defensin-1-induced wound healing advancement is achieved *in vitro* by significantly increased matrix metalloproteinase-9 (MMP-9) release and consequently enhanced keratinocytes chemotaxis and neo-vascularization *in vivo*[93]. This restorative mechanical process is fundamental to the re-establishment of an effective skin barrier following epithelial cell migration after an injury; thus, such encouraging results offer a further explanation for the use of bee products in wound medicine.

1.1.3.3 General honey features of interest: sugar content and acidity make an unfavourable environment for bacterial growth

The osmotic pressure generated upon honey application on a wound site is regarded to be a primary contributor to the pro-healing features of honey of all botanical origins. In fact, the flux of fluids along the concentration gradient due to honey's high sugar

content effectively washes the wound area from contaminants, debris and bacteria and transports nutrients while regulating the moisture balance in exudative wound[94]. Fyfe et al. reported that a negative sugar control (consisting of a saturated solution of 38.5% fructose, 33.3% glucose, 6.2% maltose and 7.3% sucrose in distilled water) inhibited the bacteria tested, although not as effectively as the compared honey samples[67]. A similar observation was made in a study by Salonen et al., with artificial honey prepared with an analogous sugar composition to Finnish honey and acidified to pH 3.5 with HCl[43]. However, a 75% sucrose (w/w) artificial honey did not show antibacterial activity against any of the bacteria tested by Matzen et al.[95]. It is therefore suggested that the high osmolality is not decisive to the medicinal properties of honey by itself, although it could contribute to them.

Honey presents an intrinsic acidic pH, typically ranging between 3-4.7, due to its organic acids content[49,50,96]. This inherent acidity is deemed to be one of the primary contributors to the anti-microbial activity of honey, as it creates an unfavourable environment for bacterial growth[97]. However, no correlation between pH and antibacterial activity was found in Stingless bee honeys[98], nor in Polish honeys against Staphylococci[99]. The acidity is also believed to play a role in the capacity of honey to restore the skin barrier properties that can be affected by various medical conditions. Inflammatory diseases (atopic-prone skin, eczema, seborrheic dermatitis), skin microbiome alteration, topical infections (candidiasis), and wounds can, in fact, compromise the epidermal acidic milieu[100]. The use of topical agents with slightly acidic pH, such as honey, can help establish and maintain skin physiological pH (in the range between 4.5 to 6.0) and the re-deposition of the “acid mantle”, which is important for optimal skin barrier function in the stratum corneum (the outermost epidermal layer)[101,102]. Topical treatment with honey could also enhance wound healing by naturally creating an unfavourable environment for proteolytic enzymes, such as elastase or matrix metalloproteinases (MMPs). These are otherwise found to be hyperactive in the alkaline environment typical of chronic and infected wounds (up to 7.5-8.9), and, as a consequence of this, the skin repair is impaired by continuous degradation of recently deposited tissue[100].

1.1.3.4 The role of phenolic compounds

Phenolic compounds critically modulate the antimicrobial properties of honey against common wound infection-causing strains, both Gram-positive, such as *Staphylococcus aureus (S. aureus)* and *Enterococcus faecalis (E. faecalis)*, and Gram-negative bacteria such as *Escherichia coli (E. coli)* and *Pseudomonas aeruginosa (P. aeruginosa)*. When tested using a disc diffusion method, phenolic acid extracts were found to inhibit bacteria at a lower minimal inhibitory concentration (MIC) than the correspondent whole honey sample extract. This shows the prominent contribution of phenolic constituents, working synergistically, to the overall honey antibacterial activity[53]. Such phenolics-mediated anti-bacterial action seems to rest on a sophisticated balance between radical scavenging and pro-oxidant functions (Figure 1.2): depending on the concurring factors (i.e. hydrogen peroxide-rich honeys and the presence of transition metals), these renowned anti-oxidant agents can, in fact, auto-generate reactive oxygen species (ROS) and activate hydroxyl radicals. This triggers propagation of the Fenton reaction[81,103], resulting in the observed antibacterial effect by bacterial damage. The direction that this equilibrium tends to also depends on the pH value: pro-oxidant action has been observed at fairly neutral pH, such as in diluted honeys[80]. The total phenolic content of honeys from Nordic countries has been shown to correlate with their inhibitory capacity against *S. aureus* at 15% dilution (r = 0.57), with buckwheat, heather, sweet clover and polyfloral honeys having the most pronounced antibacterial activity[43]. However, the total phenolic content and antioxidant activity of Scottish honeys of different botanical origins have not been found to be correlated with antibacterial activity[67]. The investigation of Bueno-Costa et al. on Brazilian honeys confirmed these findings, but a significant correlation (r=0.4424) was instead observed between total flavonoid levels and antibacterial activity against *Bacillus cereus* (*B. cereus)*[104].

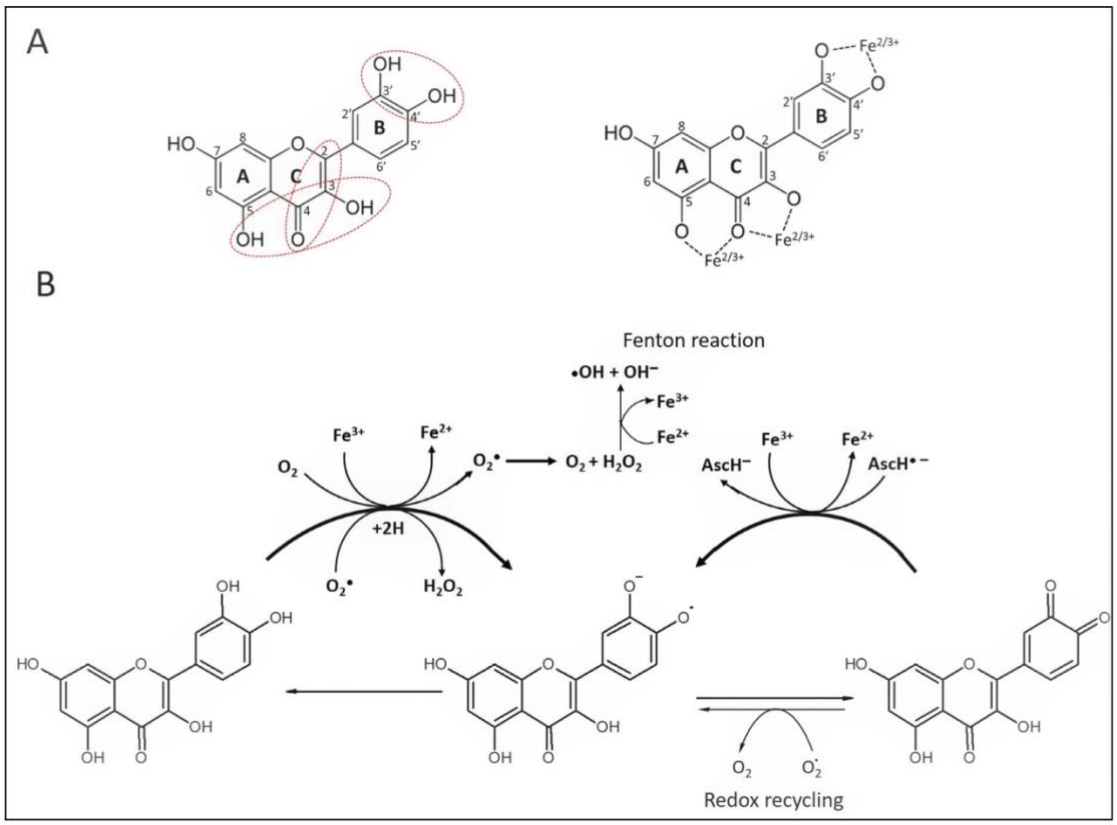


Figure 1.2 "The potential mechanism of non-enzymatic H_2O_2 production in honey by flavonoid autooxidation. (A) Structural elements required for an efficient radical scavenging activity and metal ion-chelation are circled[105,106]*. Three possible binding sites of a metal cation to a flavonoid. (B) A schematic proposal of H_2O_2 production in honey via flavonoid (quercetin) autooxidation based on literature data. Redox cycling and pro-oxidant activity of flavonoid is promoted in the presence of ascorbic acid. Ascorbic acid (AscH) is oxidized to ascorbate radical anion ($Asc^{\bullet -}$) by one-electron transfer to Fe^{3+} and its reduction to Fe^{2+}. Ascorbate radical ($Asc^{\bullet -}$) reduces quinone to semiquinone radicals propagating redox cycling." Reproduced from Metabolites 2023, 13, 526; Brudzynski et al. Unexpected Value of Honey Color for Prediction of a Non-Enzymatic H_2O_2 Production and Honey Antibacterial Activity: A Perspective*[107].

1.1.3.5 Manuka honey and methylglyoxal

Manuka honey (MH) is currently regarded as the gold standard for medical applications, and a specific unit, the Unique Manuka Factor (UMF™), has been adopted to indicate its authenticity based on the content of specific markers, such as methylglyoxal (MGO), dihydroxyacetone (DHA) and leptosperin at the time of pre-packing food quality testing[108]. MGO originates from the precursor DHA found in the nectar, and its content depends on the presence of endogenous co-factors, such as phenolic content (mainly phenyllactic acid and methoxyphenyllactic acid) and aminoacidic composition (in particular proline) that could trigger Maillard-like side reactions and environmental parameters such as storage temperature and length of time[109,110]. Given the vast attention granted to manuka honey as the only medical-grade honey recognised so far,

its fingerprint compound MGO has recently attracted increasing interest, with research focusing on its identification in other honey varieties. MGO content in honeys of different floral origins than manuka is highly variable. Terio et al. conducted a quantification of MGO in Italian honeys, showing an MGO wide range (0.4-24.01 mg/kg) with cherry (18.62 ± 3.69 mg/kg) and almond honey (17.88 ± 4.18 mg/kg) having the highest concentrations among the analysed varieties, but still significantly lower than the levels measured in manuka honey. However, this study did not include an evaluation of the antibacterial activity of the analysed honey samples, so further investigations would be needed to assess whether MGO content is relevant to the antibacterial mechanism of action of these Italian honey varieties[110]. MGO was found in high quantities (up to 166 ± mg/kg) in Nordic honey samples (mire and polyfloral) but totally absent in heather honey and in some samples of other varieties such as caraway, sweet clover, and dandelion. However, MGO content was not found to correlate with the inhibitory capacity of Nordic honeys against *P. aeruginosa* or *S. aureus* at 15% honey dilution[43]. Eleven honey samples of varieties produced from Danish flora showed a significantly lower MGO content (less than 5 μg/mL) than commercial manuka honey (54.33 μg/mL). Nonetheless, Water mint, Linden and mixed organic flora honey were shown to cause the greatest degree of bacterial growth inhibition of all the tested honey varieties, even on the Gram-negative bacteria (*P. aeruginosa* and *E. coli*) against which manuka was instead ineffective. Such activity was significantly affected by treatment with catalase, suggesting that it is ascribable mainly to peroxide-mediated mechanism[95]. The UMF™ indicator is described as linked to the antibacterial power by comparison with a reference compound, e.g. a 5 UMF™ is assigned to honey capable of inducing a microbial inhibition equal to that obtained by a 5% phenol positive control[111]. However, the MGO content in MH can increase during honey storage due to DHA conversion[109], generating incongruence with the claims reported on the labelling. Interestingly, Hixon et al. recorded the performance in the bacterial clearance test to be significantly greater in samples with lower UMF™ compared to MH of up to 20 UMF™[111]. Similar results were also observed by Girma et al.[112], which compared MIC *in vitro* against different bacteria for MH of various UMF™ values. These results highlight the urgency for a univocal criterion applicable to all honey varieties, which would be crucial in allowing a

standardised comparison of the antibacterial potency of honey across different botanical species.

Manuka honey's antibacterial effect seems to be incompatible with peroxide-mediated activity, as MGO supplementation has been shown to impede GOx activity in non-manuka honeys by morphological damage and formation of glycated derivative structure. As a result, GOx-modulated H_2O_2 production was significantly compromised in a dose-proportional fashion[113]. However, an interaction between peroxide and non-peroxide systems has been described by Henatsch et al., who studied the antibacterial activity of honey in relation not only to its content of MGO and analogous α-dicarbonyl compounds but also to their conversion into different free radicals in the presence of either hydrogen peroxide or amino acids (specifically arginine and lysine)[114]. Majtan et al. also reported how MGO structurally inactivates other peptides deemed responsible for the therapeutical applications of honey in dermatology, such as the bee-peptide defensin-1, with possible impaired antibacterial activity[115].

1.1.3.6 Honey's antibiofilm effect

Biofilms are structures in which microorganisms communities live in a self-secreted dense matrix consisting of extracellular polymeric substances (EPS) which protects them from eradication from surfaces[116,117]. In the struggle against hospital-acquired infections, biofilm eradication from chronic wounds represents a substantial challenge[118]. Different honey varieties have been tested for their antibiofilm activity. Honey in low concentrations (acacia, clover and polyfloral) has been suggested as a strategy to prevent biofilm formation by *E. coli* O157:H7, by acting against the intrinsic intercell communication systems (quorum sensing) involved in the establishment of biofilms and reducing virulence[119]. Eucalyptus honey exerted antibiofilm activity against multiresistant species due to the composite effect of osmotic pressure, H_2O_2 and defensin-1[120]. Defensin-1 (in its recombinant form) has also been shown to be an effective treatment against polymicrobial biofilms, reducing viability of *S. aureus* and *P. aeruginosa*[121]. Heather honey showed analogous effectiveness to manuka honey against biofilm formation from different species, namely *Acinetobacter baumannii (A. baumanii), E. coli, Salmonella enteritidis (S. enteritidis),* and *P. aeruginosa*. A shared key

element in the inhibition of the *P. aeruginosa* biofilm for both the honey varieties could be benzoic acid, which has been predicted by molecular docking to efficiently bind the bacterial enzyme PaDsbA1. In doing so, it would alter the protein structural arrangement, hence compromising the biological functionality of fimbriae, flagellae and adhesion factors, crucial for biofilm establishment. However, heather honey promoted *E. faecalis* and *Klebsiella pneumoniae (K. pneumoniae)* biofilms, while manuka honey was ineffective on *S. aureus*[122]. This different susceptibility of the bacterial strains to honey of different botanical origins could be considered when evaluating which honey variety to use to treat a given infected wound. Portuguese heather honey effectively inhibited *Candida tropicalis (C. tropicalis)* planktonic population at a lower concentration (MIC 12.5% w/v, Minimum Fungicidal Concentration (MFC) 50% w/v) than manuka honey (MIC 25% w/v, MFC 50% w/v). The two honey varieties were also compared on their antimicrobial action on single and multi-species biofilms of *C. tropicalis* with *P. aeruginosa*. A significant reduction in cell viability of *C. tropicalis* was observed for both heather and manuka honey at a concentration of 50% (w/v). In regard to *P. aeruginosa*, manuka honey was more effective in decreasing the cell count already at a concentration of 25% (w/v) and up to 4 log (CFU/cm^2) reduction at 50% (w/v) in the single biofilm. A significant cell inhibition compared to control was nonetheless obtained with heather honey at 50% (w/v). When assessed in association with conventional antifungal treatment, the supplementation with honey allowed the administration of a 50% lower dose of fluconazole, but higher inhibition was still achieved with honey monotherapy at 50% (w/v)[123]. Both manuka and honeydew honey effectively reduced viability of pathogens in multispecies biofilms[121], which are often found in chronic wounds, hindering their healing. Honey may also be used to advance the antibiofilm effect of other treatments, due to its ability to infiltrate the biofilm structure facilitating the penetration of the drugs. For example, the combination of chestnut honey with bacteriophages has been shown highly effective in eradicating single and multispecies biofilms of *E. coli* and *P. aeruginosa*[124].

1.1.3.7 The influence of the bee species

Ultimately, *Apis mellifera*, commonly known as honeybee, is not the only insect responsible for honey production. The species of nectar-foraging insects seems to determine the phytochemicals pattern in the ripened honey, thus leading to different effectiveness against bacterial infections. Malaysian honeydew honey has been reported to have a higher bactericidal effect against *S. aureus* and *E. coli in vitro* when produced by the stingless bee species *Heterotrigona itama* rather than *Apis cerana* and *Geniotrigona thoracica*[76]. Of the eight stingless bee honey samples analysed by Rosli et al., honey produced by *Homotrigona fimbriata* has been found to possess the highest inhibitory capacity against five bacteria: *Serratia marcescens (S. marcescens), E. coli, Bacillus subtilis (B. subtilis), Alcaligenes faecalis (A. faecalis)*, and *S. aureus*. The least active honey was produced by *H. erythrogastra* and has been shown to be inactive against the tested bacteria at all the evaluated concentrations[98]. In contrast, *H. erythrogastra* honey from Borneo was shown to be broadly active against all the bacteria strains tested by Tuksitha et al. Honey produced by *G. thoracica* has been shown to be significantly more effective than the honey samples from other stingless bees against both Gram-positive (*Staphylococcus xylosus (S. xylosus)*) and Gram-negative (*P. aeruginosa* and *Vibrio parahaemolyticus (V. parahaemolyticus)*). Interestingly, *H. itama* honey was both the least active antimicrobial agent against Gram-negative bacteria and the richest in phenolics, while significant Gram-negative bacteria inhibition was obtained with the honey samples with the highest level of flavonoids (*G.thoracica*). This has been hypothesised to be due to flavonoids-mediated disruption of the outer bacterial membrane integrity or impaired DNA synthesis[125].

1.1.4 Anti-inflammatory properties of honey

Honey has been long appreciated for its exceptional ability to de-escalate phlogosis, hence the traditional use as a medicament on chronic inflammatory skin conditions and in the management of persistent symptoms such as unremitting discomfort, itchiness often associated with skin abrasion and laceration due to scratching, all severely affecting the quality of patients' everyday life. Along with the conventional treatments prescribed to soothe the affected skin (predominantly topical corticosteroids), growing

interest has been expressed for natural remedies in the management of disorders with a main inflammatory component, such as eczematous lesions and psoriasis[126,127]. Alangari et al. researched the performance of manuka honey on atopic dermatitis (AD) patients and tried to elucidate the responsible biochemical mechanisms by AD-related *in vitro* models. Both evaluations confirmed a significant ameliorative effect by direct Medihoney™ (medical-grade MH-based commercial ointment) and MH extracts application. Inflammation was reduced, as confirmed by lower IL-4 stimulation on chemokine ligand CCL26 (a chemotactic involved in pro-inflammatory and allergic response). Also, histamine release suppression from mast cells was obtained in a concentration-related fashion[128].

ROS-mediated oxidative damage has been linked to chronic inflammation and compromise of wound healing; hence, targeting and modulating the systems responsible for aberrant inflammation is emerging as a promising strategy to promote skin repair[129,130]. Unlocking the mechanisms of the antioxidant and radical scavenging activity of honey could possibly allow the full potential of honey application to be leveraged in wound medicine. Alvarez-Suarez reported a statistically improved wound closure rate by manuka honey pre-treatment through enhanced human dermal fibroblast viability and migration. MH also showed a significant protective effect on the oxidative stress model and reduced ROS generation compared to control and artificial honey. This might be due to augmented 5'-AMP-activated protein kinase (AMPK) phosphorylation[54]. The activation of AMPK ultimately results in the upregulated expression of antioxidant enzymes such as glutathione peroxidase, glutathione reductase, and glutathione s-transferase, as also observed by Gasparrini and co-workers[131]. When assessed in a macrophagic LPS-induced inflammation model, manuka honey has been shown to modulate a series of crucial peptides (caspase 3, p38, pErk1/2, AMPK, SIRT1 and PGC1α). As a result, dose-dependent downregulation of apoptosis and augmented mitochondrial metabolism were observed, with cellular proliferation and migration[132], confirming manuka honey's ability to promote wound closure. Again, however, it should be noted that the basis for this observed effect was not explored in this study.

As with the antimicrobial properties, a greater degree of attention is reserved to manuka variety rather than other honeys in regard to their antioxidant benefits. Nonetheless, Sardinian strawberry tree (*Arbutus unedo*) honey (STH), also described as "bitter honey", is also appreciated for its exceptional phenolic content and antioxidant properties[133], and was shown to exert targeted cytotoxic performance at lower doses than MH on colon cancerous and metastatic cells, with enhanced ROS production[51]. For both STH and MH, a highly significant correlation was observed between polyphenols and flavonoid contents, and a strong relationship between these values and the Total Antioxidant Capacity of honey, suggesting how the virtuous health properties can be ascribed to the notable phytochemical composition. In particular, STH from the Berchidda area had the highest value of total polyphenols of all the samples (1.00 ± 0.02 g GAE/Kg), including MH (0.89 ± 0.01 g GAE/Kg), and its total flavonoids content was also significantly higher (108.20 ± 2.69 g CAE/Kg) than MH value (71.90 ± 0.03 g CAE/Kg). These results are in line with the values reported by Di Petrillo et al. (969.7 ± 8.5 mg GAE/kg)[56]. The same bitter honey was further analysed for its anti-proliferative effect, and the biological mechanisms inducing its protective and antineoplastic features were thoroughly disclosed by Afrin et al.[57,134]. While a full discussion on the findings of such an in-depth investigation is beyond the scope of this review, whose focus is on the honey benefits to the skin, it is worth attention that the expression of honey health-giving properties is modulated on the biological target. Cell migration was, in fact, significantly suppressed by STH pre-treatment in cancerous cells, as confirmed by lowered expression of invasion factors like MMP-2 and MMP-9, which indicates the honey capacity to inhibit the invasive potential of metastatic cells. Conversely, STH promoted the migration of non-neoplastic control cells in a wound-healing model, a promising result for skin repair applications. Likewise, oxidative stress provokes biological aberration in healthy cells, and it is regulated in physiological conditions by compounds acting as free-radical scavengers. However, ROS expression is significantly augmented by STH in a dose-proportional fashion, and the activity of antioxidant cellular enzymes is downregulated in colorectal cancer cells. Whilst this might seem paradoxical for a well-known antioxidant product, the pro-oxidative environment is intended to induce apoptosis and reduce malignant cell proliferation[134]. Similarly, in an *in vitro* study by

Minden-Birkenmaier et al.[94], despite the initial promotion of the inflammatory response, a significant reduction in superoxide release was observed after 24 h, together with inhibited chemotaxis of neutrophils and IκBα phosphorylation, indicating a significant overall dose-proportional anti-inflammatory outcome. This duality explains the broad applicability of honey for health issues characterised by distinctive pathogenesis. Furthermore, when discussing and researching the positive attributes of a medical-use agent, as for any other bioactive compounds, it is imperative to keep in mind the aphorism "Sola dosis facit venenum", i.e. the dose makes the poison. This means that different concentrations of the therapeutic agent need to be evaluated to define the cut-off dose above which the side effects are observed. It is rightfully suggested by Minden-Birkenmaier et al.[94] that a thorough evaluation of the possible release kinetics should be conducted and that a prolonged release, able to maintain a steady concentration of the active compounds in the area over an extended time frame, might be more advantageous than high concentrations administered with a burst delivery.

1.2 Honey in wound healing

1.2.1 Wounds and physiology of the healing process

The skin is the largest organ in the human body and it has a pivotal role in countless critical functions such as protecting from external agents (physical, environmental, chemical and biological), thermoregulation and balancing fluid loss, and sensory stimulation, to name but a few[135]. Briefly, the skin consists of three main layers, namely epidermis (the most superficial), dermis (a highly fibrous tissue incorporating nerves, blood and lymphatic vessels) and hypodermis (subcutaneous tissue, mainly formed by adipocytes and fibrous connective tissue)[136]. The epidermis, generally considered the primary site of the skin barrier function, is mainly formed by keratinocytes, melanocytes (responsible for skin pigmentation), and mechanoreceptors[137]. It also houses skin appendages such as hair follicles, nails, and sebaceous and sweat glands[136]. Four main sublayers, or strata, can be identified in the epidermis: the deepest is the stratum basale, responsible for continuously providing differentiating epidermal stem cells for physiological turnover and skin repair. In their differentiation process, keratinocyte

stem cells migrate through stratum spinosum, stratum granulosum (the last layer containing living cells), and finally the outermost protective layer stratum corneum, consisting of corneocytes[136,138].

After a wound is inflicted, the human body physiologically puts in place a series of steps aimed at restoring homeostasis and reconstructing the skin's barrier function. These steps comprise a sequence of partially overlapping and interdependent phases, namely coagulation, inflammation, proliferation and remodelling[29] (Figure 1.3). Promptly after the injury, initial vasoconstriction, aimed at limiting blood loss and facilitating the deposition of a clot and secretion of growth factors by platelet, is observed[139]. The inflammatory signal cascade begins with the recruitment of neutrophils, macrophages and mast cells, cytokine release, immunomodulation, protease-mediated removal of debris, and vasodilation essential for the supply of nutrients[140–142]. These build the conditions for the proliferative phase, with fibroblasts and keratinocytes active in the deposition of granulation tissue and re-epithelialisation, respectively[143,144]. Formation of new blood and lymphatic vessels (angiogenesis) also occurs[29]. Finally, with the resolution of the inflammation and the reduction of vessel permeability, the maturation phase begins when apoptosis of fibroblasts and remodelling and contraction of new extracellular matrix (ECM) and collagen occurs[145]. The result of this sophisticated healing process is a scar[146].

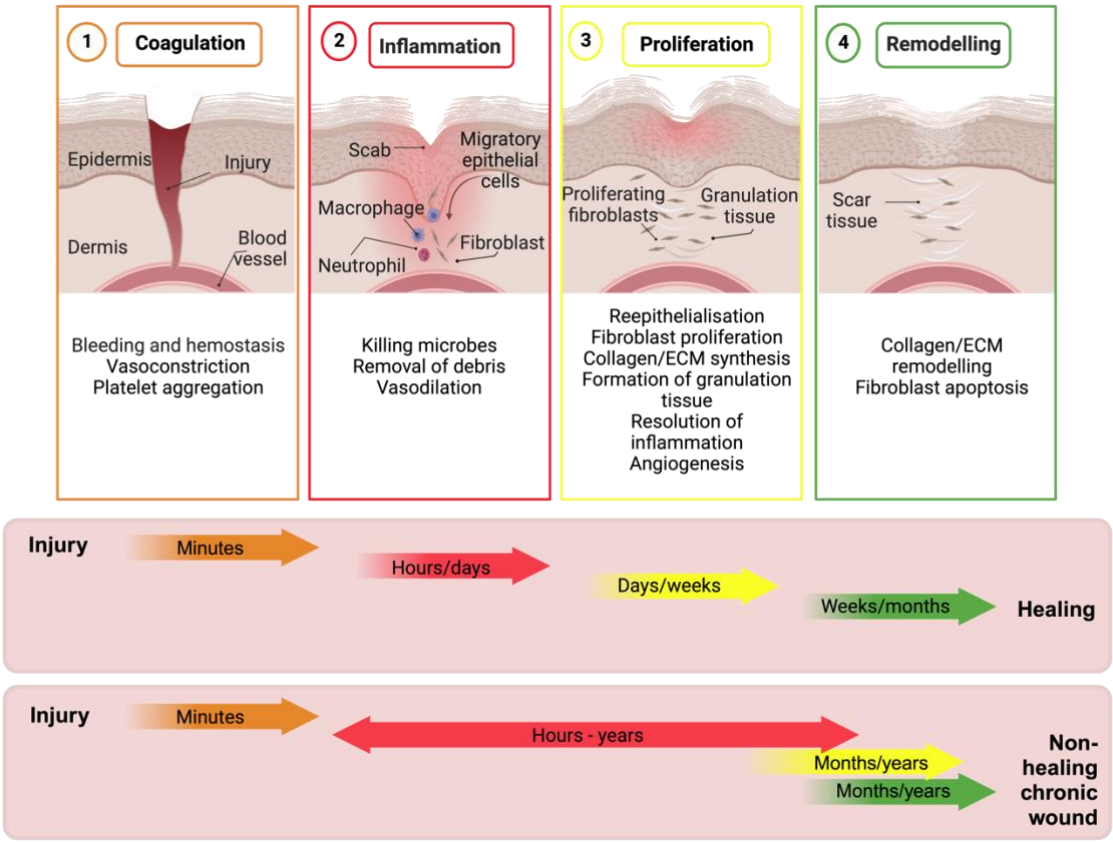


Figure 1.3 The four stages of wound healing, namely 1) bleeding and haemostasis, 2) inflammation, 3) proliferation, and 4) remodelling, with their timelines in physiological healing and non-healing chronic wounds. Adapted in part from "Wound Healing", BioRender.com (2020)[147] *and Shedoeva et al.*[38].

1.2.2 Challenges in wound healing: chronic and infected wounds

It is generally agreed that when the physiological progression of wound healing is impaired, wounds can become chronic, i.e., difficult to heal. This impairment leads to a dysregulation of the steps outlined above, and is usually characterised by a prolonged inflammatory state, characterised by high pH, overexpression of cytokines, proteases and reactive oxygen species (ROS)[29,148–151]. Chronic wounds can stall in the same healing phase for an extended period, from over 6 weeks to months and even years (Figure 1.3). This comes with considerable economic costs, such as for specialised healthcare professionals and specific wound dressings, for an extended time[152]. Furthermore, the psychological burden on the patients is particularly onerous, with excessive exudate, malodour, significant pain, impaired mobility and limited independence often leading to isolation and feelings of shame[153]. The incidence of chronic wounds is higher in elderly patients, particularly those affected by metabolic syndrome or malnutrition, and with multiple comorbidities[152,154].

One of the root causes of the chronicisation of wounds is the presence of infections, generally occurring in a wound with more than 10^5 CFU/g of tissue[155]. However, a holistic approach is essential for the management of difficult to heal wounds, and the 10^5 reference diagnostic value should only be considered in conjunction with other factors, such as host's compromised immune defence, poorly managed wound environment (such as in the case of inadequately trained healthcare professionals, low-quality exudate management, and inappropriate necrotic tissue debridement) and microbial risk factors (based on the microbial load, coexistence of different microbial species, their virulence and/or resistance)[156,157]. The organisation of bacteria in biofilm structures (characterised by aggregation and adherence to surfaces, as is often the case on prostheses) is often identified in chronic wounds, further complicating the healing process and requiring tailored strategies to achieve successful biofilm eradication while avoiding the insurgence of acute infections[158,159]. Commonly identified bacteria in wounds are *S. aureus, E. faecalis,* and *P. aeruginosa,* with a high prevalence of multidrug-resistant bacteria[157]. The continuous emergence of new resistant bacterial strains is a matter of major global concern, as reflected in the urgent calls for improved antibiotic stewardship by the World Health Organisation and the European Commission[160–162]. However, no selective pressure of antimicrobial-resistant species has been shown with natural products, such as phytochemicals, nutraceuticals and bee products, making them viable options for the management of infected wounds[38,163,164].

1.2.3 Latest advances in honey applications in wound care

Honey and other bee products have been known for centuries for their beneficial properties on several diseases and have been extensively used in traditional medicine alone or in association with other therapeutic regimens[5]. The treatment of burn lesions is amongst the numerous honey ethno-pharmacological applications, worthy of mention for their relevance to the aim of this thesis. The rationale for this usage has been illustrated in a 2015 Cochrane review that highlighted that healing time for partial thickness burns medicated with honey bandages is 4 to 5 days shorter than with conventional dressings medication (high-quality evidence). Similarly, an expedited outcome is observed on post-surgical infected wounds when treated with honey rather

than with antiseptic rinses plus gauze application, with fewer side effects (moderate-quality evidence)[165]. A 2017 Cochrane review comparing different antiseptics for the treatment of burns stated that burn injuries tend to heal faster with honey treatments than if treated with topical antibiotics (moderate certainty) or non-antibacterial unconventional medicaments (high certainty)[166]. Most of the studies summarised in these review papers were conducted using manuka honey, but since this review, other honey varieties and bee products have also been investigated, and their beneficial effects have been disclosed. For example, the association of chestnut honey and RJ in an ophthalmic formulation promoted corneal healing of a chemical burn model on rats. This was also confirmed by the significantly increased levels of α4β1-integrin on histological sections after two weeks of treatment[167]. A synergistic effect with natural products of different origins has also been reported for honey promotion of burn healing. For example, mixtures of Euphorbia honey and *A. sativum* L. in different proportions were found to be more effective compared to conventional treatments such as silver sulfadiazine and betadine, functionally reducing the time needed to achieve epithelialization and burn wound contraction, with no side effects such as allergic reactions[168]. A synergistic effect, as an increased inhibition zone *in vitro*, has also been observed for honey combined with antimicrobial drugs, suggesting a possible use of honey as a complementary aid to conventional antibiotics in wound treatment[76]. Furthermore, supplementation of honey with propolis extract has been shown to improve honey antimicrobial activity with a synergistic effect[169]. An additive effect was observed in the scratch assay with a more significant wound closure compared to the control. It has been hypothesised that these increased beneficial effects of honey might be explained by the improved antioxidant and anti-inflammatory activity upon propolis extract addition[170]. Another mechanism responsible for the ability to promote wound healing could be the propolis-mediated increased expression of AQP3 and consequent augmented Ca^{2+}, a mechanism also observed in honey[78], as described earlier in this review. These encouraging results show that honey and bee products of different botanical origins can be employed to promote more rapid healing and to effectively treat infections that colonize burns, complicating skin recovery. From the latest studies, it can, therefore, be concluded that honey application represents a valuable natural

approach in the treatment of burns, both alone or in association with alternative or conventional medicaments.

In the field of wound medicine, Diabetic Foot Ulcers (DFU) are currently a critical challenge with an alarming estimated incidence in 15% of diabetic patients, resulting in dramatic compromise of their quality of life and high mortality[171,172]. Microbial infections represent the main complication, particularly given the insurgence of antibiotic-resistant strains, together with impaired wound healing, and the aggravation of symptoms can often result in limb amputation[173]. Due to the severity of the potential consequences, urgent progress in the management of diabetic ulcerations is required, with early intervention and preventative plans of action as cardinal elements in the therapeutical strategy. Clinical use of honey as an alternative option in the treatment of DFUs is described in the medical literature, with the main limitations being the small number of patients involved in the studies and the difficulty in quantifying the dose employed in case of direct honey application, as the quantity is usually determined simply by the need to cover the wound and surrounding skin area[174–179]. Astrada et al. illustrated the case study of a large leg ulceration on a female diabetic patient with metatarsal exposure and concurrent systemic infection, requiring surgical debridement of necrotic and devitalised tissue. After two months of daily propolis-enriched Trigona honey applications, full re-epithelialisation could be observed on the wound site, which, on clinical observation, was observed to display reduced signs of inflammation[180]. Six patients diagnosed as at risk of limb amputation due to infected DFUs non-respondent to conventional therapies were successfully treated with manuka honey-containing commercial formulations. The infections, due to bacteria resistant to antibiotics, were managed in 2.6 weeks (average time), and the honey treatments also reduced malodour after just a few days of application. The use of these cost-effective medical-grade honey products, enriched with vitamins C and E, induced autolytic debridement and replacement with granulation tissue within 3.5 weeks, thus reducing the healing time[181]. In all the examined cases, honey application induced positive clinical outcomes, with improved tissue regeneration and successful wound contraction, with no allergic reactions nor maceration of the periwound area. Further studies, possibly randomised clinical trials with significantly more patients recruited, are now needed to corroborate

these promising results and support an evidence-based use of honey in the treatment of DFUs.

Honey destined for consumption as a food product contains inactive endospores and other innocuous contaminants, and so it needs to be filtered to remove the impurities[182] or decontaminated by gamma-ray exposure prior to the application on wounds in order to meet the standards for medical use[183]. Likewise, gamma radiation is employed to sterilise the hydrogels designed to treat skin injuries[184,185]. Furthermore, gamma irradiation is an essential step in the gelation of some polymer materials, as it initiates the process of crosslinking[186–188]. However, despite neutralising the total bacterial count on all the samples, this process of sterilisation does not compromise honey's efficacy against bacteria and biofilm, as demonstrated on fir honeydew honey at 10, 20 and 30 kGy[189]. Nonetheless, this treatment induced a significant dose-related reduction in small peptides such as defensin-1 at radiation doses over 10 kGy, probably by conformational alteration and aggregation, whereas higher molecular weight proteins such as GOx were not affected. Despite this report of a reduction in defensin-1, considering that the honey antimicrobial activity is ascribed to a multifactorial mechanism and that the other factors do not seem to be affected by the sterilisation, honey can be considered a safe and effective alternative option for the management of infected wounds.

Honey incorporation within scaffolds with different designs has been explored as a strategy to potentiate its medical effects on skin injuries, providing a favourable environment to facilitate complete healing across all skin strata. Typical design considerations include an easy-to-handle structure, being suitable for commercialisation and safe use for home medication. Pectin, a natural polyuronate, has been used to produce manuka honey-loaded hydrogels (Pectin Honey Hydrogels, PHH) to treat excisional wounds in rats, with significant acceleration of wound contraction compared with direct liquid honey application or control (no treatment). It is interesting to note that, at the end of the observation time, new hair follicles and organised fibrous tissue with reduced inflammation could be observed not only in the PHH group but also in the pectin blank hydrogel group[184]. This suggests that the polymer itself played an important role in achieving complete healing and indicates the paramount importance of selecting the most suitable material for the designated medical application. The

inclusion of stingless bee honey and curcumin within composite nanofibrous membranes improved their therapeutical potential in terms of the radical scavenging ability compared to the direct application of these healthful natural agents on the wound. The polymeric structures were shown to be highly cytocompatible, although in this study by Samraj. et al., the honey-loaded membrane did not significantly reduce the wound closure time compared to the conventional treatment (povidone-iodine). However, over 95% inhibition of all the bacterial strains tested was obtained upon honey addition to the gelatin membrane, while a maximum of 25% was achieved when only curcumin was loaded into the membrane[190]. Iranian honeys from three different provinces were compared by Mirzaei et al. on a rat burn model, with the thymol-rich Damavand sample showing the greatest antibacterial effect with an early onset of signs of wound recovery. Honey incorporation in an alginate scaffold further shortened the healing time (from 16 to 14 days), with remission from infection. Interestingly, in the same study, the analysis of the honey sample from the Ardabil province showed a higher level of sucrose (right below the maximum concentration allowed, above which it is indicative of adulteration) and reduced diastase activity denoting possible excessive heating, as compared to the other samples. Ardabil honey's ability to promote wound healing was lower as compared to the other samples, suggesting that honey authenticity and quality are cardinal parameters to be considered for all therapeutically effective honey varieties to ensure remarkable medical properties[191].

1.2.4 Commercially available honey-based products for skin repair

Various honey-based products are available on the market and have been approved for medical use with different indications, such as Actilite®, Activon Tulle®, Activon Tube®, L-Mesitran™, TheraHoney®, MediHoney®, Revamil®, Principelle IF™, examples of which are shown in Figure 1.4. Honey and honey-containing commercial products are currently used in clinical practice for the treatment of wounds of different aetiology. Zeleníková et al. described a statistically significant amelioration in patients aged over 65 with refractory wounds of various nature, with reduced wound size and improved pain relief (measured with the Visual Analog Scale) reported in the intervention group. This group was treated with the commercial honey dressings

Actilite® (containing a mixture of manuka honey and manuka oil, respectively 99% and 1%, shown in Figure 1.4, a)[192] for 90 days, compared to the control group[193]. A commercial ointment with 48% Medical Grade Honey (MGH - L-Mesitran™ ointment)[194] has been used in monotherapy to treat wounds of different origins (post-surgical and not) in pediatric patients. No discomfort was reported upon application, and minimal scarring was observed[195,196]. However, the antioxidant vitamins C and E and the other components (calendula officinalis, aloe vera, essential oils, lanolin) also contained in the formulation might have played a role in this positive outcome, given their intrinsic advantageous properties for skin health[197–200]. L-Mesitran™ Soft (gel containing 40% MGH supplemented with vitamin C and E) has been reported to be effective against clinical isolates of vaginal *Candida albicans* (*C. albicans*), with a minimum inhibitory concentration (MIC) of 25-50% and a minimum fungicidal effect observed at 50% (MFC). Interestingly, when raw Mexican Yucatan MGH (declared as the same honey used in the commercial product) was directly applied, no fungistatic or fungicidal effect was observed at 40% dilution (maximum concentration tested)[201]. Similar results were also reported against clinical isolates of multi-resistant *C. auris* and other Candida species responsible for nosocomial infections. A dose-dependent inhibition was achieved with L-Mesitran™ Soft on all fungal species investigated, while equal honey concentrations (Brasilian blossom honey) were not only shown to be significantly less effective in reducing fungal proliferation but even stimulated the growth of *C. albicans* and *Candida glabrata (C. glabrata)*. The antifungal effect for the raw medical grade honey was observed instead at a minimum concentration of 40%[202]. These findings suggest again that the other components of the formulations are critical to the successful clinical outcome, possibly potentiating the beneficial properties of honey[203–206]. TheraHoney® impregnated dressings[207] were compared to sustained-release ionic silver hydrophilic dressings in a prospective, double-blind, randomised clinical trial to evaluate the capacity to enhance healing of neuropathic diabetic foot ulcers and eradicate concurring infections, thus reducing hospitalisation. No statistically significant difference was observed between the two groups, indicating that both treatments are comparably effective and represent valuable options for the management of DFUs[208]. TheraHoney® gel (with medical-grade manuka honey) has been used in association with conventional antibiotic drugs to treat wounds following cochlear implants in three pediatric patients,

with remarkable promotion of surgical site repair on previously non-healing ulcerations[209]. MediHoney® paste (with Active Leptospermum Manuka Honey)[210] was successfully used as an alternative to conventional mouth rinses to topically treat oral mucositis following chemotherapy treatments in ten pediatric patients, with enhanced healing observed in 3 days, alleviated pain and reduced bleeding within 5 days[211]. Local application of MediHoney® on surgical wounds following implantation of bone-anchored hearing devices significantly improved the clinical outcome, with a reduction of the time necessary to achieve complete healing[212]. These examples of the use of honey-based products in clinical settings, despite the limited number of patients involved and the absence of a control group in the case reports, provide an indication of the increasing acknowledgement of honey-based medical devices as a valuable option to conventional treatments.

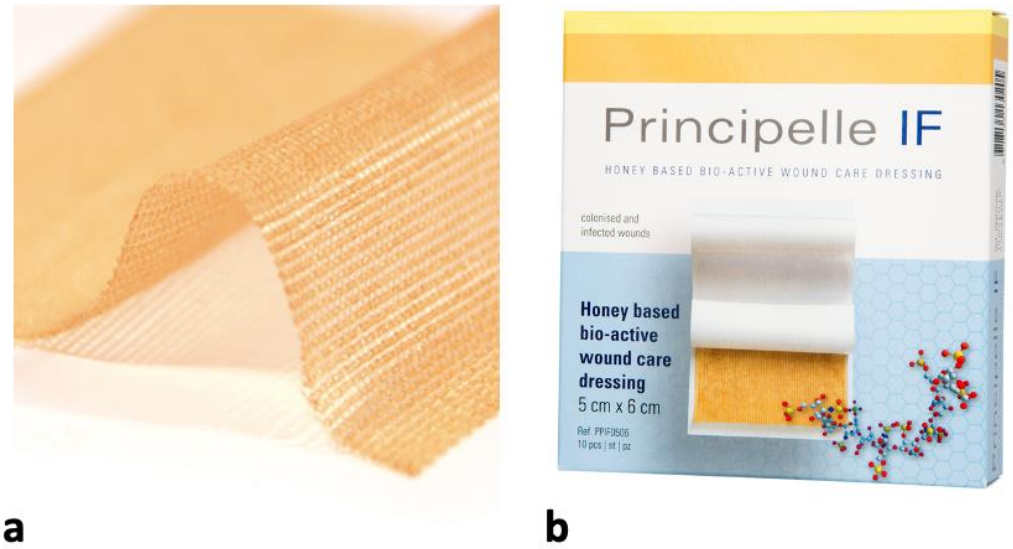


Figure 1.4 Examples of commercially available honey-based dressings for wound healing. a) Actilite® Non-adherent viscose net dressing coated with 99% manuka honey and 1% manuka oil. © Image courtesy of Brightwake Ltd (trading as Advancis Medical); b) Principelle IF® Honey-based bio-active wound care dressing containing medical grade dark buckwheat honey with no synthetic components. © Image courtesy of Principelle B.V.

1.3 Functional aspects of honey-loaded scaffolds for wound healing

Every health condition to be treated and every specific application of a therapeutic device requires specific features in order to obtain the best possible mitigation of the symptoms or achieve a full recovery. In the design process of a loaded scaffold, an accurate evaluation of the most suitable polymer to be employed depends on the

required functions, e.g., tensile strength for scaffolds destined to areas subjected to intense mechanical stress, ability to improve cell proliferation for hydrogels for wound healing, antimicrobial properties for infected wounds. For example, wider inhibition zones on a disc diffusion test were measured for chitosan-based honey hydrogels than for those fabricated with Carbopol, indicating a higher antimicrobial activity *in vitro* when equal honey concentrations were compared. Furthermore, the 75% honey-chitosan hydrogel showed better antibacterial performance than pure honey when evaluated using the disc diffusion method against four common burn-infecting bacteria and also induced the fastest burn wound closure among the tested preparations[213]. These results could be attributed to the intrinsic potential of chitosan for biomedical applications[214,215] which seemingly potentiated and improved honey's virtues.

Honey incorporation into a scaffold and its penetration into the tridimensional polymer network has also been shown to alter the mechanical performance by inducing structural modifications. This might potentially improve some functional attributes and negatively affect others; therefore, the concentration of honey has to be carefully evaluated in order to accurately tailor the scaffold's behaviour based on the specifics of the tissue to be treated. Chestnut honey incorporation on carboxymethyl cellulose (CMC) hydrogels affected the structural strength with reduced capacity to withstand compression load compared to plain CMC gels[186]. This should be kept in mind as it might assume greater importance depending on the location of the wound to be treated to ensure the hydrogel maintains structural integrity throughout the application time. Interestingly, in a study by Bonifacio et al. manuka honey was exploited as a molecular spacer in gellam gum hydrogels for cartilage implants, with improved compressive moduli and flexibility. Furthermore, these cytocompatible hydrogels induced chondrogenic differentiation with deposition of cartilage integral constituents such as collagen II, glycosaminoglycans (GAGs) and proteoglycans, which resulted in effective cartilage reconstruction. When tested against MDR *Staphylococci* pathogens typically isolated in post-arthroscopy infected joints, the scaffolds have been shown to significantly reduce the biofilm viability in comparison with controls at each time point[216]. Honey addition (up to 2%) to alginate bioink reduced its viscosity in a dose-dependent way without appreciably affecting the overall rheological features such as extrusion and printability. However, honey significantly enhanced fibroblast viability,

proliferation and adhesion when compared with the plain alginate membrane, with promising applicability to bioprint-compatible bioengineered human skin tissue substitutes[217]. These contrasting results indicate that the impact of honey incorporation on the scaffold functionality cannot be assumed and should be evaluated as a matter of course as the biomedical outcome cannot prescind from an optimised structural and mechanical performance.

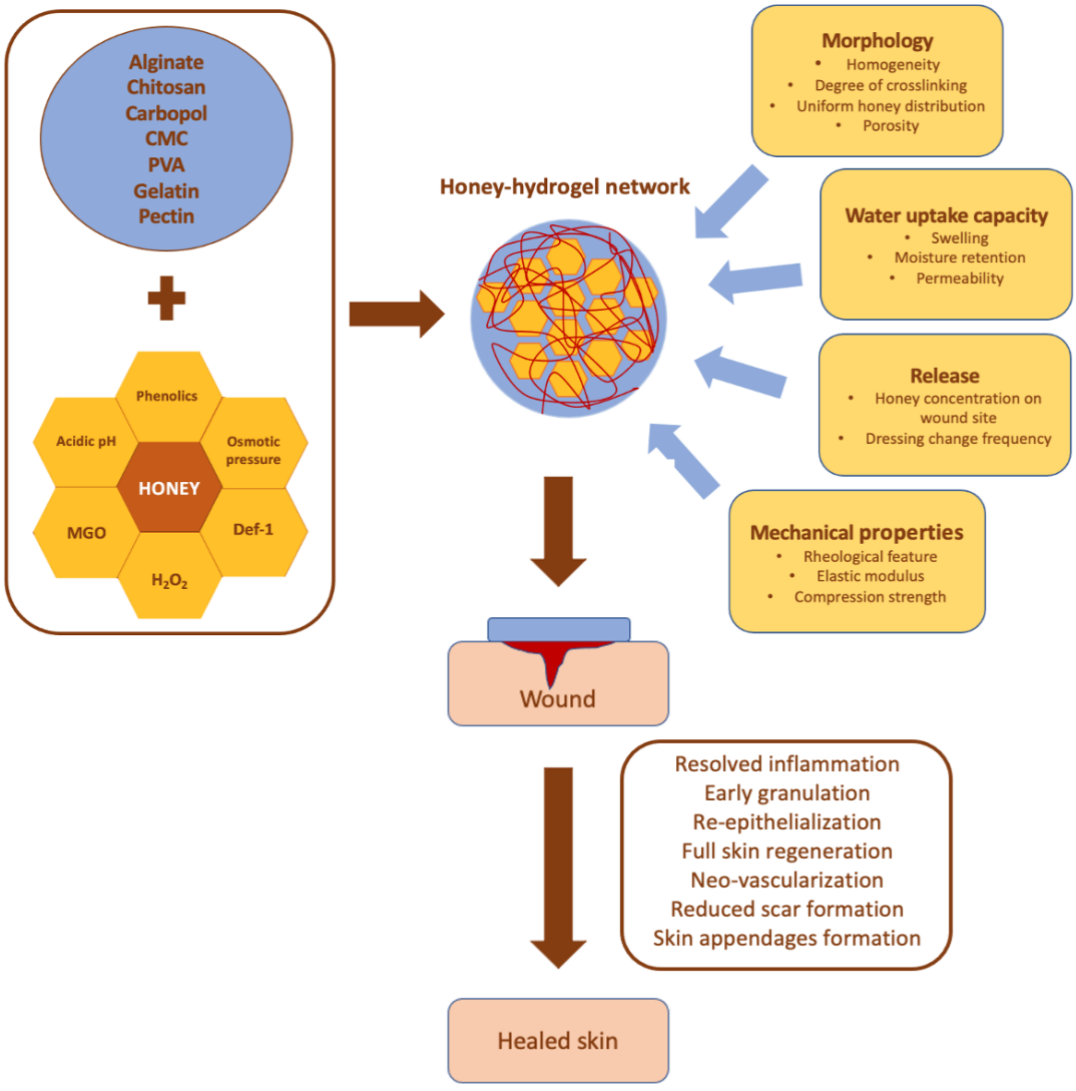


Figure 1.5 Schematic representation illustrating critical aspects for optimisation and characterisation of a honey-loaded scaffold for wound repair, with examples of polymers successfully demonstrated in published literature, as discussed in Section 1.3.

Once the honey has been incorporated within the scaffold, an in-depth characterisation of the developed formulation should be conducted in order to evaluate some critical

parameters to ensure that the treatment effectiveness is optimised, as summarised in Figure 1.5. The release rate of the medically active agent is one of these crucial aspects, as it determines important features such as the honey concentration at the wound and periwound area. It can be modulated to prevent the inconvenience of honey leaking from the scaffold, shown in Figure 1.6, and consequently reduce the dressing change frequency, with important implications on patients' adherence to the therapy, as the manipulation may cause discomfort[218]. The honey release is usually determined by indirect quantification of either sugars or markers (such as methylglyoxal for manuka honey) liberated in the medium. The maximum absorbance at specific wavelengths can be measured on a UV-Vis spectrophotometer and plotted against time[190,213]; alternatively, HPLC quantification can be employed to determine the cumulative release[216]. For highly hydrophilic and biodegradable polymeric structures, drug diffusion and scaffold degradation are considered the driving forces of the release process[190,213]. On the other hand, this process of structural degradation can be counterbalanced by an increased degree of crosslinking, i.e., chain entanglement, with improved rigidity and structural integrity of the network, hence improved honey retention[219]. For example, a significant reduction in methylglyoxal release was observed from Ca^{2+} and Mg^{2+}-crosslinked gellan gum composite hydrogels when compared with the non-crosslinked equivalents[216]. Different experimental protocols are described in the literature for the evaluation of drug release *in vitro*, with the immersion volume being variable across the papers. For honey-alginate bio-printed scaffolds, a volume of 10 mL of phosphate-buffered saline (PBS) was chosen[217]. Also Samraj. et al. carried out the *in-vitro* release test by directly submerging the membrane in PBS buffer at a controlled temperature of 37 °C, but the volume of the medium was not specified[190]. Alternatively, for amorphous honey-hydrogels, the sample can be inserted into a dialysis bag, as described by El-Kased et al.[213]. A burst release is conventionally detected at the beginning of the observation time, followed by a plateau, in hydrogels where the drug-loading was performed by direct mixing method[213,218]. However, a sustained release was observed from gelatine-based electrospun nanofibrous membranes, with 99 ± 0.5% cumulative honey release only achieved after 24 h of immersion[190]. This shows that different release kinetics can be established by employing alternative tissue engineering strategies, thus allowing for a controlled and extended release.

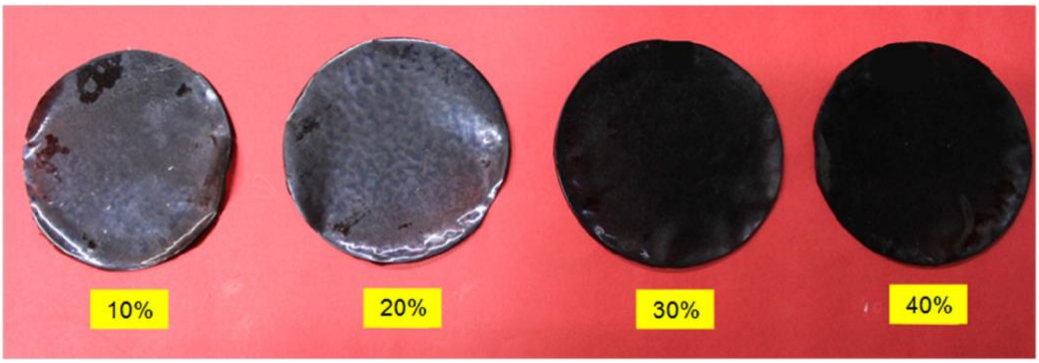


Figure 1.6 "Pictomicrographs of DNG/Ch/MH (dextran/nanosoy/glycerol/chitosan with manuka Honey) dressings at different drug concentrations (10–40%)." Reproduced from International Journal of Biological Macromolecules 120 (2018) 1581-1590; Singh et al., Scar-free healing mediated by the release of aloe vera and manuka honey from dextran bionanocomposite wound dressings[218], Copyright (2018), with permission from Elsevier.

The capacity of a wound dressing to swell and uptake a considerable volume when immersed in an extracellular fluid-resembling medium is indicative of its ability to absorb wound exudate and to maintain a curative moist environment on the affected skin area[220]. As with the evaluation of the drug release kinetics, the volume of the medium employed to assess the water absorption features of the scaffold is highly variable across the published literature. Samraj. et al. conducted the swelling study by soaking a 1x1 cm^2 sample in 50 mL of PBS at 37 °C and a maximum swelling of 500% of the original dry weight was recorded. However, no comparative evaluation was reported between the blank membrane and the drug-loaded ones, so a consideration could not be drawn on the contribution of honey to the swelling capacity of the unloaded gelatin nanofibrous membrane[190]. Azam et al. carried out the swelling test in analogous experimental conditions (PBS, 37 ± 0.5 °C) but did not specify the immersion volume. Increasing honey concentrations affected hydrogel's absorption capacity from 512 ± 21% for the blank film down to 197 ± 9% for the 10% (w/v) honey-loaded sample[221]. This is in line with the results by Sarhan et al., which described a fivefold reduction of the percentage of swelling (from 520 to 100%) when honey concentration was increased from 10 to 30% (at a fixed crosslinking level) in honey/polyvinyl alcohol/chitosan electrospun nanofibers. Honey addition also impacted the structural features of the fibres, with an increase of almost 100 nm in the mean diameter for any 10% increase in honey concentration[219]. It has been hypothesised that the frequently observed reduction in the swelling ability of hydrogels upon honey addition might be

ascribed to honey's high water solubility, causing a higher rate of degradation when the scaffold is immersed in water[219]. Another explanation might be that honey occupies the polymer's sites for hydrogen bonding, otherwise available for interactions with water molecules[221]. Conversely, honey addition to double cross-linked alginate hydrogels led to significant improvement in fluid uptake capacity up to 700% (measured in 10 mL deionised water), with an optimum observed with the 4%-honey structure which showed pronounced water absorption for 40 h followed by a plateau. This is in line with the controlled degradation kinetics, which involved non-crosslinked residuals first[222]. Analogously, the swelling ratio of honey-silk fibroin scaffolds significantly increased with higher honey concentrations, possibly due to honey hygroscopicity generating an osmotic pressure that directed the medium influx. However, the degradation rate also increased in the fibrous mats with higher honey loading, together with a reduction in compressive strength. These effects on the mechanical features could be explained by the enhanced pore size and porosity of the tridimensional structures upon incorporation of increasing honey quantities[223]. Similarly, when incorporated as an additive during the electrospinning process of silk fibroin solutions, manuka honey has been found to function as an effective hydrophilicity-enhancer, significantly increasing the water retention capacity of the control (pristine silk fibroin scaffolds) at both the honey concentrations tested (1% and 5%), up to a maximum swelling ratio of almost 400% of the initial dry weight[224].

Although a dose-dependency of honey's antibacterial and antioxidant properties has been described in the literature[94,132], when it is incorporated into a scaffold, simply increasing its concentration does not necessarily guarantee that the beneficial effects are maximised. For example, among the formulations developed by Rajput et al. for silk fibroin-based skin substitutes (loaded with multifloral honey up to 10% of the final concentration), imaged in Figure 1.7, the best pro-healing performance with reduced scar formation was obtained with the 4%-honey scaffold. This sample had an optimal microstructure, which allowed a flux of nutritious substances to the wound site, with enhanced fibroblast proliferation and migration. In particular, a full skin regeneration occurred both at the epidermal and dermal level, with a prevalence of collagen I over collagen III, angiogenesis, and formation of skin appendages such as hair follicles and

sebaceous glands after two weeks of treatment[223]. Similarly, dual cross-linked alginate hydrogels presented a disarranged crystalline structure for high honey concentrations, while honey incorporation up to 4% ensured a morphology favourable for cellular adhesion, a significantly better bactericidal activity compared to the other honey dilutions, and ultimately skin re-epithelialisation with characteristics indicative of healthy uninjured skin and minimal scar thickness[222]. Successful loading of manuka honey into dextran-based dressings was achieved for concentrations up to 20%, with uniform distribution of honey. However, when the honey concentration was above 20%, the dressings were found to be fragile, sticky and stiff. Besides these structural disadvantages, a functional drawback was also observed, as the antibacterial activity of the highly concentrated samples was also compromised, in particular against the Gram-negative *E. coli,* as demonstrated by a halved inhibition zone diameter at concentrations beyond 20%[218]. The outcome achieved with treatment can be affected not only by the intrinsic properties of that given honey variety but also by the protocol design. A factor to keep into consideration could be the number of honey applications during the observation time as well as the frequency of the dressing replacements. For example, two daily applications of thyme honey on a rat wound model induced faster healing, with a significantly higher fibroblast count and collagen deposition, and early formation of granulation tissue with angiogenesis compared to the control and the single daily application[182].

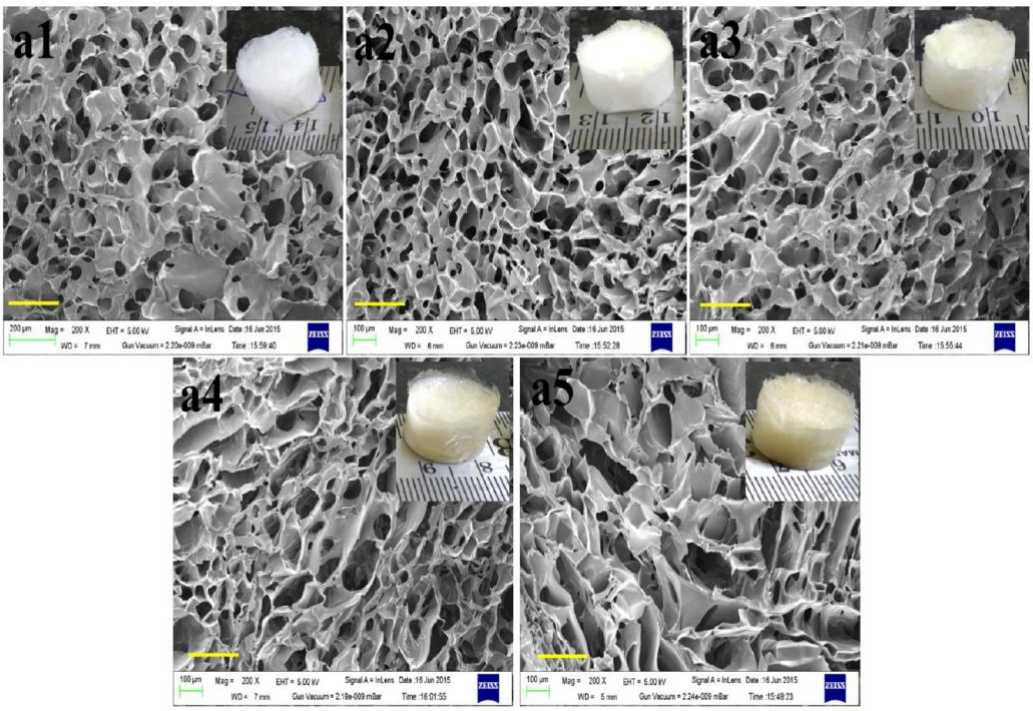


Figure 1.7 "Microphotographs by SEM showing the surface morphology of crosslinked Silk Fibroin (SF) and Honey Silk Fibroin scaffolds (a1) SF, (a2) HSF1 with 1% honey concentration, (a3) HSF2 with 2% honey concentration, (a4) HSF4 with 4% honey concentration, and (a5) HSF6 with 6% honey concentration." Reproduced from Materialia, 12 (2020), 100703; Rajput et al.; Honey loaded silk fibroin 3D porous scaffold facilitates homeostatic full-thickness wound healing[223], Copyright (2020), with permission from Elsevier.

Lastly, particular attention should be given to the possible interactions between the compound of interest and the hydrogel components, other active agents and excipients and how these can affect the pharmacokinetic aspects of the active principle, for example, by modulation of its release. For instance, it is worth mentioning that the incorporation of polyphenols within a scaffold or delivery system might restrict their antioxidant potential, as shown by diminished responsiveness to radical scavenging tests such as ABTS and DPPH of different quercetin-hydrogel formulations compared to the free flavanol[225]. Also, no statistical difference in antibacterial potency was observed between manuka honey samples with different UMF™ when these were loaded into scaffolds for medical engineering, despite a significantly different bacterial inhibition obtained with direct application of the same honeys[111].

1.4 Conclusions

Honey has been shown to be much more than a simple food product, but rather a valuable medical product with multiple mechanisms and beneficial virtues. The varying

antimicrobial, antioxidant, and anti-inflammatory properties of honey are responsible for the diverse and broad range of varieties of applications of honey being investigated as powerful topical treatments for healing and wound repair. The phenolic compounds intrinsic to honey's composition are recognised as significant contributors to its widely investigated antioxidant properties. Critically, however, whilst these phenolic compounds have been shown to modulate the antimicrobial properties of honey against common wound infections, the full breadth of determinant factors for honey antimicrobial activity is yet to be unequivocally agreed on, with peroxide-mediated mechanisms also suggested as a plausible mechanism. This complexity prompts the need for further research to explore the likely multifaceted, potentially synergistic components of honey's medicinal properties. In addition to its antibacterial properties, the anti-inflammatory properties of honey were also reviewed, and again, multiple potential mechanisms to account for this activity have been proposed. However, regardless of the ambiguities surrounding the underlying mechanical mechanisms, the evidence for the net positive impacts of honey as a component within topical wound treatments is becoming more broadly established in the literature. Whilst manuka honey is the most famous honey to be utilised in healing, as explored in this review, a number of other honey varieties are also being shown as effective in this regard. In particular, the use of honeys in the treatment of burn wounds has been robustly demonstrated to improve treatment outcomes in terms of speed of healing, skin regeneration, and reduction of scarring.

One of the most exciting aspects currently emerging in the field of honey-enhanced wound healing involves the incorporation of honey within an increasing variety of scaffolds to potentiate its medical effects on wound healing. In the second part of this review, the design considerations of effective honey-loaded scaffolds were explored. Here, too, it is important to recognise that the impact of honey incorporation on the scaffold functionality cannot be assumed, with differing configurations resulting in contrasting results. Particularly exciting is the potential for the controlled release of honey by optimising scaffold designs. Therefore, it can be concluded that advanced honey wound dressings obtained by the incorporation of honey into polymeric or fibrous structures represent a promising novel option for skin regeneration and enhanced wound healing. From the evidence collected and described here, it can be

seen that the advantageous properties of honey-loaded devices on skin medicine depend on the careful optimisation of numerous factors in order to offer a natural product with up-to-standard performance, if not improved when compared with the conventional products.

In addition to research to elucidate the underlying mechanisms driving honey's medicinal properties and the exploration of advanced design scaffolds for honey wound dressings, systematic studies with greater numbers of patients are now needed to substantiate the current evidence and further investigate the beneficial effects of honey in skin and wound healing. Bringing these key aspects together could enable medicine to fully harness the significant potential of honey to improve therapeutic wound outcomes so that sufferers of complex wounds, including chronic wounds, infected wounds, and burn wounds, can realise significantly more positive outcomes in the future.

1.5 Project aims and objectives

In this research project, the potential of selected Irish heather honey to be utilised in biomedical applications for the treatment of infected wounds is investigated.

The research question is articulated in three fundamental hypotheses, as follows:

- Honey of a specific botanical origin presents a characteristic and recognisable aroma and the identification of volatile markers can be used, in conjunction with the melissopalynological analysis, to confirm the honey variety and thus aid in the selection of varieties suitable for biomedical use;
- Selected Irish heather honey exerts relevant antimicrobial properties, comparable to those of New Zealand manuka honey, which is widely considered the gold standard of medical-grade honey;
- The incorporation of Irish heather honey into hydrogel formulations designed for wound healing can further improve the antimicrobial properties of honey.

In order to test these hypotheses, the aims of this project are:

- to characterise the honey samples in terms of physicochemical parameters and composition which could be relevant for their biomedical use;

- to evaluate if the honey samples could exert a substantial therapeutic effect by inhibiting the growth of common bacterial pathogens of wound infections;
- to develop Irish honey hydrogel wound dressings, and to assess the extent to which they can effectively treat infections.

To achieve these aims, the objectives of this project are:

- to analyse the selected honey samples in terms of physicochemical properties, to quantify their phenolic content, and to fully screen their volatile fraction (Chapter 2);
- to determine the extent of microbial contamination in the honey samples as a parameter of suitability for use as medical-grade honey (Chapter 3);
- to assess the antimicrobial properties of the Irish honey samples in comparison to manuka honey against common wound-colonising bacteria, namely a Gram-positive, *S. aureus,* and a Gram-negative, *E. coli* (Chapter 3);
- to evaluate the use of volatile emissions from pathogenic microbial species as an identification and diagnostic tool (Chapter 3);
- to design, fabricate and test functional parameters of honey-hydrogels for wound dressings (Chapter 4);
- to evaluate the potential of Irish honey-hydrogels to treat wound infections of *S. aureus* and *E. coli* (Chapter 4).

Chapter 2 Multifaceted characterisation of Irish honey marketed as heather honey: melissopalynology, physicochemical parameters, phenolic content, and aroma profile, and comparison with manuka honey

Part of this chapter has been accepted for publication. Manuscript details: Angioi, R.; Morrin, A.; White, B. Advantages of a multifaceted characterisation of honey, illustrated with Irish honey marketed as heather honey. *ACS Food Science & Technology.* https://doi.org/10.1021/acsfoodscitech.3c00475

2.1 Introduction

Honey is a highly complex matrix, containing mainly sugar and water, but also minor constituents such as minerals, amino acids, solid residues (pollen, traces of beeswax) and bioactive compounds[226,227]. These minor constituents also include the compounds that determine its organoleptic features (flavour, perfume, texture, and colour), dictating the consumer's preference for one variety over the other, and are present in small concentrations. Amongst these compounds, Volatile Organic Compounds (VOCs) are responsible for honey's aroma, to which they contribute to different levels depending on their relative volatility and odour threshold (the extent to which the volatile compound can be perceived by the human sense of smell)[228]. The investigation of honey's volatile fraction has attracted considerable interest[4,228–230] with the aim of achieving a more comprehensive understanding of honey's composition. VOC screening is widely employed with the goal of identifying markers, i.e. compounds typical of specific single-origin honey, or at least detectable in it at a significantly higher concentration than in other varieties. These markers represent a valuable aid in the determination of the botanical origin of unifloral honey, together with palynological analysis[231]. The information acquired by this technique is particularly relevant for honey varieties obtained from the nectar of plants whose pollen is underrepresented and for which the melissopalynological analysis would not be conclusive. As such, the study of the volatile fraction of honey is of advantage for commercial reasons, to better comprehend what drives the customer's choice, and to protect their rights in terms of product adulteration and fraud. Furthermore, in helping unambiguously define the botanical origin of honey, the volatile fingerprint represents a valuable tool for the selection of honey varieties with highly researched beneficial properties and promising potential in terms of biomedical applications[228,232], as thoroughly discussed in Chapter 1.

2.1.1 Source of VOCs in honey

The volatile compounds identified in honey originate from different sources, and their relative proportion in honeys can depend on various factors. From the field, including the plants and the surrounding environment (Figure 2.1, 1 and 2), from the bees and

their digestive system (Figure 2.1, 3), all the way to the hive where the nectar collected by pollinators is processed and honey is produced (Figure 2.1, 4), VOCs become part of the honey aroma and define its volatile fingerprint, as shown in Figure 2.1.

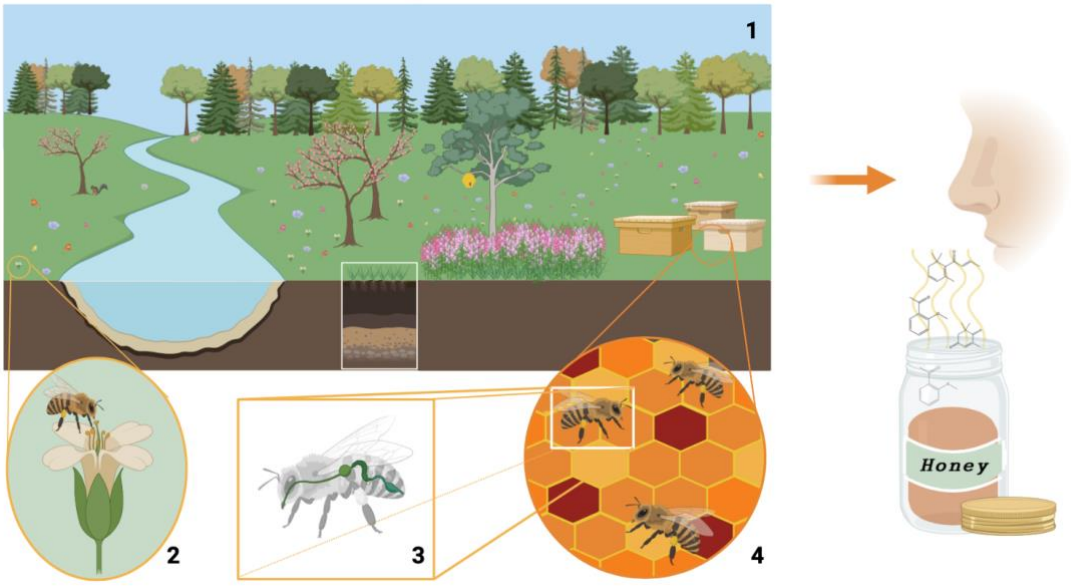


Figure 2.1 Factors contributing to honey volatile fingerprint: 1) foraged plants and surrounding environment (e.g., soil composition, other vegetation); 2) a bee foraging on a plant; 3) a bee with its digestive system highlighted; 4) nectar processing occurring in the hive. Created in BioRender.com.

The nectar of the plants visited by pollinators is the primary source of VOCs in honey[233,234]. However, even within the same botanical family, distinct volatile phytochemicals have been identified as typical of honey from one species. For example, heather honey is an umbrella term used to indicate honey derived mainly from the nectar of plants in the *Ericaceae* family, but characteristic VOCs can be used to discern the botanical origin of honey to a species-specific level[231], as further detailed in Section 2.1.2. In addition to the botanical origin, the emission of plant volatiles that influences the honey VOCs profile has been shown to depend on environmental factors, such as seasonal parameters (temperature, humidity), soil quality (nitrogen, moisture), and the coexistence of other endemic plants and invasive species[235]. Pollinators, with their essential role in producing honey, also contribute crucially to defining its organoleptic features, by carrying out the bioconversion of precursory phytochemicals into VOCs of interest. For example, the pathway of formation of terpenes in honey has been traced back to the isomers 2-isopentenyl pyrophosphate (2-IPP) and its isomer 3-isopentenyl pyrophosphate (3-IPP) synthesised both in the cytoplasm and chloroplast of the plant.

These are then converted into geranyl pyrophosphate (GPP), with further reactions mediated by enzymes in the bee stomach and saliva, leading to linalool derivatives often detected in the honey volatile fraction (including both alcohols, such as hotrienol, and aldehydes, such as lilac aldehyde)[236–238]. Finally, some VOCs detected in honey can originate from the beehive where honey is produced by bioprocessing of the nectar and is stored by the pollinators, such as heavy-weight free fatty acids and long chain hydrocarbons linked to the beeswax that the honeycomb frames are made of[234,239]. Furthermore, some VOCs originate as biotransformation products of compounds already present in nectar, such as linalool, that undergo oxidation reactions due to temperature and pH conditions in the hive[236,237,240]. Due to the variety of determinant elements contributing to shaping its volatile profile, honey as a sample presents a high level of complexity, incorporating a multitude of information (botanical and geographical origin, environmental conditions, coexisting flora, and more). Therefore, the screening of its VOCs represents a substantial contribution to unlocking honey's full identification.

2.1.2 Current knowledge in the study of Irish heather honey and its VOCs

Heather honey is an umbrella term used to indicate honey derived mainly from the nectar of plants in the *Ericaceae* family. The physicochemical features and chemical composition of heather honey from various geographical areas have been previously investigated[231,241–244], allowing the identification of relevant parameters and potential botanical origin markers for this honey variety. Terpenes and norisoprenoids represent critical components of the aroma of various natural products, such as fruit, spices, tea leaves and also honey, where they likely have a floral origin[236]. Among these compounds, VOCs originating from plant carotenoids (with the general 3,5,5-trimethyl-cyclohexenic structure, variably substituted) have been detected at high concentrations in the volatile fraction of heather honey from different countries[236]. However, even within the same botanical family, distinct volatile phytochemicals can be used to discern the botanical origin of honey at a species-specific level. For example, abscisic acid (Figure 2.2, a and b) has been described as a non-specific marker of samples from Portugal (*Erica* spp.)[244] and Poland (*Calluna vulgaris*)[241]. Similarly, C_9-norisoprenoids

such as α-isophorone, β-isophorone, and 4-oxoisophorone (Figure 2.2, c and d), have been described as distinctive of heather honey from New Zealand (*Calluna vulgaris*) and Spain (*Erica multiflora*)[242]. Guyot et al.[231] investigated heather honey samples from diverse European locations: their study identified phenylacetic acid (Figure 2.2, e) as a predominant VOC in the honey extractives from *Calluna vulgaris* honey harvested in the United Kingdom, Norway, Germany, France and Belgium, together with 3,5,5-trimethylcyclohex-2-ene derivatives. In the same study, 4-methoxybenzaldehyde (Figure 2.2, f) has been suggested as a marker of *Erica arborea* honey samples from the Mediterranean area[231].

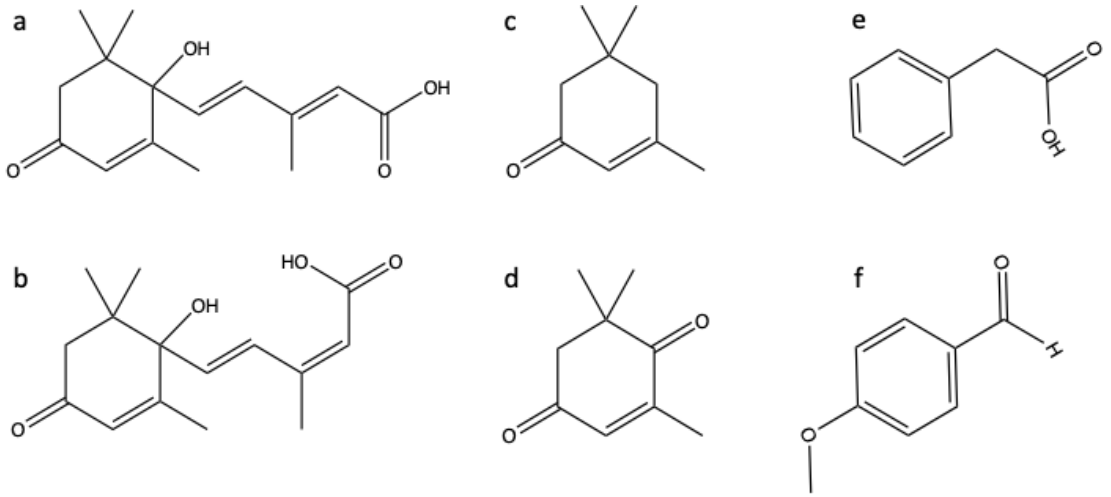


Figure 2.2 Examples of chemical structures of previously identified markers of heather honey (Ericaceae) from various geographical locations: a) 2-trans, 4-trans abscisic acid and b) 2-cis, 4-trans abscisic acid have been identified in honey harvested in Poland and Portugal; c) isophorone and d) 4-oxoisophorone reported in samples from New Zealand and Spain; e) phenylacetic acid in various European countries (such as UK, Norway, Germany); f) 4-methoxybenzaldehyde for samples harvested in the Mediterranean area.

In Ireland, there were 3000 registered beekeepers in 2020, according to the last estimation by the Agriculture and Food Development Authority[245]. Among the various honey types produced in Ireland, heather honey represents a renowned artisanal unifloral honey variety, collected at the end of the summer/early autumn. Limited research is available in the literature on the composition of Irish honey. Downey et al. investigated 50 samples of honey from Ireland provided by local beekeepers, but heather honey was not one of the honey types identified by pollen analysis[246]. Kavanagh et al. investigated several Irish honey samples, which included heather honey samples, focusing on physicochemical features, phenolic content and microbial contamination[50].

The high potential medicinal value reported of honey from the *Ericaceae* family[232] and the high market value for Irish heather honey[245] warrant interest in investigating this Irish honey. In this work, the volatile composition of Irish honey marketed as heather honey is reported for the first time. The extent to which signature VOC profiles of *Ericaceae* honey could be confirmed for Irish heather honey, the botanical origin of which was confirmed by melissopalynological analysis, is also investigated. Total phenolic content was also assessed for the Irish honey, due to its reported correlation to its biological activity (antioxidant, anti-inflammatory and antibacterial)[232], and this, along with the determined chemical parameters and VOC aroma profile of heather honey, are considered here in order to obtain a more comprehensive characterisation of this so far scarcely investigated Irish honey variety.

However, to the best of our knowledge, the screening of the volatile fraction of Irish heather honey has not been published. Given that a high variability has been reported for the honey volatile profile of the same botanical variety based on the different countries of production[247], the characterisation of the volatile composition of heather honey from the Republic of Ireland was deemed of scientific interest, and it is therefore reported in this chapter.

2.1.3 Chapter's aims

The aims of this chapter are:

- the determination of the botanical origin of the Irish honey samples, indicated by the beekeepers as heather honey;
- the characterisation of physicochemical features of Irish heather honey samples, namely pH, moisture and sugar content, and colour;
- the quantification of the total phenolic content;
- the screening of the VOCs profile to identify potential specific or non-specific markers of botanical origin;
- the comparison of chemical parameters and aroma profile of heather honey with the gold standard of honey varieties for medicinal use, manuka honey.

2.2 Materials and methods

2.2.1 Materials

Gallic acid (3,4,5-Trihydroxybenzoic acid, 97.5-102.5% by titration), Folin-Ciocalteu's phenol reagent 2N, sodium carbonate, Kaiser's glycerol gelatine (glycerin jelly) were purchased from Merck Life Science Limited (Ireland). Basic fuchsin and methanol (HPLC grade, >=99.8%) were purchased from Fisher Scientific (Ireland). Deionised water (18.2 MΩ) was obtained using PURELAB® Chorus-2 Water Purification system (ELGA LabWater, Veolia).

2.2.2 Honey samples

Five honey samples were purchased for this project (Table 2.1). Four of them were produced in Ireland and were purchased from the websites of beekeepers. Of these, three samples were marketed as heather honey (*Erica* and/or *Calluna* spp.): one from County Galway, one from the Dublin mountains, and one from the mountains between Wexford and Wicklow. The fourth sample was a blend of two types of Irish honey, namely ivy (*Hedera* spp.) and heather (*Erica* and/or *Calluna* spp.) honey. The samples of Irish honey were indicated with the codes IH1, IH2, IH3, and IH4. The final sample, of Comvita MGO 514+ (Unique Manuka Factor (UMF™) 15+) manuka honey, was purchased from a local pharmacy in Dublin (sample code MH). All honey samples were produced and purchased in 2021. Once purchased, all honey samples were stored in the fridge between 0 °C and 4 °C until analysis.

Table 2.1 Assigned sample code, year and place of production, and floral origin (based on marketing labelling) of the honey samples under analysis in this study. The samples were all purchased in 2021 and stored in the fridge between 0 °C and 4 °C until analysis.

Sample code	Year of production	Floral origin (as indicated by beekeepers)	Place of production – Country
IH1	2021	Heather (*Erica and/or Calluna* spp.)	County Galway
IH2	2021	Heather (*Erica and/or Calluna* spp.)	Dublin mountains
IH3	2021	Heather (*Erica and/or Calluna* spp.)	Wexford and Wicklow mountains
IH4	2021	Blend of Ivy (*Hedera* spp.) and Heather (*Erica and/or Calluna* spp.)	Galtee mountains, Co. Tipperary
MH	2021	Manuka MGO 514+ (UMF™ 15+) *(Leptospermum scoparium)*	New Zealand

2.2.3 Melissopalynological analysis

In order to confirm the honey samples' botanical origin provided by the beekeepers a melissopalynological analysis was performed with a method adapted from Ohe et al.[248], with slight modifications (Figure 2.3).

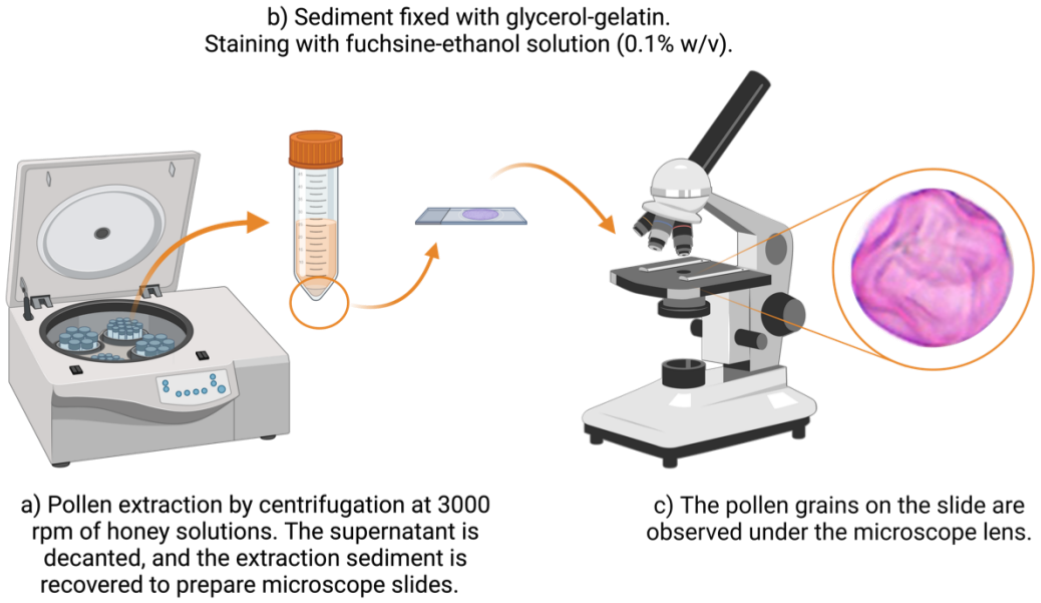


Figure 2.3 Schematic of the melissopalynological analysis for the identification of the botanical origin of honey samples. The method (whose steps are briefly described in the figure) is based on the prevalence of pollen residues naturally present in honey, identified by microscopic observation and comparison with pollen databases. The relative frequency of the pollen types was calculated as per Equation 2.1. Created in BioRender.com

Briefly, two rounds of pollen extraction (10 mins each) were performed on 10 g of honey in 20 mL deionised water by centrifugation at 3000 rpm in Eppendorf Benchtop Centrifuge 5804 (Eppendorf SE, Hamburg, Germany). The supernatant was decanted, and the pollen fraction (extraction sediment) was recovered to prepare microscope slides. The extraction sediment was fixed on the slides and dyed by the use of a glycerol-gelatin mix with a few drops of fuchsine-ethanol solution (0.1% w/v). The pollen grains were observed under the microscope Olympus CKX41 (Olympus Scientific Solutions, Tokyo, Japan), with 500 pollen grains being counted in groups of 100 grains, starting from the top left corner and following their distribution on parallel lines across the slide. The identification of the pollen genre was performed by comparison with various pollen databases available online[249–252].

The relative frequency (%) was calculated using the following equation:

$$Relative\ Frequency = \frac{The\ \ pollen\ counted\ of\ a\ particular\ species}{The\ overall\ number\ of\ pollen\ grains\ counted} * 100 \qquad (2.1)$$

Three microscopy slides were prepared for each honey sample, and the pollen percentage was averaged.

2.2.4 Methods of physicochemical analysis of honey samples

2.2.4.1 Determination of honey moisture and sugar content

The total sugar (degrees Brix, °Brix) and moisture content of honey samples were determined using an Atago hand-held refractometer MASTER-HONEY/BX (Honey moisture 12.0-30.0%, Brix 58.0-90.0%, automatic temperature compensation), as per manufacturer's instructions.

2.2.4.2 pH measurements

The pH of a honey solution (10% w/v in deionised water) was measured using a HI2210 pH meter (Hanna Instrument Ltd., UK)[50]. Three measurements were recorded for each honey sample, and the results were expressed as mean ± standard deviation.

2.2.4.3 Colour evaluation

The colour of the honey samples was determined using the method by Naab et al.[253]. A honey solution (50% w/v in deionised water) was prepared for each sample and filtered through VWR qualitative filter paper (medium filtrate rate, particle retention 5-13 µm – VWR International Ltd, Ireland). The absorbance of the samples' solutions was measured at 635 nm against a methanol blank (UV-1280 Multipurpose UV-Visible Spectrophotometer, Shimadzu Europa Gmbh). The results were expressed in the Pfund scale (mm) as the mean of three measurements ± standard deviation.

2.2.5 Determination of total phenolic content

The total phenolic content (TPC) of the honey samples was determined by the application of a modified Folin-Ciocalteu method[50]. A honey solution (10% w/v, in deionised water) was prepared for each honey sample and filtered through VWR qualitative filter paper (medium filtrate rate, particle retention 5-13 µm – VWR International Ltd, Ireland). A volume of 0.5 mL of this honey solution was mixed with 2.5

mL of 0.2 N Folin-Ciocalteu reagent, and after 5 mins, 2 mL of 75 g/L sodium carbonate solution were added. The samples were incubated for one hour at room temperature and protected from light. The absorbance of the samples was measured at 760 nm against a methanol blank (UV-1280 Multipurpose UV-Visible Spectrophotometer, Shimadzu Europa Gmbh). Quantification was based on a six-point calibration curve of gallic acid (0-200 ppm; R^2 0.9986, y = 0.0109x – 0.0034). The Limit of Detection (LOD) and Limit of Quantification (LOQ), also known as lower range limits, were calculated using the following equations[254]:

$$LOD = \frac{3.3\sigma}{S} \tag{2.2}$$

$$LOQ = \frac{10\sigma}{S} \tag{2.3}$$

where:

- σ is the standard deviation of the response based on the standard deviation of the blank
- S is the slope of the calibration curve

The values of the LOD and LOQ were estimated to be 2.28 and 6.90 ppm, respectively. The results were expressed as mg of gallic acid equivalent (GAE) per 100 g of honey, based on the following equation:

$$TPC = \frac{C * V * 100}{M} \tag{2.4}$$

where:

- TPC is the Total Phenolic Content expressed as mg GAE in 100 g of honey
- C is the concentration of gallic acid, determined from the calibration curve in ppm and expressed in mg/mL
- V is the volume of the honey solution analysed in mL
- M is the weight of honey in the sample in g

The results were expressed as the mean of three measurements ± standard deviation.

2.2.6 Screening of honey's volatile fraction

2.2.6.1 Sample preparations and VOCs extraction by Headspace – Solid Phase Micro Extraction (HS-SPME)

The extraction of the volatile compounds was carried out from the headspace of honey-saturated solutions (5 mL, 1:1 w/v in deionised water; saturated with NaCl to improve recovery) in 20 mL amber glass vials sealed with silicone-PTFE septa mounted on screw caps (Supelco Co., Bellefonte, PA, USA). Samples were equilibrated for 60 mins at 60 °C under magnetically stirring (1000 rpm). The extraction of VOCs was performed by exposing a divinylbenzene–carboxen–polydimethylsiloxane (DVB–CAR–PDMS) SPME fibre assembly (Supelco Co., Bellefonte, PA, USA) to the headspace for 40 min[255]. A control measurement was performed by exposing the SPME fibre inside a sealed empty glass vial under the same conditions described above. The fibre was previously conditioned according to the manufacturer's instructions (270 °C, 30 mins).

2.2.6.2 Gas Chromatography – Mass Spectrometry

The samples' volatile fraction was analysed using an Agilent gas chromatograph model 7820A coupled with a mass selective detector model 5977B (Agilent Technologies, Inc., Santa Clara, CA, United States). VOCs were desorbed in the injector of the gas chromatograph for 6 mins (inlet temperature: 250 °C)[255], splitless mode. The oven temperature program was as follows: the initial temperature of 70 °C was held for 2 mins, then the temperature was increased up to 110 °C at a rate of 4 °C/min, then further increased at a rate of 5 °C/min up to the final temperature of 200 °C (hold for 2 mins). Helium was employed as a gas carrier at a flow of 1 mL/min. The mass detector was operated in the electron impact ionisation mode at 70 eV, the mass scan range was *m/z* 30–400, and the ion source temperature was 230 °C. A SLB®-5ms non-polar capillary column (silphenylene polymer, virtually equivalent in polarity to poly(5% diphenyl/95% dimethylsiloxane) phase; L × I.D. = 30 m × 0.25 mm, d_f 0.25 µm) was employed for peak separation. HS-SPME followed by GC-MS was repeated four times for each honey sample.

2.2.6.3 Compound identification and interpretation of GC-MS spectra

GC-MS data were analysed using Agilent MassHunter Acquisition Data Qualitative Analysis version 10.0 (Agilent Technologies). Compounds were identified by chromatogram deconvolution algorithm, excluding ion 28 *m/z*, with an absolute area filter greater than 200000 counts. Ions were extracted from 0.3 to 0.7 *m/z*, and the base peak shape was with a 25% sharpness threshold. The obtained spectra were searched and compared with reference spectra using the NIST Mass Spectral Search Program for the NIST Tandem Mass Spectral Library Version 2.3 (built May 2017). The match score generated by the program is based on the spectral similarities between the compounds detected in the sample and reference compounds included in the database. This value was used to inform the preliminary evaluation, with a match score of at least 700 deemed sufficient to accept the identification. The compounds identified for each of the samples were then cross-matched within the four replicates, and compounds detected in at least 75% of the replicates were included in the data sets for further analysis. Finally, the compound identification was confirmed by means of their retention indices (RI), calculated based on their retention times (RT, mins) and compared with the RT of standards[255,256], as follows. Firstly, a C7–C30 saturated alkanes standard mix (1000 μg/mL in hexane; Merck Life Science Limited, Ireland) was sampled by HS-SPME, and the reference compounds were analysed with the GC-MS following the same method used for the honey samples (an exhausted SPME fibre was used because hexane exposure can damage the fibre coating). Then, the RT of the standard n-alkanes were used to calculate the experimental retention index (RI) using the Van den Dool and Kratz formula (as shown in Equation 2.2.5). This is a generalised Kovatz equation applicable not only to isothermal but also to linear temperature-programmed methods[256].

$$RI = 100\left(\frac{RT_x - RT_n}{RT_{n+1} - RT_n} + n\right) \quad (2.5)$$

where:

- RT_x is the retention time of the compound "x", of which the RI is being calculated
- RT_n and RT_{n+1} are the retention times of the standard alkanes preceding and following compound "x" in the elution order, respectively
- n is the number of carbons in the structure of the standard alkane eluting immediately before compound "x"

Finally, the calculated RI were compared with the RI reported in the NIST Chemistry WebBook - Standard Reference Database Number 69[257], and in The Pherobase – Database for Pheromones and Semiochemicals[258] for non-polar columns equivalent to the column described in Section 2.2.6.2. The identification was ultimately confirmed using a threshold-based approach, for those compounds for which the difference between experimental RI and the RI reported in the databases was less than 12 units (RI_{diff} <12)[259]. Compounds were labelled as "tentatively identified" when the difference exceeded this stated criterion (i.e., RI_{diff} >12) or when no RI reference was available on the NIST database, but the identification was supported by literature references[238,247,260–262]. The compounds identified were semi-quantified by calculating their percentage relative to the total VOCs identified (sum of the averaged values) from the GC abundancies (peak area, without correction factors). Results were reported as the mean of the values determined in the replicates where the compounds were identified (at least 75%) ± standard deviation.

2.2.7 Statistical analysis

The statistical significance of differences between samples (with α = 0.05) was calculated and graphed on GraphPad Prism (Version 9.4.0). Unpaired t-test (two-tailed) and one-way analysis of variance ANOVA (Tukey's multiple comparisons test) were used for comparisons between two groups and three groups or more, respectively. R Version 4.0.4 (The R Foundation for Statistical Computing) was used to perform data clustering and to visualise the distribution of the honey VOCs in a heatmap.

2.3 Results and Discussion

This chapter investigates different aspects of composition of four samples of Irish honey and compares them with manuka honey. Physicochemical parameters and the phenolic content are quantified. The botanical origin indicated on the labels is verified through microscopic pollen analysis and a screening of the volatile fraction (headspace) is carried out, with a focus on investigating the presence of markers of botanical origin.

2.3.1 Melissopalynological analysis for the identification of honey's botanical origin

The identification of the pollens extracted by melissopalynological analysis was based on the comparison with microscopic pictures from public pollen digital libraries[249–252]. Figure 2.4 shows some representative pictures of magnified pollen grains extracted from the samples analysed as per the method outlined in Section 2.2.3. For example, *Calluna* spp. pollen shows a characteristic shape with three (or four) sections delimited by "sulci", i.e. grooves (Figure 2.4, a-b), while the surface of the pollen of the family Asteraceae is covered by irregularly arranged spines (Figure 2.4, c), and Rosaceae pollen presents a crossed spheroidal shape (Figure 2.4, d). In total, 26 pollen types with frequency >1% were identified for these Irish honey samples (11 for IH1, 9 for IH2, 12 for IH3 and IH4).

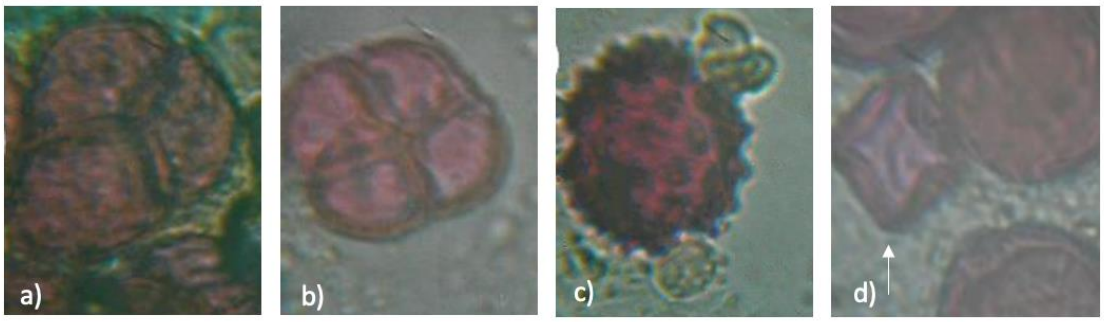


Figure 2.4 Microscopic pictures of representative pollens extracted from the Irish honey samples by melissopalynological analysis, as per method outlined in Section 2.2.3. a) and b) Calluna; c) Asteraceae; d) Rosaceae. Pictures reproduced with assistance from Dovydas Vilcinskas, BSc.

Louveaux et al.[263] suggested a method of classification of pollens based on their prevalence (quantified as a percentage of the total pollen fraction) in the honey sample, from dominant (>45%) to secondary (16-45%), then important minor (3-15%), trace (<3%) and sporadic (<1%). The presence of one dominant pollen (at least 45% of pollen from only one botanical source) is necessary for the honey sample to be identified as unifloral (single origin). Based on this classification, the pollen analysis (shown in Figure 2.5) revealed that the botanical origin declared by the beekeepers could only be confirmed for IH2, which was confirmed in this study as heather honey (73 $\pm$ 2% *Calluna* spp. pollen). For the other two Irish honey samples labelled as heather honey, the pollen from *Calluna* spp. represented only a secondary pollen (17 $\pm$ 3% and 40 $\pm$ 6% for IH1 and IH3, respectively). The most represented pollen in IH1 was from *Vicia* spp. (40 $\pm$ 2%), while for IH3 it was *Calluna* spp., although at an insufficient frequency to comply

with the standard for single-origin honey. In sample IH4, which is marketed as a mixture of ivy and heather honey, *Calluna* spp. pollen can be considered an important minor pollen (6 ± 1%). *Rosa* pollen (23 ± 3%) was the prevalent pollen in IH4, with *Arabidopsis, Agrostis and Geum* pollens being also abundant (for a cumulative value of 35%). A more comprehensive table of all the pollens identified in the honey samples, including the sporadic ones (<1% of the total pollen), can be found in Table A-1 (Appendix A).

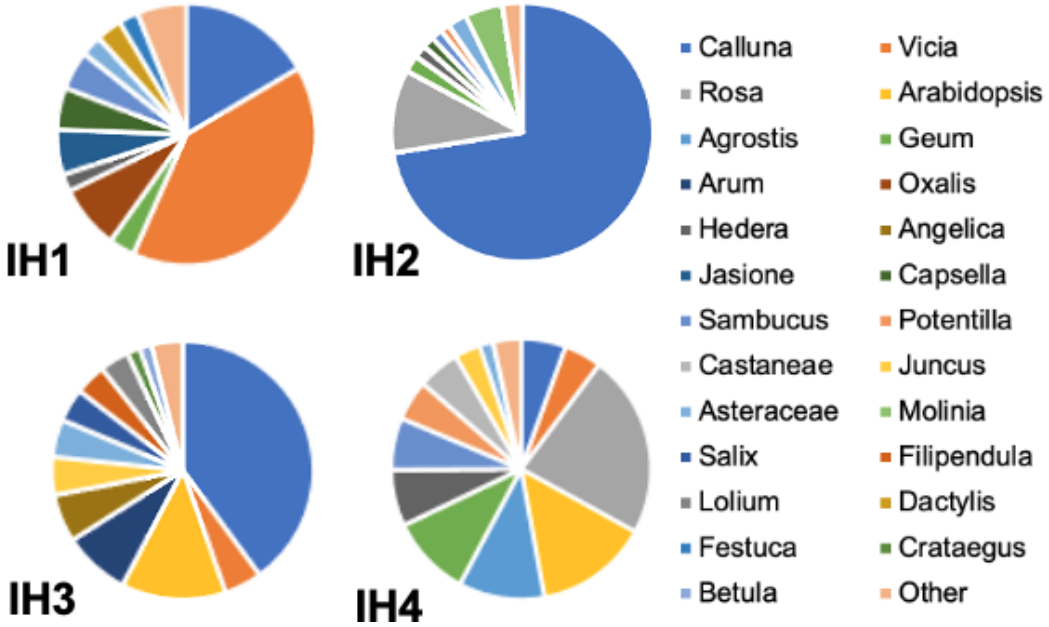


Figure 2.5 Pollen types and frequency in the Irish honey samples (namely IH1, IH2, IH3, and IH4), identified by melissopalynological analysis (method outlined in Section 2.2.3). The values shown are relative frequencies (% - average, with n=3) of each pollen type over the total number of pollen grains counted, calculated using the Equation 2.1.

It is worth considering that the pollen spectrum of heather honey can differ depending on the country where the honey is harvested, based on the plants typically coexisting with heather. In Ireland, pollen such as *Filipendula, Vicia, Castanea* or *Trifolium* (also identified here) would be commonly found during palynological analysis, while for example, pollens from Mediterranean plants like *Cistus, Olea, and Rosmarinus* accompany *Ericaceae* pollen in Spanish and Portuguese heather honey [243].

2.3.2 Physicochemical analysis of Irish honey samples

2.3.2.1 Honey moisture and sugar content

The sugar composition (total content and ratio between saccharides of different complexity) and moisture content of honey are crucial factors in assessing adulteration (due to the fraudulent practice of adding exogenous sugars) while also being

determinant for honey's rheological features and texture[44]. The sugar and water content are also important indicators of honey ripening, the complex processing of nectar into ripened honey. The sugar ratio is affected by the enzymes secreted by the bees' hypopharyngeal glands, while another crucial step in the production of ripe honey is the significant reduction of water content, from up to 70% in the raw material (nectar or honeydew) to around 20%, operated by the bees in the hive[264,265]. The standard moisture levels in honey are defined, like other relevant quality parameters, in the Codex Alimentarius[42] and in the Council directive 2001/110/EC[226], where a maximum value of 20% is set for the majority of honey varieties, with the exception of heather honey (*Calluna*) for which a value of 23% is deemed acceptable[264]. Furthermore, the moisture level reflects a seasonal trend, with a moisture content above 21% often measured in honey varieties harvested in autumn as a consequence of the wet weather conditions (*Arbutus unedo*, and various other *Ericaceae*)[266,267].

Table 2.2 Physicochemical features of the Irish honey samples (namely IH1, IH2, IH3, and IH3): total sugar (degrees Brix) and moisture content (determined using a hand-held refractometer), pH and colour (based on the Pfund scale). pH and colour value (in Pfund mm) are expressed as the average of three replicates ± standard deviation.

Sample Code	Moisture %	Brix %	pH	Colour (Pfund mm)	Colour (Pfund Scale)
IH1	21.6	76.8	4.42 ± 0.02	132 ± 2	Dark amber
IH2	19.2	79.2	4.79 ± 0.02	59.5 ± 0.8	Light amber
IH3	21.6	76.6	4.52 ± 0.01	88 ± 4	Amber
IH4	19.2	80.0	4.77 ± 0.01	90 ± 5	Amber

All the Irish honey samples analysed in this study had moisture content values between 19.2 and 21.6% (Table 2.2), thus they fall within the range established by the Codex standard for heather honey. However, as previously described in Section 2.3.1, the botanical single-origin of only one Irish sample could be confirmed as heather honey. Consequently, IH1 and IH3 should not be considered to be in conformity with the directive. The sugar content (expressed as Brix%, Table 2.2) was between 76.6 and 80%. Similarly, Kavanagh et al. reported values between 65.42 and 82.08% for Irish honey samples[50].

2.3.2.2 Honey pH

The pH of the Irish honey samples ranged between 4.42 ± 0.02 and 4.79 ± 0.02 (Table 2.2). These are in line with other reports, showing values between 4.25 (for manuka honey) and 5.0 (for heather honey)[67], with a particularly low value of 3.72 measured for Irish heather honey by Kavanagh et al.[50]. Castro-Vázquez et al.[268] observed a significant increase in heather honey acidity with the increase in storage temperature, with pH values going from 4.83 ± 0.01 for fresh honey down to 3.40 ± 0.02 for honey stored for one year at 40 °C. The typically acidic pH of honey plays a role, together with the high sugar content, in the long shelf-life of this bee product. Moreover, this parameter is of interest as the acidity of honey has been suggested to contribute to its antimicrobial effect, due to the inhibition of bacterial growth, and to its wound healing properties[228,232].

2.3.2.3 Colour evaluation

The colour of the Irish honey samples ranged between light amber (59.5 ± 0.8 mm) and dark amber (132 ± 2 mm), as shown in Table 2.2, according to the Pfund scale, which assigns higher values for progressively darker honey samples. These results are in line with the findings by Kavanagh et al. for Irish samples[50], with colour values up to 154.35 Pfund mm for heather honey and as high as 162.82 for ivy honey. The authors also reported a statistically significant positive correlation between colour and TPC, i.e. the darkest samples analysed (namely heather and manuka honey) also presented the highest content of phenolic compounds[50]. In a comparable study, the colour of Polish honey samples (measured according to the CIE L*a*b* chromatic coordinates) was highly correlated to its phenolic content and antioxidant activity, with heather and buckwheat honey characterised by a dark colour with red and yellow tones and phenolic content statistically higher than lighter honey varieties[269]. In the current study, the small sample size did not allow an evaluation of the correlation between honey colour and the content of phenolic compounds.

2.3.3 Total phenolic content (TPC)

The total phenolic content ranged between 64 ± 2 (for sample IH4) and 107 ± 4 mg GAE/100 g (for IH2), as shown in Figure 2.6. A one-way ANOVA showed that the TPC of the confirmed heather honey (sample IH2) was significantly higher than all other Irish honey samples analysed.

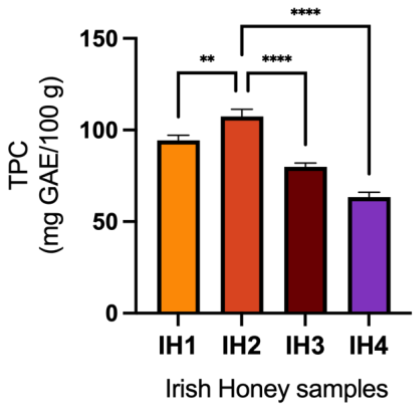


Figure 2.6 Total phenolic content (TPC) of the Irish honey samples (namely IH1, IH2, IH3, and IH3), determined by Folin Ciocalteu method. Results are expressed as mg of Gallic Acid Equivalent (GAE) in 100 g of honey (average of three measurements ± standard deviation shown as error bars).

These results are in line with what was reported by Kavanagh et al.[50], where heather honey samples had the highest TPC value of all the samples analysed (68.16 ± 2.73 mg GAE/100 g), including manuka honey (62.43 ± 10.03 mg GAE/100 g). Similarly, the TPC of 14 heather honey samples from Poland analysed by Kafarski et al.[241] ranged between 59.9 and 75.3 ± 18.3 mg GAE/100 g, with comparable results for a sample from England (74.4 ± 17.1 mg GAE/100 g). In the same study, buckwheat honey was shown to have the highest TPC (as high as 121.4 ± 11.3 mg GAE/100 g). A similar trend was reported by Kús et al.[269], with heather honey having a TPC over three-fold lower (30.62 ± 3.3 mg GAE/ 100 g) than buckwheat honey (111.30 mg GAE/100 g). However, the TPC value determined for heather honey was still significantly higher than lime, rapeseed, goldenrod and black locust honey. This is in line with what has been reported by Dezmirean et al.[270], with heather honey having almost a three times higher TPC (43.60 mg GAE/100 g) than raspberry honey (14.85 mg GAE/100 g). Higher TPC values have been described by Wilczyńska et al., with heather honey being the richest Polish honey variety in terms of phenolic compounds (between 155 and 189.52 mg GAE/100 g). A high degree of variability in the phenolic content was, however, observed in these

studies between different Polish samples, even within the same honey variety[271,272]. Even though only IH2 was confirmed as a heather honey in this study, all four of the honey samples were shown to have a TPC consistent with heather honeys studied internationally.

These results highlight how heather honey has been found to typically present high TPC, sometimes substantially higher than numerous other honey varieties. These compounds and their beneficial properties have been extensively studied, with honey samples rich in phenolic having significantly high biomedical performance in terms of anti-inflammatory, antiradical, antimicrobial and wound healing[52,62,232]. Further experiments would be required to evaluate the antioxidant activity of the honey samples examined and investigate the extent to which it correlates with the TPC.

2.3.4 Headspace fraction of VOCs in the Irish honey samples

The detection of the volatile compounds is not only dependent on aspects intrinsic to honey itself, namely its composition, but also on technical aspects like the extraction method and the selection of solvents with different polarity. This affects how some compounds are isolated at a high abundance while others are not detected, determining the chromatogram profile[4]. Employing techniques with different peculiarities (both instrumental and sensory analysis) can provide complementary information and build a database to fingerprint the full range of VOCs in honey[247,273–275]. Among the diverse techniques available, HS-SPME is widely employed for the screening of the volatile components in honey (method shown in Figure 2.7, a). The advantage of HS-SPME is that it is a solventless and versatile technique which does not require complex preparation or separate pre-concentration treatment. The sampling process is simply conducted by exposing an SPME fibre to the sample's headspace: the VOCs are absorbed on the fibre with different selectivity based on the fibre coating[247,276–278]. Nonetheless, the extraction can be optimised in terms of signal intensity and repeatability by adjusting the sampling conditions, such as equilibration time, fibre exposure time, temperature, sample/headspace volume ratio, and more[277–280]. Given these considerable advantages, HS-SPME-GC-MS was chosen for the investigation of the volatile fraction of Irish heather honey (representative Total Ion Chromatogram, TIC, and compound identification procedure shown in Figure 2.7, b-e and Figure 2.8).

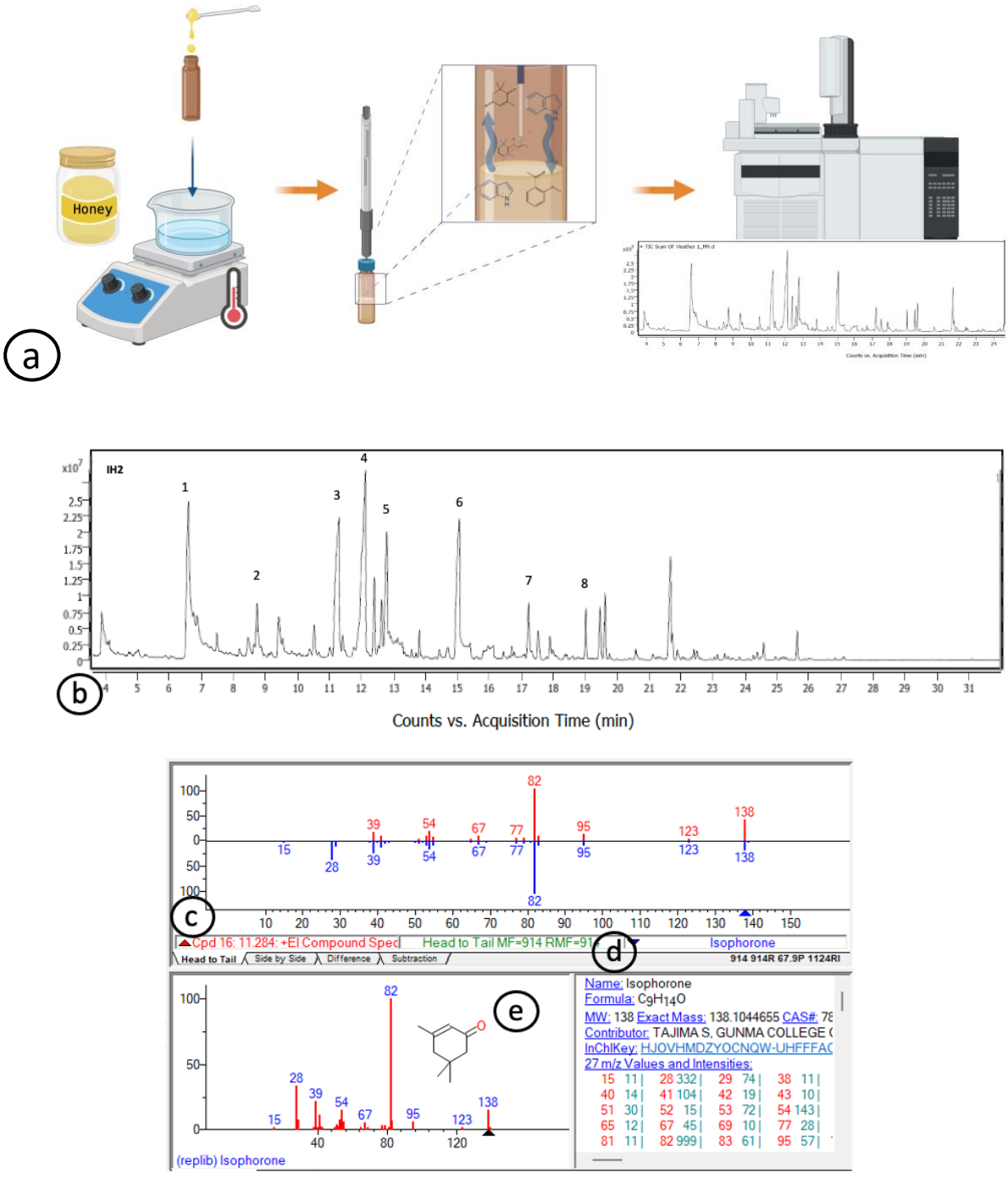


Figure 2.7 Sampling and compound identification procedure for the screening of the volatile fraction of the honey samples. a) Sample preparation and VOC extraction by Headspace – Solid Phase Micro Extraction (HS-SPME) followed by Gas Chromatography – Mass Spectrometry (GC-MS), as per method detailed in Section 2.2.6. Created in BioRender.com. b) Representative TIC, as count vs acquisition time (min), of a sample of Irish heather honey (IH2), obtained by HS-SPME followed by GC–MS. The main peaks of interest are numbered: 1) Benzaldehyde; 2) Benzeneacetaldehyde; 3) Isophorone; 4) 2-Hydroxyisophorone; 5) Benzoic acid, ethyl ester; 6) Benzeneacetic acid, ethyl ester; 7) 3,4,5-trimethyl-Phenol; 8) β-Damascenone. c) Representative MS spectra showing the fragmentation pattern of the peak eluting at a retention time of 11.284 (No. 3), identified as isophorone by comparison with reference MS spectra using the NIST Mass Spectral Search Program for the NIST23 3.0 Mass Spectral Library. c) A database match score (MF) of at least 700 was deemed sufficient to confirm the identification (here in the example, MF=914). d) Chemical structure of the compound being identified (isophorone), with details such as formula, molecular weight, contributor to the entry etc. shown on the right side of the screen.

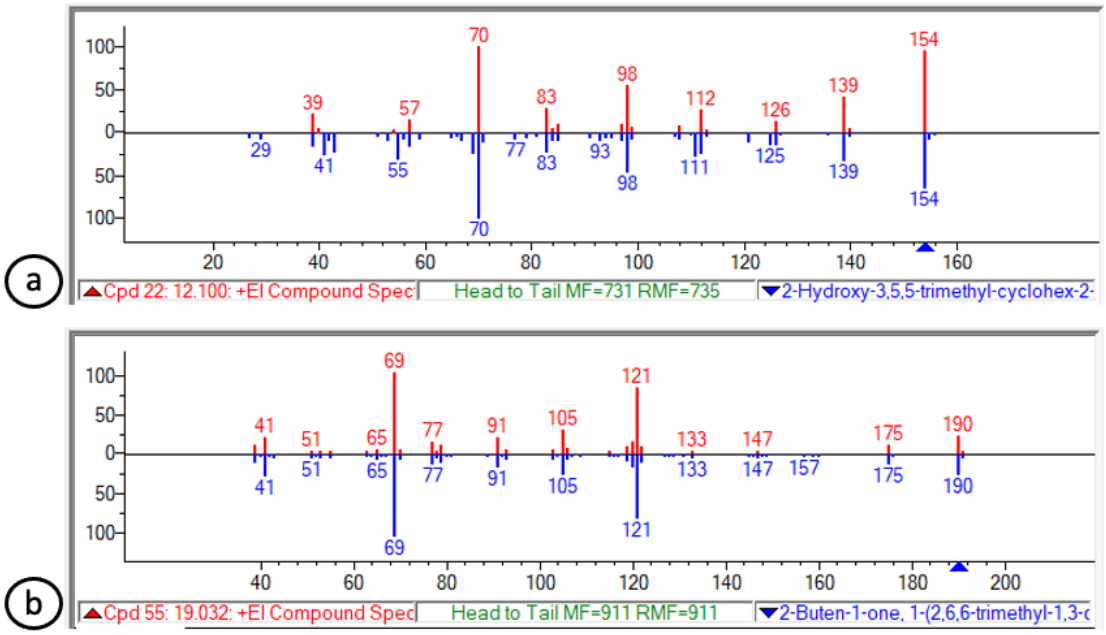


Figure 2.8 Representative MS spectra showing the fragmentation pattern of VOCs of interest of a sample of Irish heather honey (IH2), extracted by Headspace – Solid Phase Micro Extraction (HS-SPME) followed by Gas Chromatography – Mass Spectrometry (GC-MS). The detailed procedure is described in Section 2.2.6. a) 2-Hydroxyisophorone (2-Hydroxy-3,5,5-trimethyl-cyclohex-2-enone) and b) β-Damascenone (2-Buten-1-one, 1-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-, (E)-). The compound identification was carried out by comparison with reference MS spectra using the NIST Mass Spectral Search Program for the NIST23 3.0 Mass Spectral Library. A database match score (MF) of at least 700 was deemed sufficient to confirm the identification (here in the example, MF=735 and MF=911).

Similarities and differences across the dataset of the VOCs recovered from the Irish heather honey samples are visualised in the heatmap in Figure 2.9. The heatmap illustrates the total 110 VOCs identified in the Irish honey samples by assigning a colour ranging from red (highly abundant compound) to blue (compound not present) to each compound based on their abundance in each honey sample. To achieve this, the peak area values (Table A-2, Appendix A) were expressed in the logarithmic scale. The heatmap was paired with a dendrogram for hierarchical clustering, where the length of the branches of the dendrogram is proportional to the dissimilarity (i.e., longer branches mean the samples' patterns of VOC abundances are more distinguishable). The VOCs were ordered in the heatmap based on similar abundancies (see Table A-2, Appendix A for order of elution with retention time).

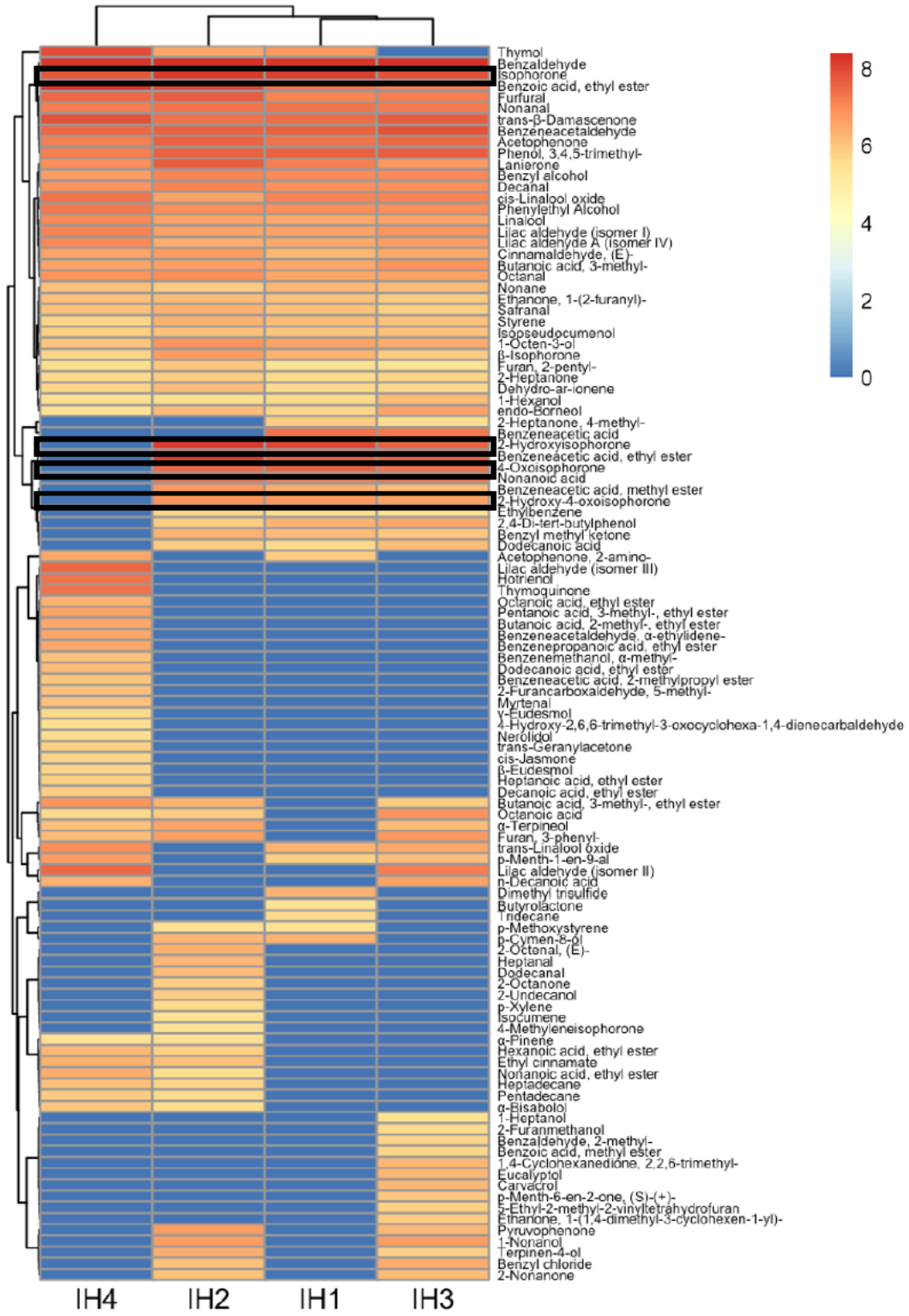


Figure 2.9 Heatmap showing the mean abundance (n = 4) of VOCs detected (rows) in each Irish honey sample (namely, IH1, IH2, IH3 and IH4, by columns). The peak area values (obtained by chromatogram deconvolution algorithm) are expressed in the logarithmic scale, with a corresponding colour ranging from red (highly abundant compound) to dark blue (compound not present). Hierarchical clustering (dendrograms) based on the similarity of VOCs abundance (by Euclidean distance). Proposed volatile chemical markers previously associated with Calluna/heather honey are highlighted by the black boxes within the heatmap.

2.3.4.1 Chemical markers of specific honey types in honey's headspace fractions

Chemical markers can be exclusive to a specific honey type or present at a significantly higher concentration than in other varieties. Therefore their identification has been suggested as a useful strategy to confirm the results of the pollen analysis, so as to facilitate the identification of honey's botanical origin. As previously detailed, the melissopalynological analysis unequivocally confirmed IH2 as the only unifloral heather honey (*Calluna* spp. pollen 73%), with IH3 also having a remarkably higher content of *Calluna* spp. pollen (40%) than IH1 and IH4 (17 and 5%, respectively). Compounds with general 3,5,5-trimethyl-cyclohexenic structure have been previously reported as chemical markers of heather honey (such as the C_9-norisoprinoids isophorone, 4-oxoisophorone, 2-hydroxyisophorone and 2-hydroxy-4-oxoisophorone)[231,242]. As can be appreciated from the colour grading in Figure 2.9, the distribution of these chemical markers (indicated by black boxes) seems to confirm the results of the pollen analysis, with unifloral heather honey (sample IH2) having the highest abundance of these markers, while IH4 showed the lowest percentage of *Calluna* spp. pollen and the lowest abundance of these heather honey markers (when present – see Table A-2 Appendix A for peak area values). However, comparing the clustering plotted in Figure 2.9 with the results reported in Figure 2.5 suggests that the composition of the volatile fraction of the honey samples analysed in this study does not completely correspond to the pollen distribution. In fact, on the basis of the melissopalynological analysis, samples IH2 and IH3 might be expected to cluster together due to the significantly higher *Calluna* spp. pollen than in samples IH1 and IH4. However, IH1 and IH3 cluster together instead, showing a higher level of similarity. Furthermore, despite sample IH3 having a higher level of pollen from *Calluna* spp. than IH1 (40 and 17%, respectively), the distribution of the suggested chemical markers does not follow the expected trend, with sample IH1 showing higher abundances of markers than IH3. This could possibly be explained by the multiple influences contributing to honey's aroma's complexity. As discussed in Section 2.1.1, plant VOCs have been suggested as the main source of honey VOCs, but more than one plant is visited by the bees in the process of collecting nectar that will be turned into honey. This results in VOCs indicative of different botanical origins being identified in honey. Furthermore, a multitude of other factors (non-plant derived) contributes to the final honey volatile fingerprint, as discussed previously. As such, while

VOC profiles can provide chemical markers to support the assignment of botanical origins, their use to exclusively determine if a honey can be classified as unifloral is not supported by this study. However, this study does support the proposition that the distribution of honey VOCs can align with the results of qualitative pollen analysis and therefore can be used to increase confidence in authentic assignment of botanical origin.

2.3.4.2 Exploration of how the VOCs identified in this study can contribute to the organoleptic properties associated with Irish honey

In addition to the identification of characteristic chemical markers, the VOC profile of honeys also reveals the distribution of typical volatile chemical classes that are considered critical to its organoleptic profile. A detailed exploration of the distribution of volatile chemical classes in different honeys known for distinctive flavour profiles can be found in the review by Machato et al[4]. For example, orange honeys, which are very sweet and floral, typically most frequently include lilac aldehydes, lilac alcohols and linalool derivatives, whilst in acacia honey, which is characterised by a sweet, beeswax and sourish flavour, benzaldehyde and a suite of ketones are most frequently detected.

A total of 110 volatile compounds were identified in the Irish honey samples in this study (53 for sample IH1, 69 for IH2, 67 for IH3, and 70 for IH4), which were categorised into seven chemical classes based on their chemical structure: alkanes, alcohols, aldehydes, ketones, carboxylic acids, and esters; compounds that did not fall into any of these categories were classified as “other” (Figure 2.10). Aldehydes and ketones are typically intensely flavoured, even at low concentrations, and the flavour profiles of honeys are attributed in part to these compounds[281]. Ketones represented the main chemical class of VOCs in samples IH1 (42%) and IH2 (39%), while aldehydes were the main VOC group in samples IH3 (47%) and IH4 (38%).

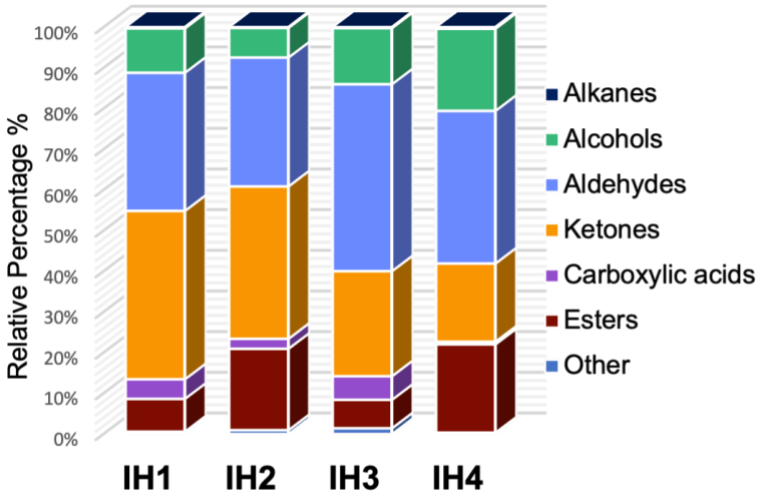


Figure 2.10 Distribution of the Volatile Organic Compounds for each honey sample into seven categories based on their chemical structures: alkanes, alcohols, aldehydes, ketones, esters, and "other". VOCs abundances for each sample are expressed as a relative percentage of the total VOCs (sum of the averaged data), normalised to a total of 100% for clarity of the visualisation.

Within each of these chemical classes however, the compound profile is distinctive for each sample, as shown in Figure 2.11 (see Table A-3, Appendix A for relative percentage values).

Aldehydes represented the main class of VOCs of the Irish honey, accounting for 32-46% (in IH2 and IH3, respectively - Figure 2.10) of the volatile fraction of the samples analysed here, with 21 compounds in total (of which 10 are present in all four samples). The most abundant aldehyde among the VOCs identified in the Irish honey samples was benzaldehyde (Figure 2.11, a), ranging between 25.9 ± 0.3% (for sample IH3) and 18 ± 1% (for sample IH4). This aromatic aldehyde is associated with a sweet, almond flavour[239] and is frequently measured in European honey varieties[240,247,282]. Benzeneacetaldehyde was detected at percentages between 8.2 ± 0.3% of the total VOCs of sample IH3 and 3.0 ± 0.2% of IH4, making it the second most abundant aldehyde (Figure 2.11, b). This aldehyde has been suggested to originate from phenylalanine by enzymatic conversion, thus its final abundance in the honey depends on the initial concentration of the aminoacidic precursor, which varies based on the botanical origin[229]. However, benzeneacetaldehyde can also be a product of the Strecker reaction between amino acids and dicarbonyl compounds, caused by thermal exposure[229,236]. As this compound can originate from different pathways, it can be difficult to estimate how

much of the measured abundance can be attributed to intrinsic factors such as the floral source, or rather post-harvesting modifications during storage, such as temperature differences in different countries and temperature variations due to seasonal trends, and also possibly during sample preparation pre-analysis.

Various furan derivatives have been suggested to be indicative of thermal exposure and prolonged storage[283–285]. A statistically significant progressive increase in the concentration of these compounds was observed in heather honey samples stored for one year at higher temperatures (20 °C and 40 °C) when compared to aliquots of the same samples fresh and refrigerated[268]. In the Irish honey samples analysed here, furfural, which is associated with a woody, sweet, fruity, soft almond flavour[258], was the most abundant compound among the furan derivatives (between $4.2 \pm 0.3\%$ of the total VOCs in IH2 and $1.6 \pm 0.6\%$ in IH1), with minor concentrations of other furan derivatives, such as furfuryl alcohol, 5-ethyl-2-methyl-2-vinyltetrahydrofuran, acetylfuran, and 2-amylfuran also identified here. It should be noted that in the headspace of the Irish honey samples analysed here, the compound 5-hydroxymethylfurfural (HMF), an indicator of excessive thermal treatment and resultant poor honey quality, was not detected.

A number of terpenic aldehydes were also detected. Of these, only two are of particular interest, safranal has been indicated as a specific compound to unifloral honey from the Ericaceae family[266], but in the Irish heather honey under study here it accounted only for a mean of 0.1% of the total VOC fraction in each sample. Another compound derived from the highly represented linalool is lilac aldehyde, a ubiquitous terpene that has been found at different frequencies in a wide variety of honey, including previous reports on heather honey[3,236,238,240,282,286,287]. In these Irish honey samples, four isomers of lilac aldehyde were identified, of which two were in all four honey samples, ranging between $0.21 \pm 0.07\%$ and $5.0 \pm 0.7\%$.

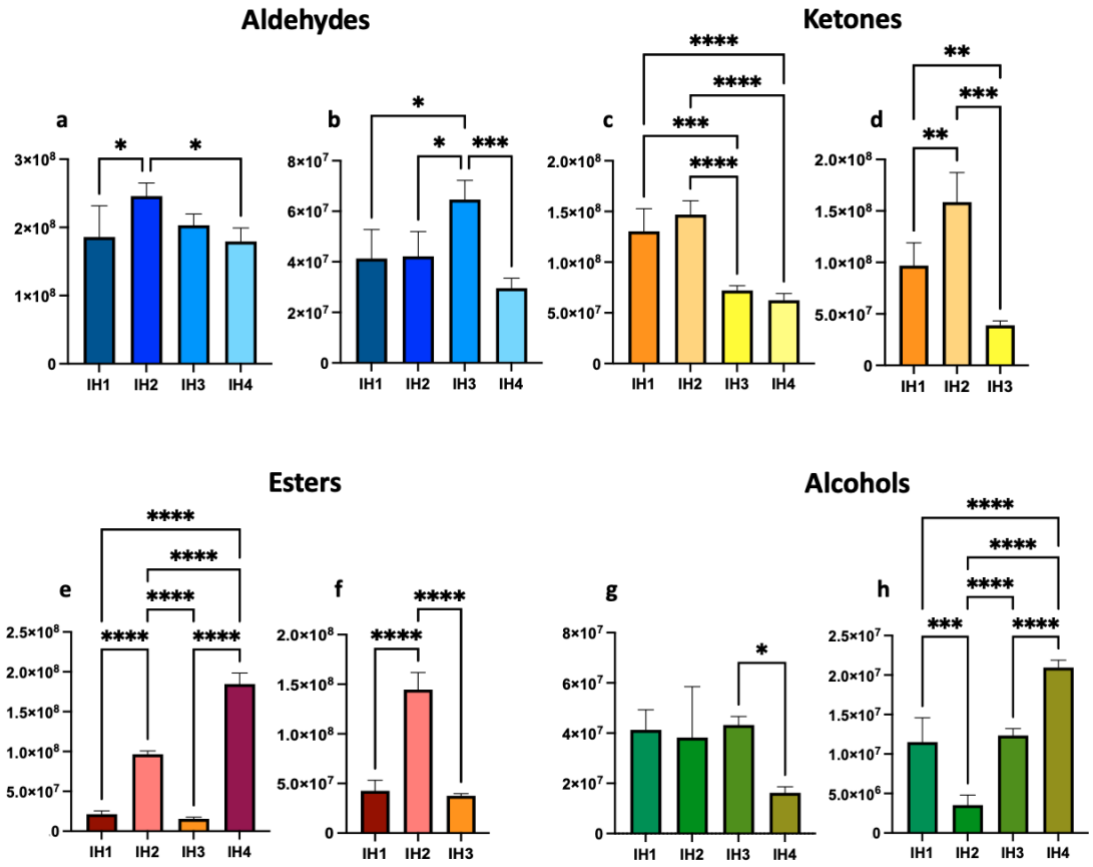


Figure 2.11 Comparisons of the peak area values in the four Irish honey samples for highly abundant headspace VOCs. The two most abundant compounds of each chemical class (as described in Figure 2.10) are shown: a) benzaldehyde; b) benzeneacetaldehyde; c) isophorone; d) 2-hydroxyisophorone; e) benzoic acid, ethyl ester; f) benzeneacetic acid, ethyl ester; g) 3,4,5-trimethyl-phenol; h) cis-linalool oxide. Values shown are averages of four replicates ± standard deviation; ordinary one-way ANOVA and Kruskal-Wallis test were used and the statistically significant differences between values are shown.

Ketones are the second most represented chemical class in the Irish honey samples (from 20% of total VOCs for IH4 up to 42% for IH1 - Figure 2.10), with 24 of them detected (7 in common in all samples). High concentrations of ketones, norisoprenoids, that are produced by the breakdown of carotenoids by enzymatic or non-enzymatic reactions (caused by temperature, light, oxygen and acidic environment)[236] have been suggested as non-specific markers of different unifloral honey varieties from the *Ericaceae* family, such as heather honey (*Calluna vulgaris*)[231,241,242,244], winter heather (Erica multiflora)[266], and strawberry tree honey (*Arbutus unedo L.*) typical of Sardinia (Italy)[59,288]. Isophorone (chemical structure in Figure 2.12), characterised as a woody, musty, spicy, camphor-like aroma[228,258] was the most abundant C_9-norisoprenoid in this study, ranging between 16 ± 1% (in IH1) and 6.4 ± 0.7% (in IH4), together with 2-hydroxyisophorone (Figure 2.12), detected at percentages as high as 11.9 ± 0.7% in IH1

and 12.9 ± 0.8% in IH2, while a percentage of 1.8 ± 0.4% was detected in IH3 (undetected in IH4) (Figure 2.11, c and d). Oxoisophorone (Figure 2.12), which also has a wood, musty, sweet aroma[258], was also identified here, at percentages between 4.5 ± 0.3% in IH1 and 2.8 ± 0.2% in IH2 (not detected in IH4).

C_{13}-norisoprenoids, unlike C_9-norisoprenoids, are typically not highly volatile and require solvent extraction techniques to be found in the VOCs fraction. β-damascenone and β-ionone are the C_{13}-norisoprenoids with the lowest odour threshold in honey, i.e. their contribution to the scent of honey is easily discernible even at low concentrations[236]. In the Irish honey samples, trans-β-damascenone (Figure 2.12), characterised by a distinctive sweet, apple-like, fruity aroma[258], was found in all 4 samples (ranging between 8 ± 1% in IH4 and 1.4 ± 0.3% in IH2) but β-ionone was not detected.

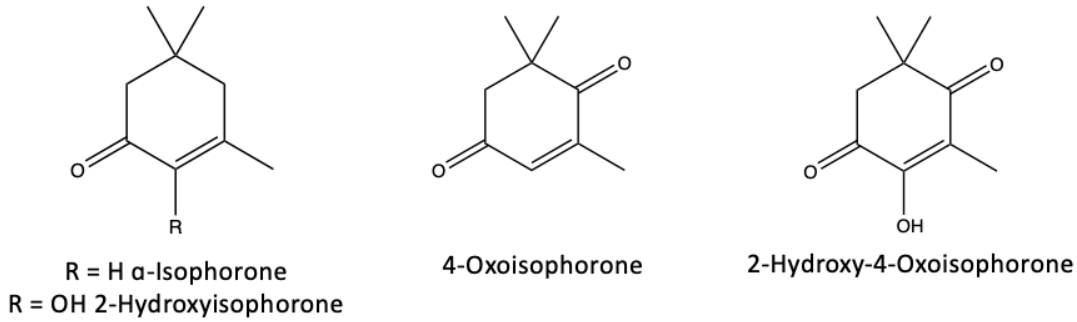


Figure 2.12 Chemical structure of the volatile markers of Irish heather honey identified by GC-MS analysis of the headspace fraction of Irish heather honey (IH2) sampled using HS-SPME, as described in Section 2.2.6.

Six carboxylic acids were identified in the samples, with IH4 having the lowest abundance and IH3 the highest (0.7 and 6%, respectively - Figure 2.10). Phenylacetic acid represented 2.6 ± 0.5% and 2.1 ± 0.8% of the VOC fraction of IH1 and IH3, respectively. Despite being suggested as a marker of honey from *Calluna vulgaris*[231], it was not detected in the unifloral heather honey sample (IH2). Its ethyl ester ethyl phenylacetate, a compound associated with sweet, fruity, floral scent[258], was, however, measured at high percentages in the samples presenting the highest level of *Calluna* spp. pollen, being the fourth most represented VOC in IH2 (12.1 ± 0.3% - 5.2 ± 0.4% in IH1 and 4.8 ± 0.2% in IH3). Ethyl phenylacetate was not present in IH4, which however presented a variety of esters that were undetected in the rest of the samples, for a total

of 1.65 ± 0.32%. In the Irish honey samples analysed in this study, esters were the third class in terms of abundance, particularly in samples IH2 and IH4 (21 and 22% of the total VOCs, respectively), with a total of 16 esters identified. The ethyl ester of benzoic acid (ethyl benzoate, associated with notes of chamomile flower and celery, with a fruity, musty odour)[258] was the most abundant ester, detected in high quantity in samples IH4 (19 ± 1%), followed by IH2 (8.1 ± 0.7% - Figure 2.11, e). By contrast, the second most abundant ester, benzeneacetic acid, ethyl ester (ethyl phenylacetate), was highest in IH2 and not detected at all in IH4 (as shown in Figure 2.11, f). A high level of ethyl esters has been suggested to be due to the esterification of aromatic carboxylic acids present in honey. These esters can, at least in part, be considered artefacts of the sample preparation, with the esterification reaction being favoured at high temperatures, such as those employed for solvent extraction[234]. Another factor that could play a role in the abundance of esters is thermal exposure of honey during its processing and storage, which can promote the alcoholic fermentation of glucose and fructose operated by yeasts providing the reagent for the esterification reactions[234].

Alcohols are also usually found in honey and are also associated with honey's flavour profile and potential medicinal properties. They were identified in percentages between 8% in IH2 and 21% in IH4 (Figure 2.10), for a total of 27 compounds (9 extracted in every sample). The alcohol 3,4,5-trimethyl-phenol (3,4,5-hemimellitenol) was highly represented in all the Irish honey samples (Figure 2.11, g - up to 5.5 ± 0.2% of the total VOCs), while thymol, characterised by a herbal, thyme, earthy aroma[258], was the most abundant alcohol in sample IH4 (9.5 ± 0.3% in IH4). Among the identified alcohols, various cyclic compounds with terpene structures were detected, such as linalool (between 0.34 ± 0.03% in IH2 and 1.1 ± 0.2% in IH4) and its derivatives, for example, borneol, terpineol, and p-cymen-8-ol. Trans-linalool oxide, accounting for 13.3% of the total VOCs of winter heather honey (*Erica multiflora*) from the Balearic Islands (Spain)[266], was detected at percentages below 1% in all the Irish samples analysed in this research; cis-linalool oxide was slightly more abundant (between 0.29 ± 0.09% and 2.2 ± 0.2% - Figure 2.11, h).

The results presented in this section showed that within the same honey type, i.e., in this case, honey labelled as "heather honey" for commercialisation, not only the abundance of the compounds but also the general distribution of the VOCs within chemical classes (typically associated with flavour profiles as well as botanical origin) can vary, which explains the variability between different brands and could justify personal preferences of consumers.

2.3.4.3 Evaluating the differing characteristics that contribute to each honey's overall characterisation

It is clear from the results of the melissopalynological analysis in Figure 2.5 that only IH2 can be classified as a unifloral heather honey. Whilst *Calluna* spp. pollen is the most represented pollen in IH3, it is not in sufficient quantities to allow a monofloral classification. *Calluna* spp. pollen is also a significant secondary pollen in IH1, but only a minor pollen in IH4. IH2, the confirmed heather honey, was also the honey found in this study to have the highest total phenolic content (TPC) Figure 2.6 though interestingly all four honey samples were shown to have TPCs consistent with both previous Irish and international studies of heather honey. The heatmap of VOC profiles of the four honeys illustrated in Figure 2.9 also indicates that the confirmed unifloral heather honey (IH2) has the highest abundance of the previously reported heather honey markers (3,5,5-trimethyl-cyclohexenic structures), while IH4 showed the lowest percentage of *Calluna* spp. pollen and the lowest abundance of these heather honey markers (when present). In the case of the Irish honey samples analysed here, previously reported markers of heather botanical origin were identified, specifically C_9-norisoprenoid compounds with 3,5,5-trimethyl-cyclohexenic general structure, such as isophorone and its structurally related compounds. These chemical markers, among the diverse VOCs contributing to honey's headspace, could enable a positive identification of heather as a constituent component of the sample being analysed. Additionally, these results confirm the deep interconnection between the complexity of the volatile fraction of honey and the numerous direct and indirect (environmental) sources of VOCs. However, a detailed comparison of the VOC clustering with melissopalynological analysis highlighted that the composition of the volatile fraction of the honey samples analysed in this study does not completely correspond to the pollen distribution, which makes sense considering

that the VOC profiles include both plant and non-plant derived factors. As such, while VOC profiles can provide chemical markers to support the assignment of botanical origins, they should not be used to exclusively determine if a honey can be classified as unifloral. That said, the presence of C_9-norisoprenoid chemical markers, representing some of the most abundant compounds in the samples, does support the use of these compounds as chemical markers for heather honey. As shown in Figure 2.9, the four compounds were among the most abundant for IH1, IH2 and IH3, and were not all present for IH4. As such, this chemical profile could represent an identification tool to complement the information from the melissopalynological analysis, which showed heather honey compositions of 17% for IH1, 73% for IH2, 40% for IH3 and only 5% for IH4. The C_9-norisoprenoid results are in line with the research of heather honey from other countries, showing for the first time that Irish heather honey is characterised by distinctive compounds that could be used, in conjunction with the pollen screening, to help in the determination of the botanical origin of this honey. It is worth noting too that the presence of these compounds is also indicative of carotenoid presence in the honeys, and carotenoids are indicated as a possible mechanism for antioxidant activity.

2.3.5 Comparison between Irish heather honey and New Zealand manuka honey

New Zealand manuka honey has gained considerable visibility due to a major campaign supported by the New Zealand government in promoting this unifloral honey variety and in setting quality standards to protect the authenticity of this product[289], namely the UMF™ Quality Assurance System[290]. In this system, a higher UMF™ value correlates with a higher methylglyoxal, which is purposed to be responsible for its health properties. UMF™ values typically range from 5+ to 25+[290]. The official recognition of its value (both in terms of physicochemical composition and health benefits, but also of potential profit) has then stimulated further investigations into its properties in the scientific community. Its highly researched antioxidant and anti-inflammatory[131,132,291], antibacterial[292–294], wound healing[54,132], and anticancer[295,296] virtues have earned manuka honey a prominent role in the bee products market. Consequently, the comparison between manuka honey and other unifloral honey varieties with promising physicochemical features and composition has attracted considerable attention[232]. Besides the primary interest for honey commercialisation as a food product, the aim of

this comparison is to investigate if locally sourced and more affordable alternatives to manuka honey are appropriate for the development of medical-grade honey products[297–299]. Among European honey varieties, heather honey has been shown to possess promising properties, comparable to manuka honey, both in terms of physicochemical features, and biomedical and antimicrobial performance[50,122,123,300,301]. Given that the worldwide acclaim for manuka honey's properties is accompanied by increasing research into alternative honey varieties providing comparable performance, here the results of the characterisation of manuka honey (sample MH, conducted according to the methods outlined in Section 2.2) are described. Furthermore, the resulting data are also compared with the unifloral Irish heather honey (sample H2), with particular attention to the VOCs profile in order to identify distinctive compounds in their volatile fractions.

2.3.5.1 Manuka honey physicochemical features and phenolic content

The identification of the pollens extracted from the honey samples (method detailed in Section 2.2.3) was based on the comparison with microscopic pictures from pollen digital libraries[249–252]. The melissopalynological analysis (Figure 2.13, a) corroborated the botanical single origin that the sample was marketed as, with *Leptospermum* pollen (with its typical triangular shape, shown in Figure 2.13, b) being dominant, namely 71 $\pm$ 5%, i.e. >45% of the total pollen fraction. Sample MH can thus be considered an authentic unifloral manuka honey. 16 pollen types were identified in total in sample MH, of which 11 were sporadic (<1%) according to the classification by Louveaux et al.[263] (as reported in Table A-4 in Appendix A). Important minor pollens (frequency between 4 $\pm$ 1% and 13 $\pm$ 1%) were from *Lotus, Salix* and *Trifolium*; traces of *Rosaceae* pollen were also found (2.3 $\pm$ 0.5%).

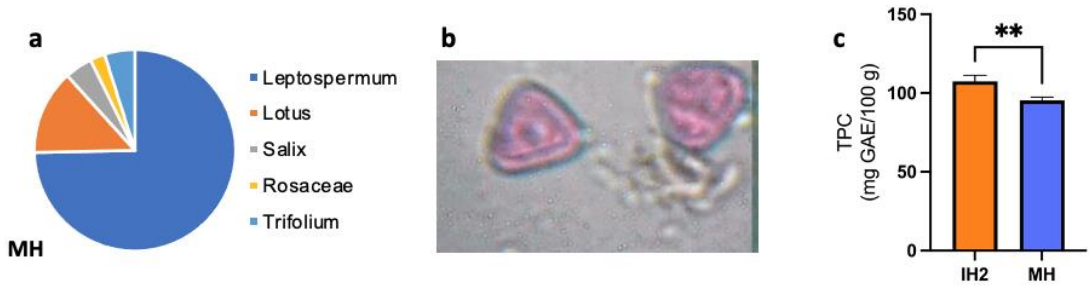


Figure 2.13 Summary of the properties of manuka honey (MH) sample. a) Pollen types and frequency, identified by melissopalynological analysis. b) Microscopic pictures of representative Leptospermum pollens extracted from the manuka honey sample by melissopalynological analysis, as per method outlined in Section 2.2.3. c) Comparison of the Total phenolic content (TPC) of heather honey (sample IH2) and manuka honey (sample MH). The results, determined by Folin Ciocalteu method, are expressed as mg of Gallic Acid Equivalent (GAE) in 100 g of honey (average of three measurements ± standard deviation shown as error bars).

The phenolic content of MH (UMF™ 15+) (95 ± 2 mg GAE/100 g) was significantly lower than that of the Irish heather honey sample IH2 (p-value 0.0091), as shown in Figure 2.13, b. Interestingly, this is not the first report of Irish heather honey having a higher level of phenolic compounds (68.16 ± 2.73 mg GAE/100 g) than manuka honey (62.43 ± 10.03 mg GAE/100 g)[50]. Similar values were also found by Zhang et al.[302], with manuka honey UMF™ 12+ and UMF™ 20+ having 66.14 ± 0.92 and 83.54 ± 0.23 mg GAE/100 g.

Table 2.3 Physicochemical properties of manuka honey: total sugar (degrees Brix) and moisture content (determined using a hand-held refractometer), pH and colour (based on the Pfund scale). pH and colour value (in Pfund mm) are expressed as the average of three replicates ± standard deviation.

Sample Code	Moisture %	Brix %	pH	Colour (Pfund mm)	Colour (Pfund Scale)
MH	19.2	79.0	4.22 ± 0.03	149 ± 3	Dark amber

The results of the physicochemical characterisation of MH are summarised in Table 2.3. The values of water and Brix% sugar content (19.2 and 79.0%, respectively) were found to conform to the legal thresholds[42]. The pH of the honey solution was 4.22 ± 0.03, similarly to the value measured in manuka honey by Fyfe et al.[67]. A pH range between 4.29 and 4.53 was recorded by Kavanagh et al.[50], with manuka honey samples found to be, together with heather honey, the least acidic of samples of various botanical and geographical origins.

Finally, from a visual perspective, manuka honey presents a dark amber colour, as confirmed by the Pfund classification, with a measured value of 149 ± 3 mm. Comparable values for manuka honey (between 165.94 and 186.68 mm) were reported in a similar study[50], with the colour values correlating with the TPC.

2.3.5.2 Characteristic VOCs of manuka honey

94 volatile compounds were identified in the headspace of the sample MH (see Appendix A, Table A-5 for peak area and Table A-6 for relative percentages), analysed by HS-SPME followed by GC-MS as detailed in Sections 2.2.6 and 2.2.6.2. The volatile compound 2-methoxyacetophenone (Figure 2.14), which is associated with powdery, anise, almond, and phenolic aroma features, was the most abundant VOC in the headspace of sample MH (24 $\pm$ 5%), in line with previous studies reporting it among the main components of the extracts of manuka honey[234,260]. It was suggested that 2-methoxyacetophenone could originate from the tree sap since it was not identified in manuka flower extracts, in a study aimed at comparing the VOC profile of honey and its nectar source[234]. 2-methoxyacetophenone has also been found to dominate the headspace of manuka honey by Beitlich et al.[261], which recommended its identification as a discriminant criterion to distinguish manuka and kanuka honey (*Kunzea ericoides*, in which 2-methoxyacetophenone was detected at a considerably lower percentage). The two honey varieties are often mistaken due to the similarity in the pollen which makes the identification by palynological analysis inconclusive[303].

In order to disentangle such ambiguities in the identification of manuka honey and to distinguish it from the honey derived from other *Leptospermum* species, the Ministry of Primary Industries of the New Zealand government published a directive (MPI Technical Paper 2017/30)[304] presenting an official method to assess the authenticity of manuka honey from New Zealand by identification of four markers. This technical paper outlines the specific procedure for the quantification of three aromatic acids (together with 2-methoxyacetophenone), namely 3-phenyllactic acid, 2-methoxybenzoic acid, and 4-hydroxyphenyllacetic acid, by liquid chromatography-tandem mass spectrometry (LCMS/MS). Following the method of the MPI directive, Rückriemen et al.[305] suggested that significant differences in the concentration of these markers in commercial manuka honey samples could be ascribed to different geographical production sites (i.e. honey

from *Leptospermum scoparium* produced in Australia instead of New Zealand)[305]. 3-phenyllactic acid and 2-methoxybenzoic acid have also been measured at high concentrations in the SPME extracts of manuka honey analysed by GC/MS[260]. However, in the present work, neither of these aromatic acids was detected in the headspace of sample MH. This is in line with the results of the screening of manuka honey by HS-SPME-GC/MS by Beitlich et al.[261], where these three markers were also not reported, and two other VOCs were identified instead as specific to manuka honey. These proposed markers were 2'-hydroxyacetophenone and 2-methylbenzofuran (see Figure 2.14 for the chemical structure), which in the current study represented $3.5 \pm 0.4\%$ and $3.3 \pm 0.6\%$ of the total VOC fraction in MH's headspace, respectively.

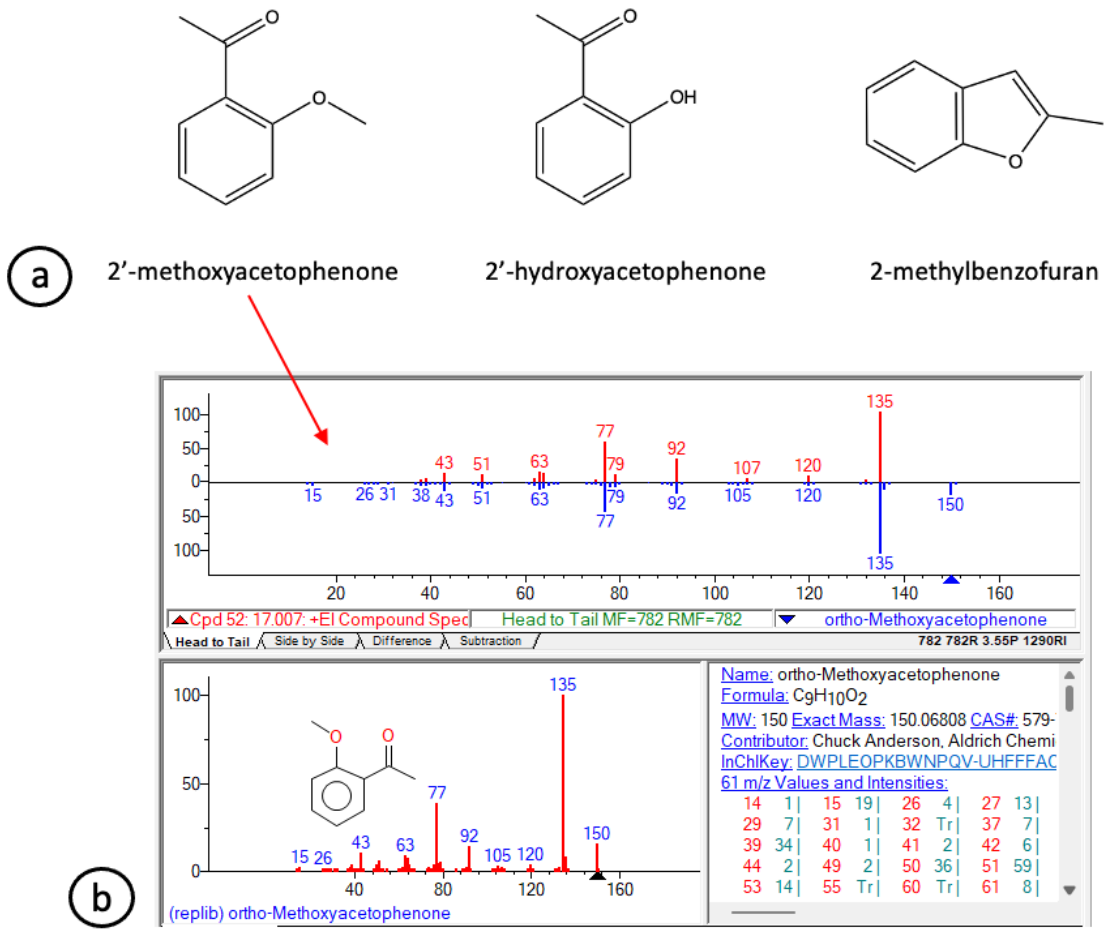


Figure 2.14 a) Chemical structures of the volatile compounds of interest extracted in sample MH by Headspace – Solid Phase Micro Extraction (HS-SPME) followed by Gas Chromatography – Mass Spectrometry (GC-MS), as per method detailed in Section 2.2.6 b) Representative MS spectra showing the fragmentation pattern of the compound identified as 2'-methoxyacetophenone, compared with reference MS spectra using the NIST Mass Spectral Search Program for the NIST23 3.0 Mass Spectral Library. A database match score (MF) of at least 700 was deemed sufficient to confirm the identification (here in the example, MF=782).

Remarkably high concentrations (between 20 and 53.5%)[260,306] of the methyl ester of syringic acid (4-hydroxy-3,5-dimethoxybenzoic acid) were reported in studies employing GC-MS for the analysis of manuka honey extractives. The aromatic acid was also identified at high concentrations in manuka honey extracts by Tan et al.[234]. However, in the present study, only methyl syringate (and not its correspondent acid) was detected in the headspace of sample MH, and at negligible percentages ($0.24 \pm 0.03\%$). Similarly, in a study investigating volatile and non-volatile compounds in manuka honey, neither syringic acid nor methyl syringate was reported among the VOCs identified in the headspace of manuka honey[261]. Interestingly, in the same study, methyl syringate was identified as a predominant component in the extracts of kanuka honey (an emerging New Zealand honey) analysed by HPLC and thus suggested, with other relevant characteristic compounds, as a marker to discriminate kanuka from manuka honey[261]. Moreover, the occurrence of methyl syringate has been reported in various other honey types[238,307], such as asphodel honey for which it represents a specific marker of botanical origin[55,274].

The melissopalynological analysis, whose results are presented in Section 2.3.5.1, together with the identification of a high abundance of volatile chemical compounds previously reported in the literature as chemical markers of manuka honey (namely 2-methoxyacetophenone, 2'-hydroxyacetophenone and 2-methylbenzofuran), allow us to confirm the botanical origin that the honey sample MH was marketed with and confidently identify it as manuka honey.

2.3.5.3 Comparison between the volatile profile of samples IH2 and MH

The proportion of VOCs belonging to different chemical classes is similar between samples MH and IH2 for most groups (as shown in Figure 2.15), with comparable values for alkanes, alcohols, aldehydes, and ketones, but substantial differences in the content of carboxylic acids, esters and more complex molecules (grouped as "other").

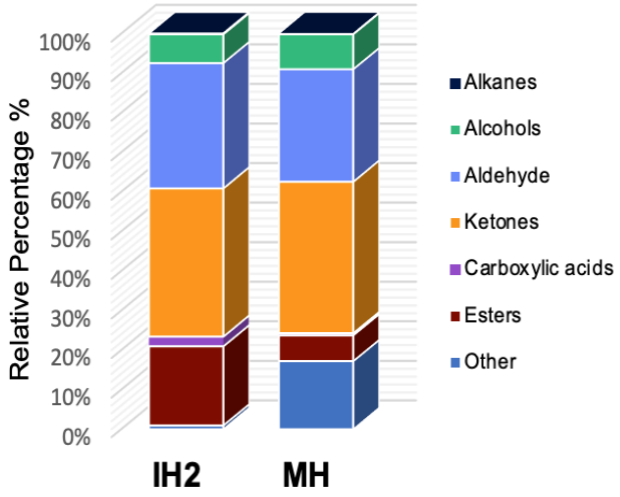


Figure 2.15 Comparison between Irish heather honey (IH2) and manuka honey (MH). Distribution of their Volatile Organic Compounds into seven categories based on their chemical structures: alkanes, alcohols, aldehydes, ketones, carboxylic acids, esters, and "other". Data are expressed as a relative percentage of the total VOCs (averaged).

The headspace of both samples, MH and IH2, was dominated by ketones. However, as extensively discussed in Sections 2.3.4 and 2.3.5.2, these two classes were characterised by markers specific for each single-origin variety. 2-methoxyacetophenone and 2'-hydroxyacetophenone, typical of manuka honey aroma, together with 2'-methylacetophenone (4.3 ± 0.4%) and other minor related compounds (3'-methoxyacetophenone, 4'-methoxyacetophenone and 2'-hydroxy-6'-methoxyacetophenone) were absent in heather honey. Similarly, compounds such as isophorone, 2-hydroxyisophorone and 4-oxoisophorone, detected in sample MH at percentages below 1.2%, were highly abundant in Irish heather honey for which they can be considered chemical markers.

Aldehydes were the second most represented class of VOCs in manuka honey, with various compounds widely detected in different honey varieties, including previous reports on the VOCs of manuka honey[261,262], and the Irish honey samples in the present study, such as benzaldehyde, benzeneacetaldehyde, and the isomers of lilac aldehydes. Some major differences in the headspace composition for this class were noted, such as the concentration of benzaldehyde in the headspace of sample MH being substantially lower than in sample IH2 (8.5 ± 0.4% and 21 ± 1%, respectively). Conversely, the linear aldehyde nonanal (associated with aroma features such as fruity, floral, waxy, sweet, melon, soapy, fatty, lavender, citrus fruit) represented 6.7 ± 0.8% of the total volatile

fraction of sample MH, while it only accounted for 1.7 ± 0.4% of the VOCs in sample IH2. Its occurrence in manuka honey was first reported by Anand et al.[262], where it accounted for 3.87% of the total VOCs fraction, and it was also identified at mean percentages between 8.25 and 12.4% in other varieties of *Leptospermum* honey such as jelly bush honey, for which it was suggested as a chemical marker. Furfural (4.3 ± 0.4%) and other furan derivatives such as 5-methyl-2-furfural (0.06 ± 0.01%), acetylfuran (0.70 ± 0.04%) and 2-acetyl-5-methylfuran (0.19 ± 0.01%) were detected at values in line with the percentages measured for the Irish honey samples (see Table A-2 in Appendix A). Similarly, furfural and 2-acetyl-5-methylfuran have been detected by Anand et al. in manuka honey at concentrations of 0.21 and 0.31%, respectively[262]. These compounds in honey are indicative of heat exposure and long storage, which could affect honey's sensory features, such as its taste and aroma, as previously described in Section 2.3.4. The compromise of its organoleptic attributes can impact the marketing value of honey, which justifies the interest in effective ways to monitor the abundance of these compounds[48,308,309]. Gras et al. developed a new chromatographic method to quantify furfural and 5-methyl-2-furfural in honey samples and validated it with manuka honey samples: substantial increases in the concentration of these two compounds were observed, when compared to fresh honey, in heat-treated honey (40 °C for 4 weeks) and in a honey sample deemed not suitable for commercialisation by a quality control laboratory due to an unpleasant taste[308]. Finally, it is worth mentioning the presence of myrtenal (3.0 ± 0.2%) in manuka honey, which was absent in sample IH2. Its occurrence in honey is limited to a few varieties, such as manuka honey and clover[310] and willow honey[311]. Furthermore, this aldehyde has been previously reported by Makowicz et al.[275] as a characteristic compound of unifloral ivy honey (at percentages between 1.27 and 1.48% of the total VOC fraction). This is in line with our study where, among the Irish honeys, myrtenal (0.12 ± 0.02%) was only detected in sample IH4, which is marketed as a blend of ivy and heather honey.

The third most abundant class of VOCs was that of alcohols, with 17 compounds in both MH and IH2. Within this class, cis-linalool oxide (4.1 ± 0.4%) and trans-linalool oxide (1.4 ± 0.3%) dominated the headspace in MH; the former was also identified in IH2 but at a significantly lower percentage (0.29 ± 0.09%), while the latter was absent. Interestingly,

cis-linalool oxide was one of the main components of the headspace of New Zealand unifloral honey according to Beitlich et al.[261], but while it only represented up to 1% of the total VOCs in manuka honey, its abundance in kanuka and jelly bush was remarkably higher (up to 45%). While 3,4,5-trimethyl-phenol was the main alcohol in IH2 ($3 \pm 1\%$), it only accounted for $0.72 \pm 0.02\%$ of the headspace fraction of MH, which is in line with the low percentages previously measured in manuka honey[261].

Despite previous reports of high contents of aromatic acids in manuka honey extractives[234,260] and the identification of possible specific markers such as 3-phenyllactic acid, 2-methoxybenzoic acid, and 4-hydroxyphenyllacetic acid[304], the class of carboxylic acids only accounted for a total $0.6 \pm 0.1\%$ of the headspace fraction of sample MH, with benzoic acid being the only aromatic. As already discussed in Section 2.3.5.2, this difference in the extracted compounds, possibly in part also due to a certain variability of the samples, can be ascribed to the employment of a different technique in this study, HS-SPME-GC/MS, as previously observed in the literature, with other studies on the headspace of manuka honey only reporting minor concentrations of carboxylic acids[310].

The VOC profile of manuka honey and heather honey in this study differed significantly in terms of the distributions of esters, which were over three times more represented in the volatile fraction of IH2 than in MH. The most abundant ester in MH was the methyl ester of 3,5-dimethoxybenzoic acid, with a percentage of $5.9 \pm 0.4\%$. This is in line with a previous report by Daher et al.[260], where the same compound was found in the SPME extractives of two manuka honey samples, accounting for 6.6 and 0.8% of the total aromatic compounds identified. A considerably higher percentage of methyl 3,5-dimethoxybenzoate (26.67% of the total VOCs identified) was measured in the headspace of manuka honey by Anand et al.[262], which proposed it as a non-specific marker for manuka honey. In the same study, this compound was also identified in two other honey varieties typical of Australia, *Agastache* and jarrah honey, at mean concentrations below 1.14 and 6.69%, respectively.

Finally, the major difference in the distribution of VOC between samples MH and IH2 resides in the diverse class, labelled in the present study as “other” for convenience, which includes different types of phytochemicals such as terpenes and heterocyclic

compounds like benzopyrans and benzofurans. Among these distinctive compounds, 2,3-dimethoxynaphthalene was one of the most abundant VOCs in MH, with a mean percentage of 4 ± 2% of the total volatile fraction. The first occurrence of 2,3-dimethoxynaphthalene was reported in honey, specifically in two samples of manuka honey (0.3 and 0.1% of the total VOC area), by Daher et al.[260]. Trans-edulan (2H-1-benzopyran, 3,5,6,8a-tetrahydro-2,5,5,8a-tetramethyl-, trans) was also highly concentrated in the manuka honey sample analysed in the current study, detected in MH at a percentage of 6.6 ± 0.9%. However, the reports of its presence in honey are scarce and, to the best of our knowledge, do not include manuka honey. Trans-edulan has been previously found in the headspace of honey samples of various botanical origins (pine, erica, thyme) at percentages between 0.01 and 0.21%, with similar concentrations of its isomer cis-edulan[312]. Among the terpene derivatives, the monoterpenes D-Limonene and α-pinene were identified in MH at percentages of 0.05 ± 0.01% and 0.15 ± 0.03%, respectively. A-pinene, previously detected at a high relative frequency in Mediterranean *Erica multiflora* honey (3.7%)[266], was detected in IH2 only at a percentage of 0.028 ± 0.002%. It is worth mentioning that a broad variety of cyclic sesquiterpenes (C_{15}-norisoprenoids) were identified in our sample of manuka honey, such as cadinene, cadalene, caryophyllene, cubenene, α-calacorene, α-corocalene and more (as shown in Appendix A, Table A-5), while these compounds were absent in IH2. Interestingly, in a study by Tan et al.[234], the extractives from the flowers and honey of *Leptospermum scoparium* were compared and it was highlighted how monoterpenes (C_{10}-compounds) such α-pinene and linalool, and sesquiterpenes like cadinene and caryophyllene, detected in the flower extractives, were not found in manuka honey. The absence of these compounds in manuka honey is in contrast with our study, but it can possibly be explained by the intrinsic differences in selectivity due to the employment of two different methods, with different VOCs extracted by the solvent technique[234] when compared to the honey headspace examined in the present study. These secondary metabolites, particularly sesquiterpenes, have also been reported to dominate the volatile emissions of manuka plants (foliage) and their essential oils[235,313]. Moreover, the proportion of selected sesquiterpenes and monoterpenoids has been used to discriminate various clones and Comvita cultivars of manuka plants[314], further

supporting their importance in the volatile profile of *Leptospermum scoparium*. Furthermore, variation in the emission of these compounds has been shown to provide valuable insights into the environmental conditions (temperature, herbivory, nitrogen and soil quality) affecting manuka plants[235]. Lastly, the emission of sesquiterpenes has been suggested to play an important role in the frequency at which pollinators visit the plant, mediating relevant signalling on the plant's physiological and health state, particularly in harsh environmental conditions[315]. These reports confirm the deep interconnection between the complexity of the volatile fraction of honey and the numerous direct and indirect (environmental) sources of VOCs, as already described in Section 2.1.1.

2.3.6 Final considerations on the characterisation of honey samples of different botanical origin

Collating the findings of the different, but complementary, analysis techniques, provides a unique, insight into the composition of the different honeys, shown in Table 2.4. As discussed above, through melissopalynological analysis, the botanical origin was confirmed only for one Irish sample (IH2) identified as true unifloral heather honey, and for manuka honey. A degree of variability in the profile was observed even within the Irish honeys marketed with the same honey variety, in part ascribable to the effect of environmental factors. Sample IH2 was most similar from a pollen perspective to IH3. However, in terms of TPC, it was most similar to IH1. While some previous studies have drawn correlations between colour and TPC, this is not confirmed here, where of the three honeys with the highest TPC, two (IH2 and MH) are dark amber while the other is light amber. In terms of VOC profiles, specific compounds, previously identified as chemical markers of botanical origin, were identified in the aroma profile of the samples. The headspace of manuka honey was dominated by the markers 2-methoxyacetophenone and 2'-hydroxyacetophenone, absent in the Irish honey samples, together with 2-methylbenzofuran. The three honey samples with *Calluna* spp. as a major pollen contributor were found to contain the isophorone, 2-hydroxyisophorone and 4-oxoisophorone compounds suggested as heather honey markers. However, the most similar honeys in terms of overall VOC profile (IH1 and IH3) were not the two honeys with the highest *Calluna* spp. contribution (IH2 and IH3). An

extensive study on the volatile composition of the Irish flora and corresponding honey varieties would be needed to evaluate to what extent the aroma of a unifloral honey is determined by the nectar of one specific plant and how the minor pollens are indicative of the contribution of other plants to honey's volatile fingerprint. With the support of statistical investigations applicable to bigger and more representative sample sizes, it could be elucidated whether the honey VOCs distribution correlates with the pollen frequency and how much the pollen distribution could be used to confidently predict the presence of chemical markers of botanical origin, or vice versa.

*Table 2.4 Summary of the most relevant characteristics of the four Irish honeys (IH1, IH2, IH3, and IH4) and manuka honey samples analysed in this study, illustrating the results of melissopalynological analysis for botanical origin determination, physicochemical analysis, total phenolic content and volatile fraction. *Percentage of expected pollen to confirm botanical origin from marketing label.*

	IH1	**IH2**	**IH3**	**IH4**	**MH**
Beekeeper-indicated floral origin	Heather (*Erica* and/or *Calluna* spp.)	Heather (*Erica* and/or *Calluna* spp.)	Heather (*Erica* and/or *Calluna* spp.)	Blend of Ivy (*Hedera* spp.) and Heather (*Erica and/or Calluna* spp.)	Manuka honey (*Leptospermum* spp.)
Pollen analysis*	17 ± 3% *Calluna* spp. pollen	73 ± 2% *Calluna* spp. pollen	40 ± 6% *Calluna* spp. pollen	6 ± 1% *Calluna* spp. pollen	71 ± 5% *Leptospermum* spp. pollen
Unifloral honey?	No	Yes	No	No	Yes
Total Phenolic Content (mg GAE/100 g)	94 ± 3	107 ± 4	80 ± 2	63 ± 2	95 ± 2
Colour (Pfund scale)	Dark Amber	Light Amber	Amber	Amber	Dark Amber
Acidity (pH)	4.42 ± 0.02	4.79 ± 0.02	4.52 ± 0.01	4.77 ± 0.01	4.22 ± 0.03
Chemical markers of botanical origin	Yes	Yes	Yes	No	Yes
Most common relative VOCs (with associated aroma descriptors[258])	Benzaldehyde (Burnt sugar, Almond, Woody)				
	Isophorone (woody, musty)	Isophorone (woody, musty)	Isophorone (woody, musty)	Ethyl benzoate (Chamomile flower, Celery, Fruity, Musty, Tea)	2-methoxy-acetophenone (Powdery, Anise, Almond, Phenolic)
	2-Hydroxy-isophorone	Ethyl phenylacetate (Sweet, Waxy, Fruity, Spicy, Cinnamon, Floral, Anise)	trans-β-Damascenone (sweet, apple-like, fruity aroma)	Thymol (Herbal, Thyme, Earthy)	Nonanal (Fruity, Floral, Waxy, Sweet, Melon, Soapy, Fatty, Lavender, Citrus fruit)

2.4 Conclusions

In this study, a screening of the composition of four samples of Irish honey, marketed as heather honey, was conducted and compared to that of manuka honey. The compliance with values established by official directives for moisture and sugar content was verified. While these parameters are regulated by international standards (aimed at preventing spoilage and ensuring the safety of consumers), honey's organoleptic properties result from the simultaneous influence of a multitude of diverse factors beyond these standard parameters. Firstly, the total content of phenolic compounds was quantified, showing that unifloral heather honey presented phenolic levels higher than MH here and reported values of the gold standard of medical-grade honey, manuka honey. The comparability of the TPC result of the Irish heather honey to manuka honey warrants further investigations into its antioxidant activity and potential benefits for biomedical applications. This study is, to the best of our knowledge, the first report on the volatile fraction of Irish heather honey and sets the foundation for a broader screening of the VOCs of Irish honey. All the honey samples are shown to have distinctive VOC profiles, both in terms of the different classes of VOC present and in terms of the individual compounds within each VOC class. The identification of markers from the volatile fraction is suggested here to be complementary to the melissopalynological analysis in supporting the assignment of the floral origin. As both the VOC profiles and physicochemical parameters impact the organoleptic and potential health benefits of honey, it is clear that they need to be considered in addition to melissopalynological analysis and TPC when evaluating honeys. Overall, a multifaceted characterisation is required to obtain a comprehensive honey profile of the potential contributory factors that impact honey's organoleptic properties and potential medicinal uses.

Chapter 3 Assessment of the antibacterial activity of four Irish honey samples, marketed as heather honey, when compared to manuka honey

This work was carried out by R. Angioi in collaboration with Dr. Linda Holland (School of Biotechnology, Dublin City University).

3.1 Introduction

3.1.1 Honey antibacterial properties: more than just manuka honey

Honey-based products for medical use containing honey defined as "medical-grade"[232,316] are commercially available, but across all these products, manuka honey is currently the primary honey variety used. It is the only variety to have a designated system to express its quality and its antibacterial effect, namely the Unique Manuka Factor (UMF™), which is based on the content of methylglyoxal (MGO)[108]. However, recent research indicates that neither the MGO content nor the "medical-grade honey" indication necessarily guarantees a net higher antibacterial activity. For example, when tested against 128 isolates, the antibacterial effect of the manuka honey samples did not reflect the performance expected based on their UMF™ (5+, 10+, and 15+), with the sample with higher UMF™ being the least active sample and the sample with UMF™ 5+ and 10+ being equally effective[112]. A similar lack of correlation between MGO content and antibacterial activity was observed in another study investigating the bactericidal activity of manuka honey against *Clostridioides difficile*[317]. Similarly, three commercially available medical-grade honeys were found to be less effective than honeydew, linden and acacia honey from Slovakia against *S. aureus* and *P. aeruginosa*[318]. These examples highlight how the beneficial biological effects of honey cannot be attributed to one single compound or denomination. This makes sense when one considers the complexity of the mechanism of the antibacterial effect of honey. Whilst honey has been widely used in medicine for centuries, the development of an international quality standard to comprehensively represent the biological value of all honey varieties and to inform the selection of honey safe and suitable for biomedical applications is of critical importance to continue its use in modern medicine[318,319].

Different features in honey's multifaceted composition, namely osmolarity, pH, glucose oxidase, hydrogen peroxide, phenolic compounds, MGO, and defensin-1 (as detailed in Chapter 1) have been identified to be responsible for the spectrum of inhibition against a diverse set of microbial targets[232,319,320]. For example, in a recent study by Anthimidou et al.[321], honey solutions were treated with the enzymes responsible for the inactivation of H_2O_2 and proteins (catalase and proteinase K, respectively) to assess if either were potential antibacterial agents in Greek and Cypriot honey samples. Interestingly, in this

study, it was observed how, in a honey blend from origan and clover, H_2O_2 played a dominant role in the growth inhibition of *S. aureus*, while peptides were responsible for the antibacterial activity against *P. aeruginosa*[321]. In a study by Bucekova et al.[318], several honey samples exerted an antibacterial effect comparable to that of artificial honey (a supersaturated sugar solution intended to mimic the composition of honey) against *S. aureus* and *P. aeruginosa*. This indicated that the osmotic effect was crucial to their antibacterial effect, with *P. aeruginosa* being particularly susceptible[318]. However, in other studies, no bacterial growth inhibition was exerted by artificial honey (often used as a negative control), suggesting that some other mechanisms apart from the sugar level must be responsible for the antibacterial activity of honey[302,316]. These examples demonstrate how different antibacterial mechanisms of action occur even in the same honey variety, with different susceptibility for different bacterial species. This suggests that different honey varieties could all represent valuable resources in treating infections of different natures and colonised by different microorganisms, based on the honey spectrum of action.

Therefore, despite the indubitable biomedical value of manuka honey, demonstrated by a vast body of research[292–294], several other honey varieties (buckwheat, heather, strawberry tree, and honeydew, to name a few) have been the focus of recent studies, and have been shown to offer promising performance in medical applications, comparable to that of manuka honey[52,232,319]. Moreover, promoting local excellence, while helping to unlock the full potential of all honey types, represents a more sustainable choice. Among the European varieties, heather honey (either *Calluna* or *Erica* sp.) has been attracting increasing interest for its biomedical properties, namely antifungal, antibacterial and antibiofilm activity[122,301,322], chemoprotective effect against mutagenic agents[323], and antioxidant activity[324]. Within the *Ericaceae* family, different parts of the plant *Calluna vulgaris,* commonly known as heather, and their extracts have been traditionally used in ethnopharmacology for their biological properties, such as anti-inflammatory, antioxidant, and antibacterial activity[325–331]. Moreover, *Calluna vulgaris* is a nectariferous plant, with its honey being highly appreciated by consumers and widely researched for its potential in medical applications[325]. For example, of twenty-eight honeys tested by Carnwath et al.[316], Scottish heather honey produced the strongest antibacterial effect against ten bacterial

isolates from equine wounds, performing even better than the medical-grade manuka honey products tested in the same study. Turkish heather honey has been shown to possess a comparable antibacterial activity to manuka honey against several strains, with higher growth inhibition induced against *Helicobacter pylori*[332]. Heather honey from Northern Ireland has been tested against community-acquired methicillin-resistant *S. aureus* (CA-MRSA), showing a remarkable inhibitory effect with no bacterial colonies detected after 24-hour incubation[333]. Heather honey has also been shown to have complementary efficacy to manuka honey in its effect to inhibit biofilm formation in different bacterial species[122]. In another study aimed at investigating planktonic growth of both single and mixed-biofilms of *C. tropicalis* and *P. aeruginosa,* both heather and manuka honey were comparably effective[123]. As the potential of *Calluna vulgaris* honey from many locations is being investigated globally, this chapter will assess the antibacterial activity of four samples of Irish honey, marketed as heather honey, against two common wound pathogens and compare their effect to that of a manuka honey sample.

3.1.2 A standard for medical-grade honey: microbial contamination in honey

Hermanns et al.[183] have suggested five critical requirements for honey to be indicated as "medical-grade", namely (1) organic and free of toxic contaminants, (2) produced and handled according to strict manufacturing practices, (3) sterile, (4) its use being implemented in clinical practice with validated operating procedures, and (5) with physicochemical features. While the study of chemical contaminants and beekeeping practices are of significant relevance for the quality of honey[334–340], they are beyond the scope of this thesis and will not be further discussed in this chapter, which will focus on the microbiological aspects of honey (contamination and antibacterial activity).

Honey can naturally support the growth of various microbial species, which has been attributed to physicochemical parameters such as moisture and pH[341–343]. The primary source of microbial contamination in honey is of an environmental nature, i.e., it originates from plants, and from the foraging bees and other pollinators (nectar, pollen, dust, soil, etc.)[342]. Furthermore, as for any food product, secondary contamination due to collection and handling (from equipment, food handlers, cross-contamination, etc.) may occur, though this is avoidable by adopting good manufacturing practices and

following the general principles of food hygiene[342,344] (Figure 3.1). While a certain level of contamination is safe for oral consumption of honey (except for infants under 12 months of age, due to the presence of spores of *Clostridium botulinum*)[345], it needs to be evaluated prior to medical applications of honey. The most common microorganisms detected in honey are yeasts and spore-forming bacteria that can survive unfavourable growth conditions such as honey's high sugar concentration and low acidity[342], with a higher variety of microorganisms described in multifloral honeys[346]. Interestingly, bacterial isolates from honey have been investigated for their production of antibacterial compounds, namely peptides and bacteriocins, and have been shown to act as natural antibacterial agents against foodborne pathogens[346–348]. However, a thorough discussion on the identification of microbial contaminants found in honey is beyond the scope of this thesis; therefore, for a comprehensive dissertation on the microorganisms identified in honey, the readers are referred to the exhaustive reviews by Silva et al.[341] and Snowdon et al.[342].

In the context of investigating honey as a potential treatment for infected wounds, it is critical to investigate both the extent and the potential impact of microbial contamination, due to the need to ensure the safety of treated patients. It has been proposed that the microorganisms contaminating honey could play a role in the composite antibacterial activity of this bee product. However, an aseptic technique is usually considered essential to the management of wounds[349,350]; and raw honey is not automatically sterile. Therefore, to obtain an adequate standard of sterility for medical-grade honey, different methods of sterilisation of honey have been evaluated in the literature. Gamma irradiation is unanimously considered the preferred method and has been shown to preserve honey's composition and biological properties[183,189,351,352]. Due to cost and availability, however, it is not always available. Ozone treatment has been patented as an alternative method to sterilise unheated raw honey, with the capacity to reduce microbial counts in honey to 100 colony-forming units (CFU)/g or less[183,353]. UV treatment has also recently been explored as an option to reduce the microbial load in honey, but further studies are required to fully understand the advantages and limitations of this method[354–356]. The focus of this thesis is the investigation of Irish heather honey for application on infected wounds. Therefore, in line with aseptic clinical practice, and with the proposed standards for medical-grade honey[183,318], the microbial

contamination of the honey samples was investigated. Furthermore, for samples found to be contaminated by microbial species, sterilisation was carried out. Due to its reported use in the literature[53,99,297,357], and to being an economical and easily accessible method, filter sterilisation has been chosen as the preferred method of sterilisation in this chapter.

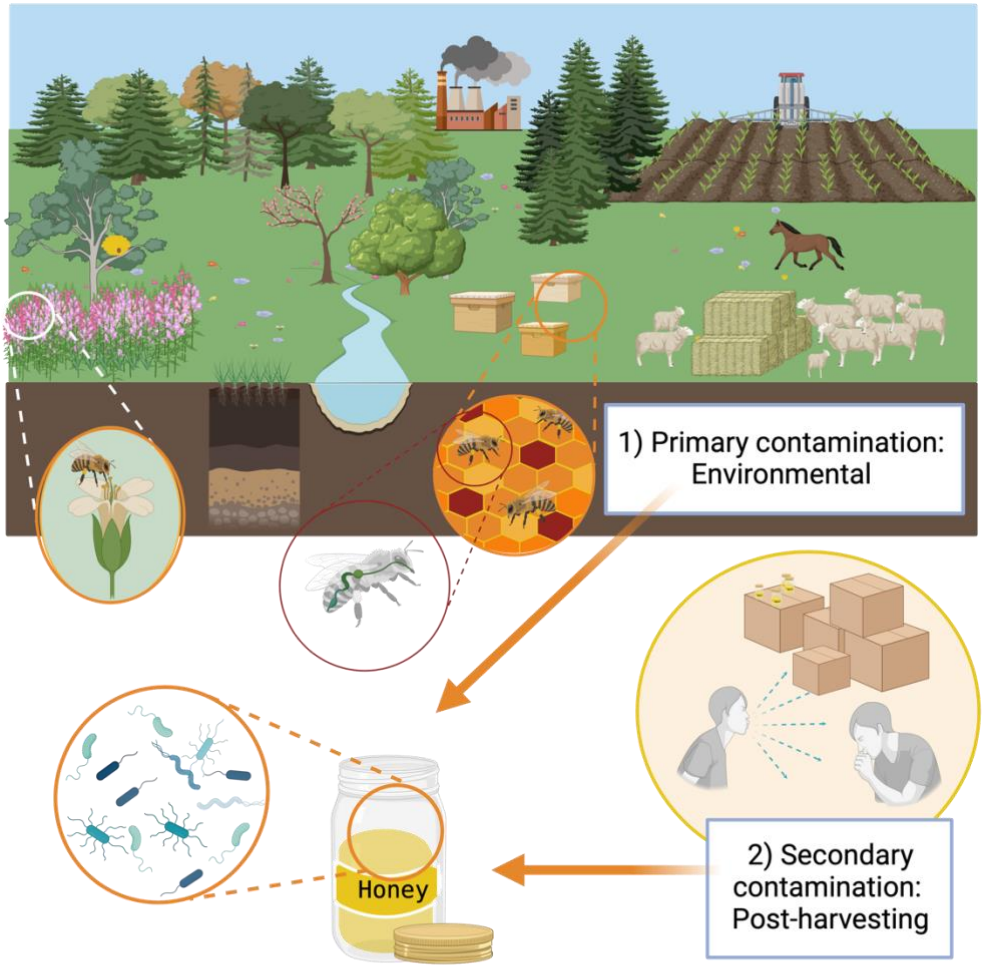


Figure 3.1 Microorganisms in honey. The sources of microbial contamination in honey are 1) environmental, originating from nectar, pollen, water, air, beehive, bees and their digestive system; 2) due to poor manufacturing practices during collection and handling, such as cross-contamination, contaminated equipment, food handlers (coughing and sneezing). Created with Biorender.com.

3.1.3 Infection-associated bacterial Volatile Organic Compounds (VOCs)

Wound infections represent a social and economic burden, contributing to delayed recovery and chronicisation of wounds, with increased hospitalisation, higher amputation rates and higher mortality[152]. Preventative measures, such as wound hygiene and appropriate dressing selection, are key to avoiding bacterial colonisation of the wound and are pivotal to successful healing outcomes[158,358,359]. When the recommended prophylactic practices are not implemented (and sometimes despite it), wound infections can occur. While redness, pain, heat and oedema (due to the host inflammatory response), and also malodour, exudate thickness and colour, and purulence, are still considered the primary clinical indicators of infections, the importance of more subtle, often overlooked signs has more recently been acknowledged as integral to early diagnosis of infection[156,360]. For example, wound enlargement and bridging of the wound edges (such as in tissue laceration), could be used, in conjunction with classic, more evident signs, to identify infections and prevent, with early antibacterial measures, delayed wound healing[361]. Based on these clinical observations, empirical prescription of broad-spectrum antibiotics (without suitable microbial susceptibility tests) is a common practice but poses a serious risk, particularly in vulnerable patients[362,363]. The empirical prescription often results in inappropriate and indiscriminate antibiotic usage, which represents one of the root causes of the global emergency of antimicrobial resistance, as highlighted by the World Health Organisation and European Commission, calling for antibiotic stewardship measures and improved techniques for the diagnosis of infections[160–162]. Therefore, the design of state-of-the-art diagnostic tools for real-time detection of infections and discrimination of the microbial species involved has been suggested as an alternative to conventional time-consuming laboratory methods (plate cultures, Gram-staining, biopsies, antibiogram, etc.)[364]. A rapid non-invasive assessment of infections represents a valuable resource in improving the precision in prescribing antibiotics and tackling the antimicrobial resistance challenge, and the need for it is reflected in the interest in the research field, as shown in comprehensive reviews[365–367] and a designated database of microbial VOCs[368]. As a result of their characteristic metabolism, bacteria emit VOCs (more or less specific to bacteria only)[367], which enables great potential for diagnostic research. For example, breath analysis has drawn significant attention due to the ease

of sampling directly from the exhaled air, with breath tests already a standard procedure to diagnose *Helicobacter pylori* infections, but also widely researched in pneumology, for the diagnosis of airway infections[369,370], and in dentistry, for example, in the case of halitosis and dental abscesses[371].

Foul odour represents one of the most unsettling and discomforting symptoms of wounds, often inducing patients to social isolation. Malodour has been empirically used to diagnose wound infections and to monitor wound healing progression for centuries[372], but its use has been more recently implemented with the development of instrumental methods and the design of electronic systems such as e-noses and wearable devices for point-of-care diagnosis with bedside clinical applicability[373–377]. Furthermore, similar methods based on volatile emissions have found application in clinical studies where they have been shown to be able to discriminate compounds indicative of skin lesions when compared with VOCs isolated from healthy skin areas on the same patient[378–381]. However, the chemical environment in a wound could be highly complex, including physiological skin volatiles from the intact surrounding areas, wound VOCs not derived from the infections, and the indoor environment (as in a hospital room). As such, the background volatilome of the host needs to be evaluated accounting for its physiological microbiome, and how these are affected by age, sex, metabolic and hormonal state, comorbidities, and medications (in particular, antibiotics previously prescribed for another infection), etc[367,382]. Similarly, in the research laboratory practice, considerably big variations in volatile emission have been observed due to experimental growth conditions[376], with parameters that could affect VOC emissions often overlooked in the literature, such as culture media, growth phase, and genomic variations in bacterial strains[367]. Therefore, in the developmental phase of a new method, it is important to match the VOC data with the results of conventional methods (microbiology swab, wound biopsy, plate culturing, antibiotic sensitivity tests, etc.) to confirm the observations and build a reference database[378]. To this aim, in this chapter, the VOC data were analysed together with the information from the growth curve study, to enable monitoring of variations in metabolite emission over time during the bacterial growth. Furthermore, VOC data and growth curves were both evaluated to assess bacterial growth inhibition in the presence of honey. Some reports available in the literature employ bacterial VOC variations to monitor microbial response to therapy,

with VOC reduction observed, in a dose-dependent fashion, upon antibiotic treatment[367,383,384]. However, to the best of our knowledge, this is the first report of bacterial VOC emissions being monitored to evaluate the susceptibility of the examined bacteria to the honey treatment.

While the detection of microbial VOC *in vitro* has not been shown to be directly translatable to standardised diagnostic methods in clinical practice yet, building a library of characteristic microbial VOCs is a crucial step forward to fulfil the aim of developing robust and reproducible yet rapid and non-invasive diagnostic tools to better patients' quality of care. Therefore, this chapter presents a proof-of-concept study which has potential in the interdisciplinary application of analytical chemistry and microbiology to monitor the progression of wound infections.

3.1.4 Chapter's aims

The aims of this chapter are:

- Preliminary assessment of the degree of contamination of the honey samples;
- Evaluation of the antibacterial activity of the honey samples;
- Identification of the bacterial volatile emissions of *S. aureus* and *E. coli* during different growth phases over an observation time of 24 h;
- Identification of significant switches in the volatilome of bacteria due to the honey-induced growth inhibition.

3.2 Materials and methods

3.2.1 Materials

Phosphate Buffered Saline (PBS, tablets) was purchased from Merck Life Science Limited (Ireland). Trypticase Soy Agar (TSA), Saboured Dextrose Agar (SDA), and Trypticase Soy Broth (TSB) were purchased from Fisher Scientific (Ireland). Nunc™ MicroWell™ 96-Well, Nunclon Delta-Treated, Flat-Bottom Microplate were purchased from Thermo fisher, Denmark.

3.2.2 Instrumentation

Sample handling and preparation were performed in an NU-425-300E Class II biosafety cabinet (NuAire Lab Equipment, Plymouth, USA). A MIR-153 incubator (Sanyo Electric Co., Ltd.) and an Excella E24 Incubator Shaker (New Brunswick Scientific, Eppendorf AG, Germany) were used to incubate samples at a controlled temperature of 37 °C. A UV-3100PC Spectrophotometer (VWR International LLC., Avantor) was used to measure the OD600 of liquid bacterial cultures. A Tecan Infinite 200 plate reader paired with the Tecan i-control 2.0.10.0 software (Tecan Trading AG, Switzerland) was used to read the absorbance of samples in 96-well plates.

3.2.3 Honey samples

Five honey samples were analysed in this project, as outlined in Chapter 2, Section 2.2.2 (see Table 2.1). For the experiments carried out in this chapter, a new jar (same batch, and same production and purchase year, namely 2021) was used, which was opened exclusively in the Class II biosafety cabinet to avoid contamination.

3.2.4 Microbial contamination assessment

The microbial contamination of the honey samples was assessed according to the methods from Gomes et al.[385], with some modifications. Honey solutions were prepared by dissolving 1 g of honey in 9 mL of PBS and decimal dilutions were prepared in the same solvents from each of these stock solutions. A screening of the microbial contamination was carried out by plating the honey solutions in TSA and incubating them for 48 h at 37 °C[50]. Moulds and yeast were quantified in SDA with the addition of ampicillin (80 μg/mL) and amoxicillin (50 μg/mL) to inhibit the growth of bacteria (incubation at 30 °C for 7 days). Negative controls were carried out with sterile PBS. The experiments were conducted in triplicate and results were expressed as CFU per gram of honey (CFU/g), calculated as follows:

$$\frac{CFU}{g} = \frac{No.of\ colonies}{V} * DF \quad (3.1)$$

where

- V is the volume (in mL) of the honey solution spread on the agar plate

- DF is the dilution factor, calculated as the ratio of the volume of the initial stock solution (V_1) to the volume of the dilute solution (V_2), i.e., V_1/V_2.

3.2.5 Honey antimicrobial properties

3.2.5.1 Bacterial strains studied

Two bacterial strains were studied: *S. aureus* (DSM799) and *E. coli* (DSM30083). All bacterial isolates were obtained from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH. Each strain was streaked individually on TSA plates and incubated at 37 °C overnight. Overnight liquid cultures were prepared in 5 mL of TSB and kept overnight in a shaking incubator (37 °C, 180 rpm).

3.2.5.2 Determination of honey's Minimum Inhibitory Concentration (MIC) by broth microdilution method

The Minimum Inhibitory Concentration (MIC), defined as the lowest concentration of an antimicrobial agent that exerts 100% growth inhibition of microbial cultures, was determined by the broth microdilution method as described by Anthimidou et al.[321] with slight modifications (Figure 3.2). Solutions of the honey samples in TSB were prepared by a twofold dilution starting from a 50% stock solution (range of honey concentrations: 50-0.78% w/v). The solutions of the samples found to be contaminated by microbial species (see Section 3.2.4) were filter-sterilised (pore size 0.2 μm) to avoid proliferation of contaminant species present in the honey during incubation. Based on OD600, *S. aureus* overnight cultures grown in TSB were adjusted to an OD1 of 10^8 CFU/mL. A volume of 10 μL of the bacterial culture was added to 190 μL of honey solution, obtaining a final bacterial concentration of approximately 10^7 CFU/mL. A positive control was performed with the antibiotic oxacillin and amoxicillin, for *S. aureus* and *E. coli*, respectively. Negative controls consisted of the untreated honey solutions at the same concentration as the inoculated honey samples (Control 1, or C1), TSB only (C2) and inoculated TSB (10 μL of bacterial culture in 190 μL of TSB; C3). The OD was measured at 600 nm[297] after 24 h of incubation at 37 °C in static incubator (without shaking).

To evaluate the inhibition exerted by each honey sample, firstly, the OD of each untreated honey solution (C1) was subtracted from the OD of each inoculated sample at the same concentration. Then, the growth inhibition obtained with each honey concentration was calculated using the following equation[321]:

$$\%Inhibition = \left(1 - \frac{OD_X}{OD_C}\right) 100 \quad (3.2)$$

where OD_x was the OD of the inoculated samples and OD_c was the OD of C3.

The results are reported as the average value of six replicates (two technical replicates for each of three biological replicates).

3.2.5.3 Minimum Bactericidal Concentration (MBC)

The minimum bactericidal concentration (MBC) was determined as in Figure 3.2 by sampling from the 96-well plate used for the broth microdilution assay using a VP407AH multi-blot replicator (V&P Scientific, Inc., San Diego. USA). The replicator was gently pressed on a TSA plate which was then incubated at 37 °C for 24 h[302]. The MBC was then defined as the lowest concentration of honey that allowed no bacterial growth.

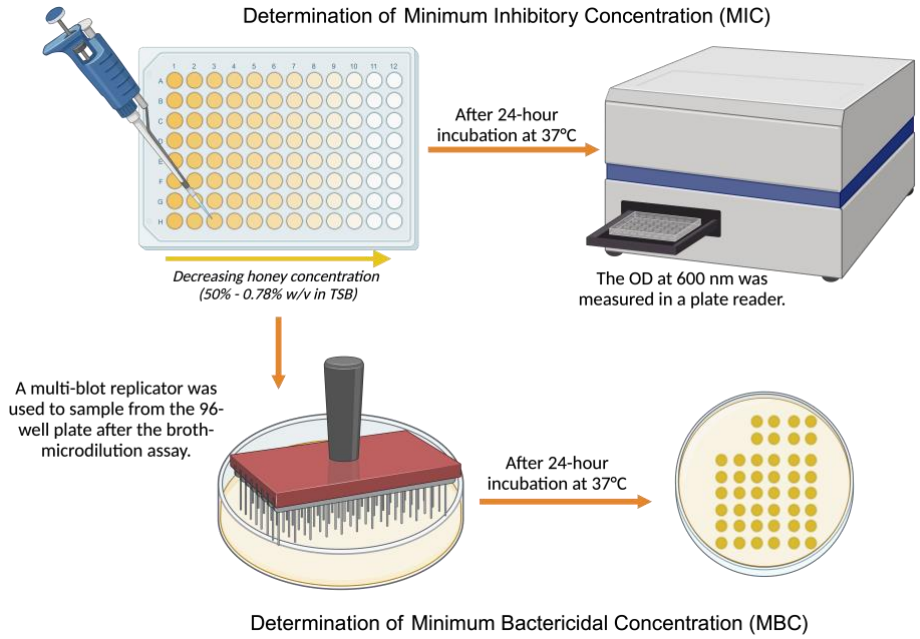


Figure 3.2 Methods of evaluation of the antibacterial properties of honey expressed as Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC), respectively top and bottom of the illustration. The MIC is assessed with the broth microdilution method, with honey solutions prepared in TSB by twofold dilutions starting from a 50% stock solution (range of honey concentrations: 50-0.78% w/v). The MBC was determined by sampling the 96-well plate using a multi-blot replicator which was then gently pressed on a TSA plate. Created in BioRender.com.

3.2.6 Bacterial volatile sampling and identification

3.2.6.1 Sample preparation and VOCs extraction by Headspace – Solid Phase Micro Extraction (HS-SPME)

Based on OD600, the overnight cultures of *S. aureus* and *E. coli* were adjusted to an OD1 of approximately 10^8 CFU/mL (Figure 3.3, Part 1). Overnight cultures were then diluted in TSB to a final cell count of approximately 10^7 CFU/mL in a final volume of 5 mL in 20 mL headspace vials which were then sealed with silicone-polytetrafluoroethylene septa mounted on screw caps (Supelco Co., Bellefonte, PA, USA). The extraction of the volatile compounds was carried out at predefined regular intervals (1, 2, 4, 6, 8, and 24 h) during an incubation time of 24 h (37 °C, 180 rpm). The extraction was performed by temporarily transferring the vials to a standard incubator at 37 °C and exposing a divinylbenzene–carboxen–polydimethylsiloxane (DVB–CAR–PDMS) SPME fibre assembly (Supelco Co., Bellefonte, PA, USA) to the headspace of the bacterial liquid cultures (Figure 3.3, Part 2). After a sampling time of 15 mins, the fibre was retracted and the SPME assembly was removed from the vial. A control measurement was performed by exposing the SPME fibre to the headspace of the TSB media under the same conditions described above. The fibre was conditioned according to the manufacturer's instructions (270 °C, 30 mins) daily prior to the analysis.

In order to evaluate the effect of bacterial growth on the VOC emission, a growth curve study was performed according to the method by Fitzgerald et al. with some modifications[386]. The OD600 was measured at the same intervals of the VOC sampling (1, 2, 4, 6, 8, and 24 h), and OD values were expressed as the average value of six replicates (two technical replicates for each of three biological replicates) ± standard deviation.

3.2.6.2 Effect of honey on bacterial growth and volatile emissions

In order to assess to what extent honey could affect bacterial growth and the emission of bacterial signature volatile compounds, the growth study and VOCs study were performed under the same conditions detailed in Section 3.2.6.1, replacing the TSB media with a 50% (w/v) honey solution of samples IH2 (unifloral Irish heather honey) and MH (manuka honey) in TSB. A volume of 250 μL of the bacterial culture (adjusted

to an OD1 of approximately 10^8 CFU/mL) was added to 4750 µL of honey solution, obtaining a final bacterial concentration of approximately 10^7 CFU/mL in a volume of 5 mL (in 20 mL headspace vials). Control measurements were performed by exposing the SPME fibre to the headspace of a 50% (w/v) honey solution (in TSB).

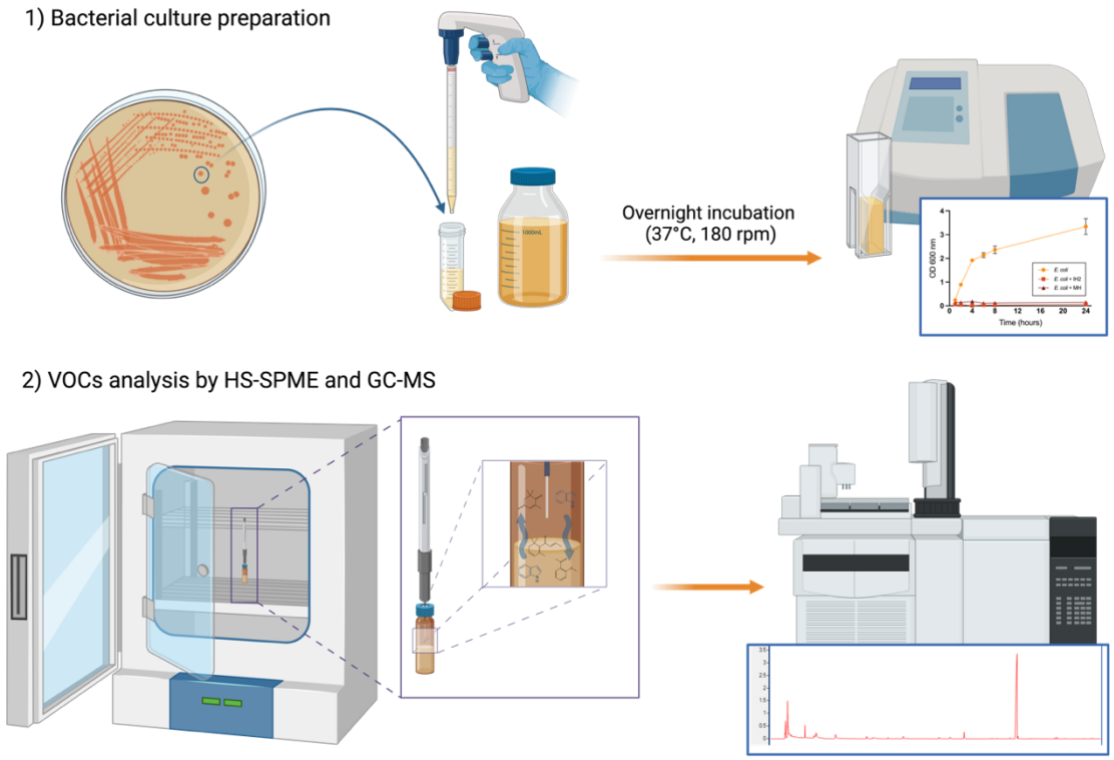


Figure 3.3 Method of bacterial volatile sampling and identification. 1) Bacterial culture preparation and growth curve study based on optical density. 2) VOC extraction by Headspace – Solid Phase Micro Extraction (HS-SPME) followed by Gas Chromatography – Mass Spectrometry (GC-MS). The detailed procedure is described in Section 3.2.6. Created in BioRender.com.

3.2.6.3 Gas Chromatography – Mass Spectrometry

The samples' volatile fraction was analysed using an Agilent gas chromatograph model 7820A coupled with a mass selective detector model 5977B (Agilent Technologies, Inc., Santa Clara, CA, United States). VOCs were desorbed by inserting the fibre assembly in the injector of the gas chromatograph and exposing the fibre for 5 mins (inlet temperature: 250 °C), splitless mode. The oven temperature program was as follows: the initial temperature of 40 °C was held for 4 mins, then the temperature was increased up to 42 °C at a rate of 0.5 °C/min, then further increased at a rate of 3 °C/min up to 75 °C, 4 °C/min up to 110 °C, 5 °C/min up to 130 °C, and 7 °C/min up to the final temperature of 200 °C, for a total run time of 42 mins. Helium was employed as a gas carrier at a flow

of 1 mL/min. The mass detector was operated in the electron impact ionisation mode at 70 eV, the mass scan range was *m/z* 30–400, and the ion source temperature was 230 °C. A SLB®-5ms non-polar capillary column (silphenylene polymer, virtually equivalent in polarity to poly(5% diphenyl/95% dimethylsiloxane) phase; L × I.D. = 30 m × 0.25 mm, df 0.25 µm) was employed for peak separation. HS-SPME followed by GC-MS was repeated four times for each honey sample.

3.2.6.4 Compound identification and interpretation of GC-MS spectra

The compound identification was carried out according to the method described in Chapter 2, Section 2.2.6.3, with some modifications. GC-MS data were analysed using Agilent MassHunter Acquisition Data Qualitative Analysis version 10.0 (Agilent Technologies). Compounds were identified using a chromatogram deconvolution algorithm, with an absolute area filter greater or equal to 5000 counts, a relative area filter of 1% of the largest peak, an absolute height of 500 counts and a relative height of 2% of the largest peak. The obtained spectra were searched and compared with reference spectra using the NIST Mass Spectral Search Program for the NIST23 3.0 Mass Spectral Library. A database match score of at least 700 was deemed sufficient to accept the identification. The compounds identified for each of the samples were then cross-matched within the four replicates, and compounds detected in at least 75% of the replicates were included in the data sets for further analysis. The retention times (RT, mins) of standard n-alkanes (C7–C30) were used to calculate the experimental retention index (RI) using the Van den Dool and Kratz formula (see Chapter 2, Section 2.2.6.3, Equation 2.5)[256]. Finally, the calculated RI were compared with the RI reported in the NIST Chemistry WebBook - Standard Reference Database Number 69[257], for non-polar columns equivalent to the column described in Section 3.2.6.3. The identification was ultimately confirmed for those compounds for which the difference between experimental RI and the RI reported in the databases was less than 12 units (RI_{diff} <12)[259].

3.2.7 Statistical analysis

The statistical analysis was carried out in Prism Version 10.0.3 (GraphPad Software, Inc.). Unpaired t-test (two-tailed) and one-way analysis of variance ANOVA were used for comparisons between two groups and three groups or more, respectively. The post-hoc

analysis was performed using Tukey's, Bonferroni, and Dunnett's methods for multiple comparisons, with 0.05 as the alpha threshold (95% confidence interval). The level of significance in statistical analysis is coded as follows: nonsignificant $p > 0.05$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$. In the graphs used in this chapter, only statistically significant differences are displayed.

3.3 Results and discussion

This chapter investigates the microbiology of the honey samples, i.e. the extent of microbial contamination and the antibacterial properties of the samples in comparison to manuka honey against *S. aureus* and *E. coli*, with the quantification of MIC and MBC. A new approach is tested to evaluate honey's antibacterial activity by tracking bacterial volatile metabolites.

3.3.1 Evaluation of the degree of microbial contamination in the honey samples

Of the samples under evaluation in this study, three (IH1, IH2 and MH) did not show any contamination in either medium, TSA for general screening of the microbial load, or SDA for the quantification of moulds and yeasts. Samples IH3 and IH4 had a growth of 166 and 66 CFU/g in TSA, and 100 and 166 CFU/g in SDA, respectively (Table 3.1).

Table 3.1 Microbial contamination (expressed as Colony Forming Unit per gram of honey as per Equation 3.1) in the Irish honey samples (IH1, IH2, IH3 and IH4) and manuka honey (MH). The honey samples were diluted to an initial concentration of 10% w/v in PBS, then serial decimal dilutions were prepared and plated in tryptone soy agar (TSA - incubation at 37 °C for 48h) and Saboured Dextrose Agar (SDA - incubation at 30 °C for 7 days). The experiments were conducted in triplicates. Negative controls were carried out with sterile PBS.

	Honey samples				
CFU/g	**IH1**	**IH2**	**IH3**	**IH4**	**MH**
Microbial load (TSA)	-	-	166	66	-
Moulds and yeasts (SDA)	-	-	100	166	-

A high degree of variability has been reported in the contamination level of honey (measured based on the total plate counts)[342] and there is a lack of official international standards defining acceptable values. Hermanns et al.[183] suggested 10 bacterial CFU and

10 fungal and mould CFU as the maximum microbial contamination level acceptable in honey to be destined for medical use. The MERCOSUR (Southern Common Market) commission established a higher limit of 1.0 x 10^2 CFU/g[387]. Kanavagh et al.[50] reported that Irish honey samples, stored in a refrigerator at 4 °C until analysis, showed negligible microbial contamination, with growth below 4 CFU/g of honey for aerobic mesophiles, moulds and yeasts. Similar microbial contamination has been observed for honey samples of different botanical origins from Portugal (below 10 CFU/g for aerobic mesophiles and up to 2.2 x 10^1 ± 2.89 CFU/g for moulds and yeasts)[385] and for Eucalyptus honey from Ecuador (below 10 CFU/g)[388]. Higher degrees of microbial contamination have been measured in Portuguese heather honey (*Erica* sp.) by Estevinho et al. (1.3× 10^2 ± 7.5 × 10^1 CFU/g of aerobic mesophiles and 5.5 CFU/g of moulds and yeasts)[389] and by Feás et al. (2.0 x 10^2 ± 1.0 x 10^2 CFU/g of aerobic mesophiles and 1.3 x 10 ± 2.5 CFU/g of moulds and yeasts)[390], in line with the contamination observed in this thesis. These values are lower than those measured for Argentinian multifloral honey with up to 10^3 CFU/g, with average CFU/g values between 244 and 500 CFU/g, and moulds and yeasts level up to 470 CFU/g[391]. Even higher microbial contaminations were measured by Migdał et al.[351], with values of up to 460000 CFU of aerobic bacteria and up to 900 CFU of yeasts and moulds per gram of honey. Exposure of the honey samples to higher storage temperatures has been shown to promote microbial growth, as reported by Kavanagh et al.[50] for honey samples that were kept at 27 °C for a week before analysis. Nonetheless, low heat treatment (45 °C) for up to 120 mins has been shown to significantly reduce the microbial activity in honey due to the moisture reduction induced[343]. However, no significant correlation was observed between pH, moisture and phenolic content and the microbial contamination measured in Irish honey samples, regardless of the storage conditions[50]. The high variability in honey microbial contamination calls once again for the establishment of international quality standards stating acceptable levels for both food consumption and medical applications of honey. This could ensure the safety of consumers and patients and build a solid sense of trust, both in healthcare practitioners and in patients, in the use of honey for the treatment of infections in medical practice.

The identification of the microbial species observed in the total plate count is beyond the scope of this study and has not been pursued. Instead, the focus of this chapter was

the evaluation of honey's antibacterial activity against common wound pathogens, namely *S. aureus* and *E. coli*. Therefore, in order to fully appreciate the inhibitory effect of honey against the bacteria, samples IH3 and IH4, which showed microbial growth, were filter-sterilised prior to the assessment of their antibacterial properties in the assays below.

3.3.2 Assessment of honey's antibacterial activity against *S. aureus* and *E. coli*

3.3.2.1 Determination of the Minimum Inhibitory Concentration (MIC) of honey necessary to induce an antibacterial effect

All the honey samples at a concentration of 50% (w/v) produced a notable bacterial growth inhibition (of at least 93%) against *S. aureus* (Figure 3.4). Manuka honey induced a statistically higher inhibition also at lower concentrations (25 and 12.5%, w/v) when compared to the Irish honey samples, while for concentrations lower than 12.5% (w/v), the inhibition drastically decreased to 26% or less (Figure 3.4, b). No linear trend based on the concentration of the honey solutions emerged at concentrations below 25% (w/v) for any of the honey samples, as previously observed by other authors[302]. For concentrations ranging from 25 to 0.78% (w/v), the degree of inhibition ranged between 19 and 45%.

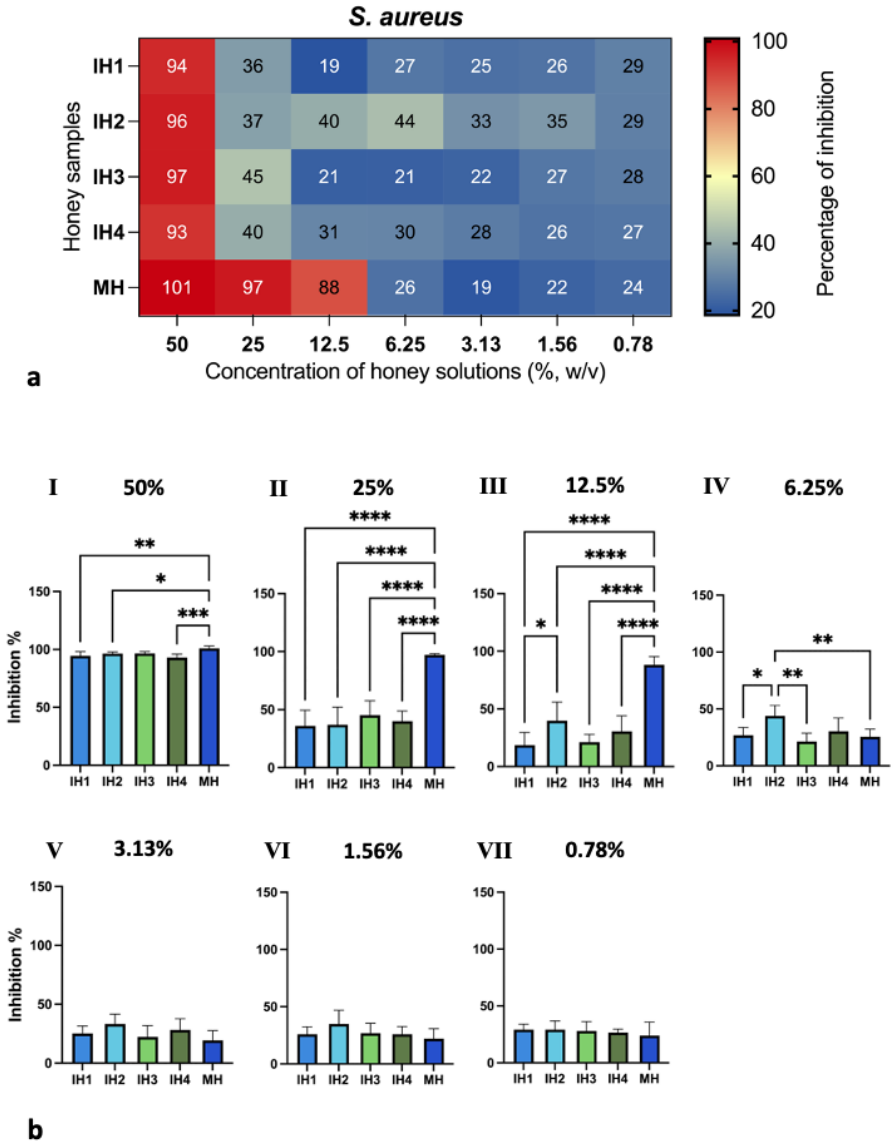


Figure 3.4 Summary of the results of the broth microdilution method to determine the Minimum Inhibitory Concentration (MIC) of the Irish honey (samples IH1, IH2, IH3 and IH4) and manuka honey (MH) against S. aureus. The honey solutions, prepared in TSB, were tested at the range of concentrations of 50-0.78% w/v. The honey-induced bacterial growth inhibition is expressed as a relative percentage per Equation 3.2. The values, shown in the form of a heatmap (a) and bar charts (b), are the average of six replicates (two technical replicates for each of the three biological replicates). Statistically significant differences between the honey samples at each given concentration are displayed in b), with standard deviations shown as error bars.

E. coli showed a higher susceptibility to the honey treatment than *S. aureus,* with percentages of inhibition above 98 and 80% for honey concentrations of 50 and 25% (w/v), respectively (Figure 3.5). At a honey concentration of 12.5% (w/v), manuka honey still induced growth inhibition of 77% against *E. coli*, while the antibacterial activity of the Irish honey samples decreased to 52-56%. For both honey concentrations of 50 and 25% (w/v), a statistically significant difference was observed between manuka honey

and each of the Irish samples (Figure 3.5). At concentrations of 6.25% (w/v) and lower, the antibacterial activity was comparable (between 50 and 41%) for both Irish honey and manuka honey. For both *S. aureus* and *E. coli,* full growth inhibition was achieved in the positive control, indicating fully sensitivity of the bacteria to the antibiotics.

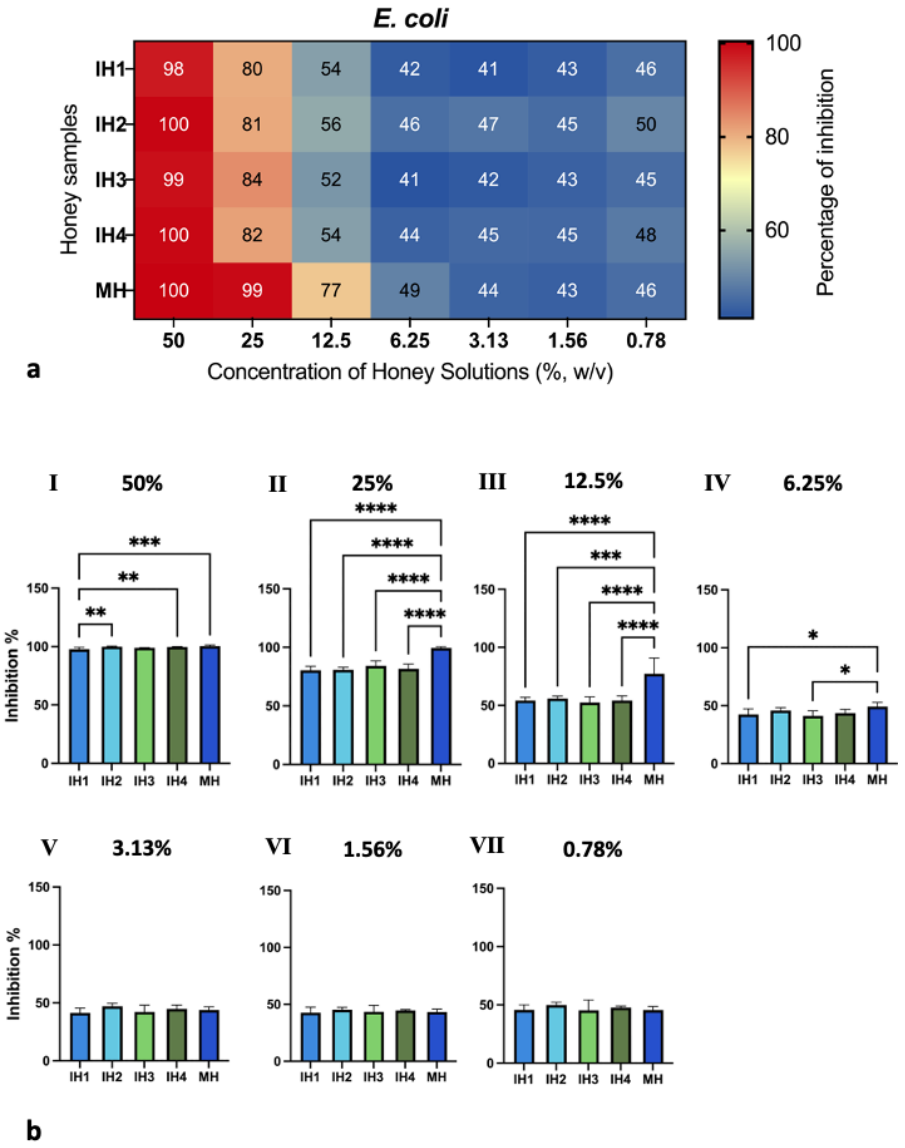


Figure 3.5 Summary of the results of the broth microdilution method to determine the Minimum Inhibitory Concentration (MIC) of the Irish honey (samples IH1, IH2, IH3 and IH4) and manuka honey (MH) against E. coli. The honey solutions, prepared in TSB, were tested at the range of concentrations of 50-0.78% w/v. The honey-induced bacterial growth inhibition is expressed as a relative percentage per Equation 3.2. The values, shown in the form of a heatmap (a) and bar charts (b), are the average of six replicates (two technical replicates for each of the three biological replicates). Statistically significant differences between the honey samples at each given concentration are displayed in b), with standard deviations shown as error bars.

Considerably lower MIC values for Portuguese heather honey (*Calluna vulgaris*) were measured by Dezmirean et al.[300], with honey concentrations between 2.1 and 7.4% needed to inhibit different bacterial strains. Similarly, Scottish heather honey had a MIC between >2% and 6% (v/v) against ten common bacterial equine wound pathogens, whereas the average MIC range of the other samples tested in the same study was between >2 and 16% (v/v), including medical-grade manuka honey[316]. A broader range of MIC values (between 4.69 and 18.75%, w/v) was measured for ten heather honey samples of unspecified geographical origin by Osés et al.[392]. The MIC values measured here for Irish honey are higher than the average values of 28.6 and 19.2% quantified by Bucekova et al.[318] for commercial honeys from supermarkets and local shops in Slovakia, respectively, against *S. aureus*. A broad range of MIC_{90} (honey concentration capable of inducing a 90% bacterial growth inhibition) was observed by Zhang et al.[302] for twelve Chinese honey varieties analysed, namely between 2.5 and 25% (w/v) against *S. aureus* and between 3.1 and >50% (w/v) against *E. coli.* Many reports in the literature have shown that the antibacterial effect of manuka honey was comparable to that of other honey varieties. For example, Polish honeydew honey was comparably more effective than manuka honey against Gram-positive bacteria (*S. aureus* and *E. faecalis*) and, at generally higher concentrations, against Gram-negative bacteria (*P. aeruginosa* and various *Salmonella* strains) in both agar diffusion test and broth microdilution assay. However, in the same study, other honey varieties such as buckwheat, linden and multifloral, showed limited inhibition of bacterial growth *in vitro*[298]. Conversely, Deng et al.[297] reported that Chinese buckwheat honey exerted a higher antibacterial effect than manuka honey in the agar well diffusion assay, with a steady increase in inhibition of the growth of *S. aureus* colonies with a progressive increase in the honey solution concentration. However, for both manuka and buckwheat honey, solutions of at least 70% (w/v) were necessary to inhibit *P. aeruginosa.* Similarly to these two studies, higher susceptibility of Gram-positive bacteria to the honey treatment than Gram-negative bacteria has been observed in other research[302,321,388,393]. Nonetheless, in the case of Rubus honey, higher MICs were needed to inhibit Gram-positive bacteria such as *S. aureus* and *S. epidermidis*[394]. Similarly, all the samples evaluated in this thesis have shown higher antibacterial activity against the Gram-negative *E. coli* than the Gram-positive *S. aureus*. It is important to highlight that the significant differences in MIC

values observed in the literature could, at least partially, be ascribed to experimental conditions, such as the different media used (for example, Mueller Hilton, antibiotic medium broth, Lysogeny broth, or nutrient broth – Oxoid), plate incubation conditions, and the initial bacterial load[297,298,300,302,392]. Finally, the technique used to determine the inhibition, either visually based on the turbidity of solution[298,300,316,392] or by instrumental quantification[297], is crucial to an accurate evaluation of honey's antibacterial activity.

Another widely used method to assess honey's antibacterial properties *in vitro* is the agar well diffusion test. However, various research has highlighted a different antibacterial activity when the MIC was determined as opposed to when honey was tested in the agar well diffusion (when comparing the same honey sample against the same bacterial strain). For example, in a study by Anthimidou et al.[321], Greek heather honey produced no zone of inhibition in the agar culture of *P. aeruginosa*, while a MIC of 12.5% (v/v) (equivalent to manuka honey) was measured by spectrophotometric assay against the same bacteria. Similarly, in a study by Deng et al.[297], a MIC of 22.5% was quantified by broth dilution method for both manuka and buckwheat honey against *S. aureus* and *P. aeruginosa.* Nonetheless, this result was not confirmed in the agar well diffusion tests, where the two honey varieties differed significantly in their degree of inhibition. This method presents several limitations, such as the impaired diffusion of bioactive macromolecules through the agar[302,320,395], which could in turn possibly be due to a different viscosity of the honey varieties[320]. Therefore, due to the abovementioned limitations, this assay was not adopted for the evaluation of the antibacterial activity of the honey samples in this thesis.

3.3.2.2 Determination of the bactericidal effect of honey (Minimum Bactericidal Concentration, MBC)

For all the honey samples, a concentration above 50% was shown to be required to induce a bactericidal effect against *S. aureus*, while a bactericidal effect was obtained against *E. coli* at a honey concentration of 50%. However, for manuka honey, a concentration of 25% (w/v) was observed to be sufficient to produce a bactericidal effect against both *S. aureus* and *E. coli* (Table 3.2). This shows that the inhibition of the growth of both bacteria by the Irish honey samples observed in the broth microdilution

assay (Section 3.3.2.1) at honey concentrations above the MBC was due to a bacteriostatic effect rather than a bactericidal effect.

Table 3.2 Minimum concentration of honey (expressed as a percentage % w/v, in TSB) necessary to induce bactericidal effect against S. aureus and E. coli. The honey solutions, prepared in TSB, were tested at the range of concentrations of 50-0.78% w/v. The values shown are the average of six replicates (two technical replicates for each of the three biological replicates).

	Honey samples (w/v% in TSB)				
	IH1	**IH2**	**IH3**	**IH4**	**MH**
S. aureus	>50	>50	>50	>50	25
E. coli	50	50	50	50	25

Our results are in line with what was reported in a recent study by Zhang et al.[302], where most of the 12 Chinese honey varieties tested had an MBC higher than 50% (w/v) against *S. aureus, E. coli* and *C. albicans*, including manuka honey (12+ and 20+). Similar values were also measured for Malaysian honey, namely acacia, and pineapple; however, unlike what was observed for Irish honey in this chapter, lower MBCs were measured for *S. aureus* than for *E. coli*[357]. A bactericidal effect on *S. aureus* has been observed by Osés et al.[392] for heather honey at significantly lower concentrations (between 4.69 and 9.38%, w/v) than the MBC measured here for Irish heather honey. However, it is of interest that a certain degree of variability can be observed even between samples of the same botanical origin, with one heather honey sample analysed in the same study having an MBC of 37.5% (w/v)[392], indicating that other factors (environmental, handling and processing) other than the floral origin might play a crucial role in determining the antibacterial effect of honey.

3.3.3 Proof-of-concept study on the potential of monitoring bacterial Volatile Organic Compounds (VOCs) to assess the antibacterial activity of honey

3.3.3.1 Fundamental considerations for the design of the study

Monitoring volatile emissions from pathogenic microbial species is emerging as a valuable alternative strategy for the rapid diagnosis of infections and evaluation of antibacterial treatments, as presented in Section 3.1.3. In order to achieve a comprehensive understanding of the significance of VOC data in the design of metabolomic studies, it is crucial to include validation parameters, such as culture media

controls and monitoring of the bacterial growth phase during VOC sampling[367]. Therefore, in this chapter, the VOC information was analysed together with the growth curve study (carried out based on optical density), to assess if the emission of characteristic compounds and their variation in concentrations throughout the observation time was in line with the bacterial growth phases. Furthermore, a background measurement of the growth media headspace was carried out as a control. *E. coli* was selected as a representative bacterial species for the evaluation of the method adopted in this chapter due to its volatilome being previously researched and its higher susceptibility to the honey treatment, as shown in Section 3.3.2.1 and 3.3.2.2. When a bacterial colony is suspended in a liquid nutrient medium, the bacteria will rapidly reproduce and the cell count will increase typically following a kinetic consisting of four phases: lag phase, where the bacteria are adapting to the fresh media; log phase, where the exponential growth occurs; stationary where a plateau is reached, due to nutrients depletion and accumulation of waste metabolites, and finally, death phase[396]. Figure 3.6 shows the growth curve of *E. coli* in TSB over 24 h of observation: while the lag and death phases are not visible here (occurring outside of the observation time), the exponential phase can be appreciated from 1 h of incubation up to 4 h, followed by a steady increase continuing up until 24 h of incubation.

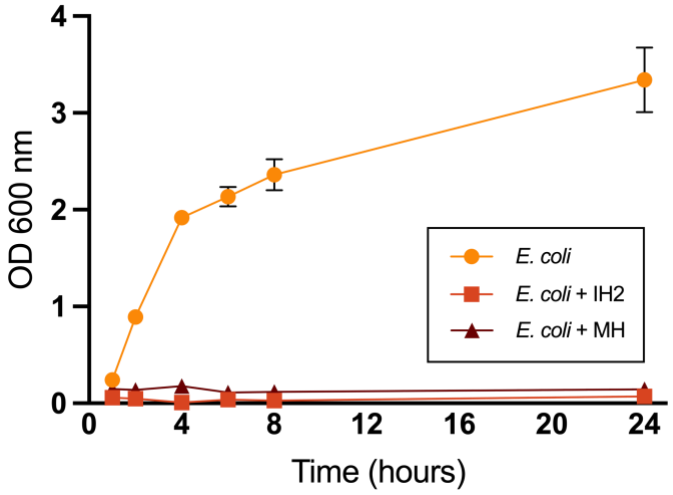


Figure 3.6 Bacterial growth curve of E. coli based on OD600 measurements at 1, 2, 4, 6, 8, and 24 h of incubation (37 °C, 180 rpm). The graph shows control growth (E. coli in TSB) and test growth in the presence of samples IH2 and MH (honey solutions at 50% w/v in TSB). The values shown are the average of two technical replicates for each of the three biological replicates (for a total of n=6) ± standard deviations.

Both Irish heather honey (sample IH2) and manuka honey (MH) induced a statistically significant bacterial growth inhibition against *E. coli* during the 24-hour incubation in the growth curve study, as shown in Figure 3.6. Specifically, when compared to the untreated control, IH2 significantly reduced the growth of *E. coli* already at 1 hour (p value = 0.0005); the inhibition was then significant at all time points (p value = <0.0001). The inhibitory effect of MH against *E. coli* could be observed after 2 h of incubation (p value = <0.0001) and remained equally statistically significant. The results of this growth curve study confirm the antibacterial effect exerted by H2 and MH against the growth of *E. coli*, as shown in the experiments reported in Section 3.3.2.

3.3.3.2 Screening of the bacterial volatilome to identify distinctive compounds

Bacteria possess a characteristic metabolism, with the emission of distinctive VOCs that have the potential to be used to detect an infection. Some of these VOCs, such as ethanol, acetic acid and isopentanol (3-methylbutanol), have been shown to be emitted by various pathogens, and may only have diagnostic values for infections without discriminating against the bacterial species[367]. Some other VOCs are emitted specifically by one bacterial species, or at a significantly higher abundance than by other species, thus they can be used as an aid to discriminate between bacterial species of concern. However, in order to implement such a diagnostic strategy, prior data gathering of background physiological VOCs is of critical importance, so as to be able to discriminate concentration trends and value ranges indicative of bacterial metabolism and infection. In a laboratory setting, this can be achieved with control measurements of growth media. Figure 3.7 shows a side-by-side visualisation of the headspace fraction of the *E. coli* liquid culture in TSB (prepared as per the method outlined in 3.2.6.1) and of the TSB media control (also listed in Appendix B, Table B-1 and Table B-2).

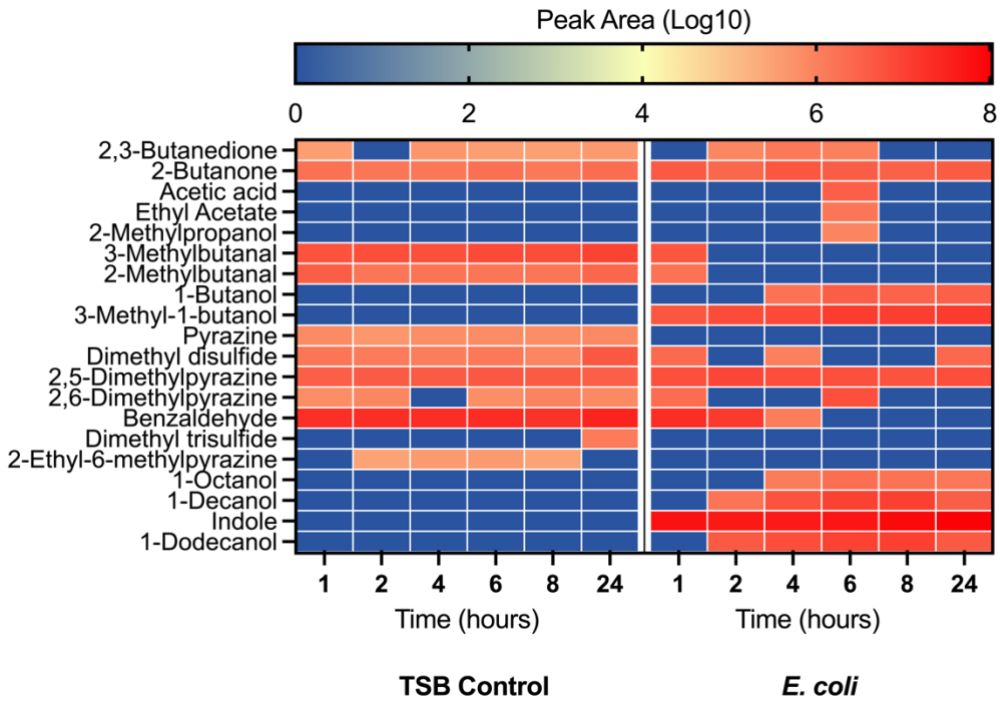


Figure 3.7 Heatmap summarising the volatile emissions from E. coli (in TSB) and TSB media control (combined list of VOCs). VOC sampling was performed by HS-SPME followed by GC-MS at 1, 2, 4, 6, 8, and 24 h of incubation (37 °C, 180 rpm). The peak area values (obtained by chromatogram deconvolution algorithm) are expressed in the logarithmic scale, with a corresponding colour ranging from red (highly abundant compound) to dark blue (compound not present). The values shown are the average of four biological replicates.

It can be noted from Figure 3.7 that, when compared to the control, indole, obtained by enzymatic deamination of the essential amino acid L-tryptophan (by tryptophanase)[367], dominated the headspace of the *E. coli* culture at all the bacterial growth phases (see Figure 3.8 for a representative chromatogram and identification procedure). This is in line with the literature reports indicating indole as a marker of *E. coli* infections[259,367,386,397,398]. Indole has also been suggested to play a role in the establishment of *E. coli* biofilms, by acting as a signalling agent in the modulation of biofilm-regulating factors[399,400].

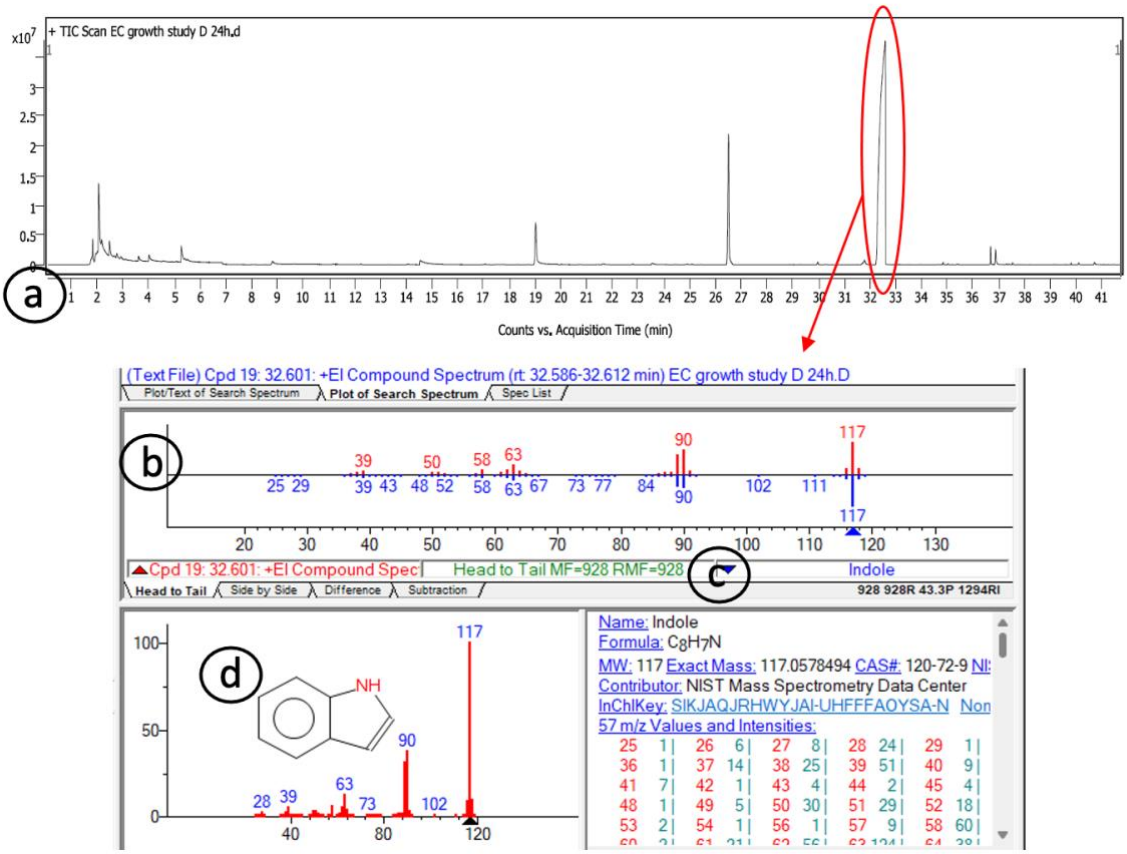


Figure 3.8 Supporting information for the identification procedure of markers of bacterial growth of E. coli. a) Representative chromatogram (count vs. acquisition time) of the volatile fraction of E. coli obtained by Headspace – Solid Phase Micro Extraction (HS-SPME) followed by Gas Chromatography – Mass Spectrometry (GC-MS) after 24 hours of incubation (37 ˚C, 180 rpm). The detailed experimental procedure is described in Section 3.2.6. The peak eluting at a retention time of 32.601 was the most abundant in the sample's headspace. b) The compound identification was carried out by comparison with reference MS spectra using the NIST Mass Spectral Search Program for the NIST23 3.0 Mass Spectral Library, which confirmed the compound as indole, previously identified as marker of E. coli. c) A database match score (MF) of at least 700 was deemed sufficient to confirm the identification (here in the example, MF=928). d) Chemical structure of the compound being identified (here indole), with details such as formula, molecular weight, CAS number, contributor to the entry etc. shown on the right side of the screen.

Various linear alcohols were emitted during the exponential and stationary growth phase of *E. coli* (Figure 3.7), namely, 1-decanol (Figure 3.9) and 1-dodecanol (after 2 h of incubation) and butanol and 1-octanol (after 4 h of incubation, in line with previous reports[259,401]. These alcohols are produced by either fatty acid metabolism or glucose metabolism, with a degree of variability in chain length observed across different strains and culture media used[386]. Due to their antibacterial effect[402], the emission of these alcohols by *E. coli* might play a role in the competitive inhibition of other bacterial species[367].

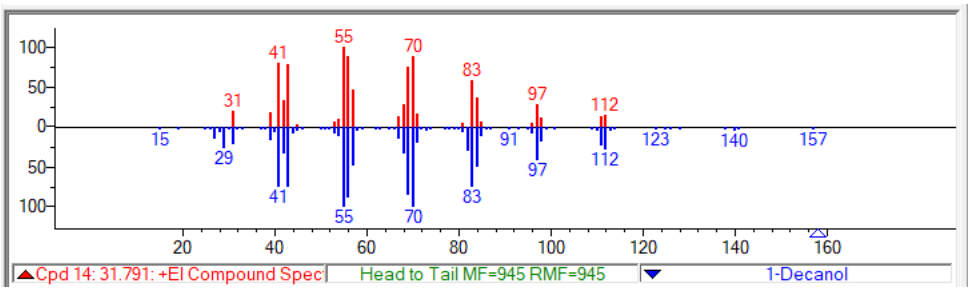


Figure 3.9 Representative MS spectra showing the fragmentation pattern of 1-Decanol which is a volatile metabolite of bacterial growth of E. coli, extracted by Headspace – Solid Phase Micro Extraction (HS-SPME) followed by Gas Chromatography – Mass Spectrometry (GC-MS). The detailed procedure is described in Section 3.2.6. The compound identification was carried out by comparison with reference MS spectra using the NIST Mass Spectral Search Program for the NIST23 3.0 Mass Spectral Library. A database match score (MF) of at least 700 was deemed sufficient to confirm the identification (here in the example, MF=945).

It is important to notice that not only the increase in the concentration of a compound but also its reduction can be indicative of infections as it may signify different metabolic processes with different prevalence across the four phases of bacterial growth. Aldehydes such as methylpropanal, 3-methylbutanal and 2-methylbutanal are products of the metabolism of amino acids, and in turn, these aldehydes represent the precursors of esters and ketones[403]. In this study, 3-methylbutanal and 2-methylbutanal were detected in the growth media (TSB) at all incubation times but were absent in the *E. coli* headspace after 2 h of incubation (as shown in Figure 3.7). Likewise, benzaldehyde concentrations in the *E. coli* headspace progressively decreased over time, until the compound was not detectable after 4 h of incubation, whereas it was detected at high concentration in the TSB control headspace. Similarly, pyrazine was detected in the TSB control but was totally absent in the *E. coli* samples. The decrease in concentration compared to the media observed here, and previously reported by other authors[259,404], suggests that these compounds provided in the nutrition broth were used up in the bacterial metabolism during the exponential growth phase, with possible conversion to other compounds. For example, the emission of 3-methylbutanol by multiple bacterial species has been suggested to be the result of the reduction reaction of 3-methylbutanal[404]. Similarly, the ketone 2-butanone, present in the TSB headspace at all sampling times, was detected in the *E. coli* samples at concentrations up to three times higher than in the media control, suggesting an additional emission by the bacteria. However, these compounds are not proposed as markers of *E. coli*, as they have been

previously suggested as markers of *S. aureus* (2-butanone) and *P. aeruginosa* (2-methylbutanal)[367].

Dimethyl disulfide is frequently associated with Gram-negative infections, and like other small sulfur-containing molecules (such as methyl mercaptan or dimethyl sulfide), it has been linked to inflammatory responses in animal models, suggesting they might be used as indicators of high virulence[405]. Dimethyl disulfide was detected here at different stages of incubation, in line with the time-dependent production by *E. coli* that has been previously described in the literature by other authors[397,406]. However, dimethyl disulfide was also detected across the entire incubation time of the culture media control (TSB), as previously reported by Fitzgerald et al.[259,386]; therefore, this compound will not be considered further as an indicator of *E. coli* growth in this chapter.

3.3.3.3 Evaluation of bacterial growth inhibition by monitoring VOC emissions

The identification of characteristic bacterial VOCs, achieved through the screening of their volatilome in comparison with the background environment, is of paramount importance to be able to confidently develop a diagnostic strategy. Based on the findings outlined in Section 3.3.3.2, five compounds were selected as potential markers of *E. coli* to be used to monitor the inhibitory effect of the honey samples IH2 and MH, namely indole and the linear alcohols butanol, 1-octanol, 1-decanol and 1-dodecanol emitted by the untreated *E. coli* culture (full list of the VOC emissions of samples and controls is available in Appendix B). Despite not being specific to *E. coli* volatilome, the emissions of 3-methylbutanol were also considered for this aim as a general infection marker, considering it is emitted by various bacterial species[367].

The predominant VOC emitted by *E. coli*, indole, was detected in the honey-treated samples (Figure 3.10), but with a statistically significant reduction in peak area induced by both Irish heather honey (IH2) and manuka honey (MH) from after 2 h of incubation, with ultimately no indole detected after 24 h of incubation in the MH-treated culture (Figure 3.11). This is in line with the trend shown in the growth curve (Figure 3.6), suggesting that the emission kinetics of indole by *E. coli* over time can be used as a parameter to evaluate bacterial growth.

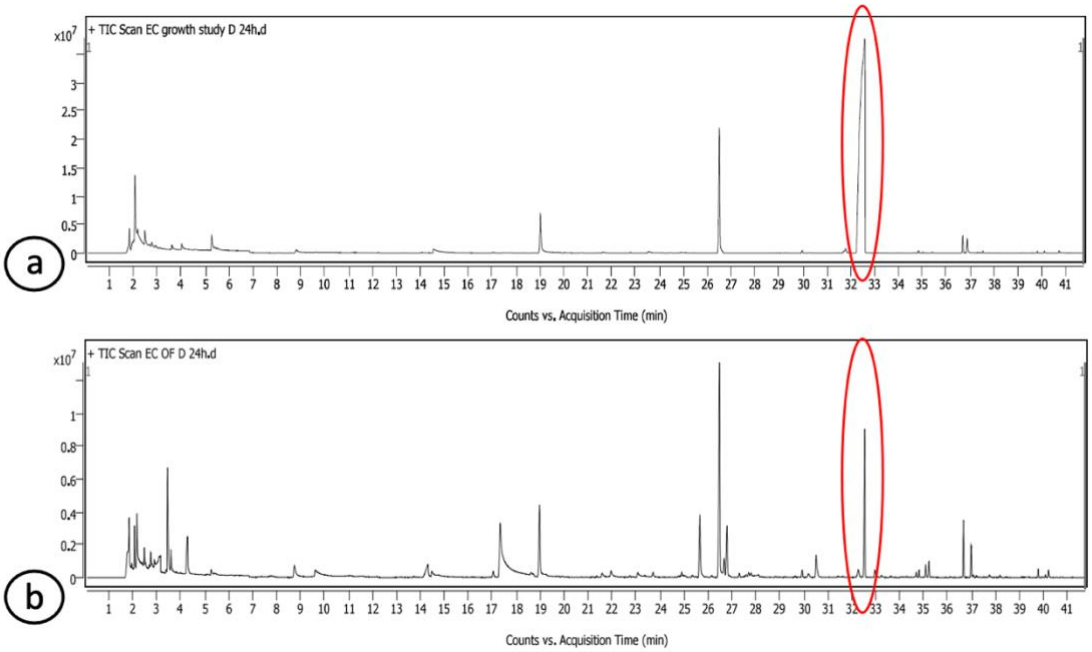


Figure 3.10 Evaluation of the effect of honey-induced growth inhibition on the emission of metabolites of bacterial growth of E. coli. Comparison of representative chromatograms (count vs. acquisition time) obtained by Headspace – Solid Phase Micro Extraction (HS-SPME) followed by Gas Chromatography – Mass Spectrometry (GC-MS) after 24 hours of incubation (37 ˚C, 180 rpm). The detailed procedure is described in Section 3.2.6. a) E. coli liquid culture (control growth study) b) E. coli treated with unifloral Irish heather honey (sample IH2). The marked peak is indole, predominant volatile compound of E. coli headspace and suggested markers, with area significantly reduced in b).

Interestingly, all the linear alcohols emitted by the *E. coli* culture (butanol, 1-octanol, 1-decanol and 1-dodecanol) were absent in the honey-treated samples (as shown by the deep blue colour in Figure 3.11), suggesting that when *E. coli* is inhibited by IH2 and MH and its metabolism is affected, alcohol production is impaired. Finally, a statistically significant reduction in emission of the non-specific bacterial volatile 3-methylbutanol was measured in the honey-treated samples at all sampling times, when compared to the untreated *E. coli* culture.

The significant reduction in emission of all the distinctive bacterial VOCs, typically emitted by *E. coli*, observed in the honey-treated samples, confirmed the considerable level of bacterial growth inhibition already described in Section 3.3.2 of this chapter, induced by both Irish heather honey and manuka honey.

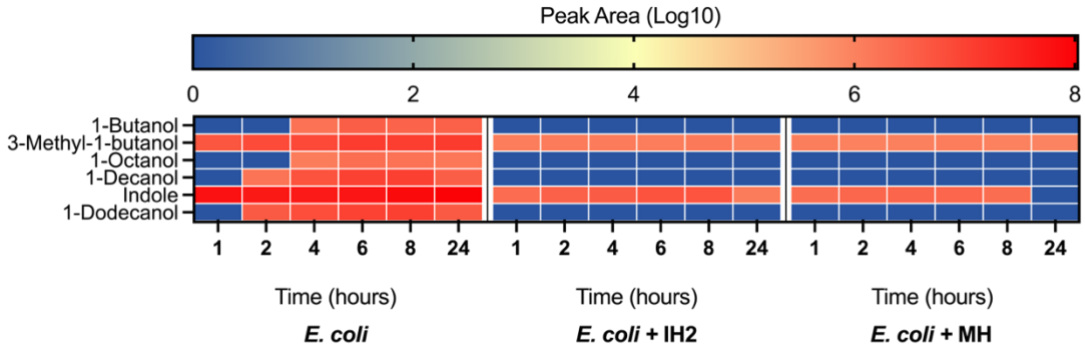


Figure 3.11 Heatmap summarising the emissions of relevant VOCs from untreated E. coli liquid culture (in TSB - control), E. coli cultured in the presence of Irish heather honey (IH2) and E. coli with manuka honey (MH) - honey solutions at 50% w/v in TSB, sample prepared as detailed in Section 3.2.6.2. VOC sampling was performed by HS-SPME followed by GC-MS at 1, 2, 4, 6, 8, and 24 h of incubation (37 °C, 180 rpm). The peak area values (obtained by chromatogram deconvolution algorithm) are expressed in the logarithmic scale, with a corresponding colour ranging from red (highly abundant compound) to dark blue (compound not present). The values shown are the average of four biological replicates.

3.4 Conclusions

This chapter explored the potential of Irish honey varieties as antibacterial agents in comparison to manuka honey. All honey varieties have been shown to provide excellent antibacterial properties, comparable to those of the gold standard of medical-grade honey, manuka honey, as shown by the results of the broth microdilution test, of the assessment of the bactericidal effect and of the growth curve study. Exploring locally sourced honey varieties is important as it could represent a more sustainable option due to easier accessibility. Furthermore, despite manuka honey being more effective at lower concentrations, multiple honey varieties should be considered as complementary options and investigated for their potential to offer greater coverage against a variety of microorganisms.

The design of analytical methods that employ bacterial volatile emissions with diagnostic value requires the identification of distinctive markers and the defining of a range of concentrations indicating colonisation and infection. The proof-of-concept study presented here in Section 3.3.3 has shown the potential of monitoring bacterial VOCs as a tool not only to assess bacterial growth and detect infections but also to evaluate the effect of an antibacterial treatment, namely Irish heather honey and manuka honey. Nonetheless, further optimisation will be needed, specifically by expanding the database of bacterial signature compounds through repeating the experiments with different bacteria, different strains of the same bacterial species, and

different media. This will allow us to appreciate the high degree of variability in the bacterial volatilome and enhance the discriminatory power of the method by considering more than one infection marker for each bacterium.

Overall, the results presented in this chapter show that Irish heather honey has the potential to be used as a biomedical treatment for infections. Locally sourced honey varieties could represent a more sustainable option due to easier accessibility and offer greater coverage against a variety of microorganisms than reliance on only one medical-grade honey variety.

Chapter 4 Design, fabrication, and characterisation of honey-alginate hydrogels with antibacterial activity against common wound pathogens

4.1 Introduction

Hydrogels are three-dimensional (3D) macromolecular networks formed by crosslinking, either physical (non-covalent ionic interaction) or chemical (by covalent bonds), of linear polymer chains[407]. They are characterised by a high percentage of water (up to 90% of their composition in weight), remarkable liquid uptake capacity due to hydrophilic functional groups (with a relative percentage of swelling up to 1000%) and biocompatibility[408].

Hydrogels can have a diverse set of uses, spanning from environmental engineering to the development of sensors, from bioengineering to 3D printing, but their primary application is in the biomedical field, where they can be used in drug delivery, tissue replacement, implants and injectables, diagnostic imaging, wound healing and more[409,410]. The application for which hydrogels are designed dictates the required features to show satisfactory performance. Among the characteristics that make hydrogels suitable for applications in wound healing, which represents the focus of this chapter, is the management of copious exudate emission, which can be of up to 12 L/m^2 of exudate daily, as described for venous leg ulcers[411], easy removal without abrasion (to avoid secondary injury) and antibacterial action for infected lesions[412]. Furthermore, hydrogels have been proven to have high similarity to the extracellular matrix, making them ideal for delivering cells and growth factors for tissue regeneration and engineering[408]. Finally, hydrogels' performance should be tailored to guarantee resistance to mechanical stress that they might be subjected to when applied to a body in motion[413].

Each constituent polymer and, consequently, each resulting hydrogel, based on the different composition, will present a combination of some of these positive features but also inevitably some limitations. For example, synthetic covalent hydrogels have long been considered more stable to mechanical stress than hydrogels from natural polymers, although significant progress has been recently made to improve the mechanical performance of natural hydrogels[413,414]. Polyelectrolytes of natural origin, especially when linked by ionic interactions, generally show higher compatibility than those of synthetic origin but suffer more batch-to-batch variations in the production of the polymer material[407]. When considering what polymers to choose for this study,

whose main aim was the development of hydrogels for the treatment of wounds, we defined essential requirements features such as high biocompatibility, high liquid uptake capacity, easy formulation, and affordability. Based on these considerations, polymers of natural origin crosslinked by ionic interactions were preferred.

Among the various natural polymers investigated in the literature, sodium alginate has been chosen for the development of the hydrogels in this study due to its remarkable properties that make it ideal for promoting wound healing, as confirmed by the several alginate-based wound cover products already commercially available[408]. Sodium alginate is a very versatile material, as it also finds numerous applications in diverse technical sectors, from the food industry, edible coatings and packaging[415–418] to wastewater treatments and environmental science[419–422]. Nonetheless, a significant amount of research on alginate has been dedicated to its potential in the biomedical field, such as drug delivery[423], tissue engineering[424] and wound healing[425,426]. Another widely researched polymer of natural origin used in the development of hydrogels is chitosan. Similarly to alginate, chitosan has been employed in a variety of fields such as agriculture and agronomy, water filtration, organic chemistry as a catalyst, dentistry and drug delivery[427–429]. In the biomedical field, chitosan has been extensively researched for its wound healing applications, showing excellent hemostatic and antimicrobial properties, and it is biocompatible and biodegradable[430–434]. A combination of these two polymers has been investigated to develop the hydrogels formulated in this chapter.

4.1.1 Gelation mechanisms of alginate and chitosan

The ionic interaction between alginate and chitosan to give complex polyelectrolyte gels has been amply investigated for different applications such as food packaging[435] and biomedicals such as tissue engineering for the central nervous system[436], bones[437] and skin[438]. At a molecular level, sodium alginate is a polysaccharide consisting of the sodium salts of a mixture of polyuronic acids [$(C_6H_8O_6)n$] obtained from algae belonging predominantly to the Phaeophyceae. It is slowly soluble in water forming a viscous, colloidal solution[439]. Two (1–4)-linked epimers, β-d-mannuronic acid (M units) and α-l-guluronic acid (G units), form sodium alginate's unbranched chains of anionic polyelectrolyte. It can form copolymers with irregular sequences of the monomers, and different proportions of the two epimers are also possible, with high

variability based on the species of the algae or seaweed that the alginate is derived from[440,441]. The primary mechanism of gel formation is due to ionic interactions between a highly specific site in the residues of guluronic acid and Ca^{2+} from different sources or other divalent or trivalent cations[442–444] (Figure 4.1). The higher the number of residues of guluronic acid in the alginate structure, the higher the concentration of calcium ions required to reduce the hydrogel's solubility[445]. In other words, not only Ca^{2+} concentration but also the ratio between mannuronic and guluronic acid in the alginate chain significantly affects physicochemical properties such as film thickness, swelling degree, and moisture content[441,445]. When calcium ions are added to the solution, these bivalent ions replace sodium ions, but due to its double charge, Ca^{2+} can bind two polymer strands, giving the so-called "egg-box" model (Figure 4.1), a particular type of complexation that is believed to be typical of also other kinds of polyuronates such as pectin[446]. Two main strategies have been reported to induce gelation in alginate solutions with calcium, namely external and internal gelation methods, which result in different distributions of crosslinking sites through the network[447]. In the external gelation method, a highly soluble calcium salt solution is used, from which Ca^{2+} ions are readily available to react with the alginate chains of a scaffold immersed in it. It is often employed to prepare small structures such as alginate beads[447,448]. This method produces scaffolds with a higher degree of crosslinking on the surface, where the Ca^{2+} ions react first with alginate, than on the inside. The internal gelation method requires the dispersion of a low-solubility calcium salt into the alginate solution, from which the Ca^{2+} ions are then released internally by a controlled change in pH[449].

From a structural point of view, chitosan hydrochloride is the chloride salt of an unbranched binary heteropolysaccharide consisting of the two units N-acetyl-D-glucosamine and D-glucosamine (represented with random frequency within the polymer chain)[441]. It is obtained by partial deacetylation of chitin (extracted from shrimp and crab shells), normally leading to a degree of deacetylation of 70.0 to 95.0%[439]. Chitosan has often been chemically crosslinked using glutaraldehyde[450–452], and more recently, genipin has been used as a crosslinker due to its biocompatibility[453–455]. The interaction between chitosan and alginate is possible because while alginate's guluronate residues have been shown as crucial to the calcium-induced gelation of alginate solutions, the negatively charged mannuronic units have been reported to

present the optimal conformation and distance to interact with the positively charged chitosan[441,456,457], giving what is best described as a connected double network with internetwork crosslinking[458] (Figure 4.1). Consequently, in the pH range between their respective pK_a values, the polycationic chitosan and polyanionic alginate will interact by ionic crosslinking forming polymeric networks[441]. The pK_a values for the amino groups of chitosan range between 6.17 and 6.51, with lower degrees of deacetylation in the chitosan chains leading to higher pK_a values and only minor variations depending on the molecular weight[459]. The pK_a value of the carboxyl groups in alginate is typically around 3.5. Therefore, the optimal pH for the interaction is between 3.5 and 6, approximately.

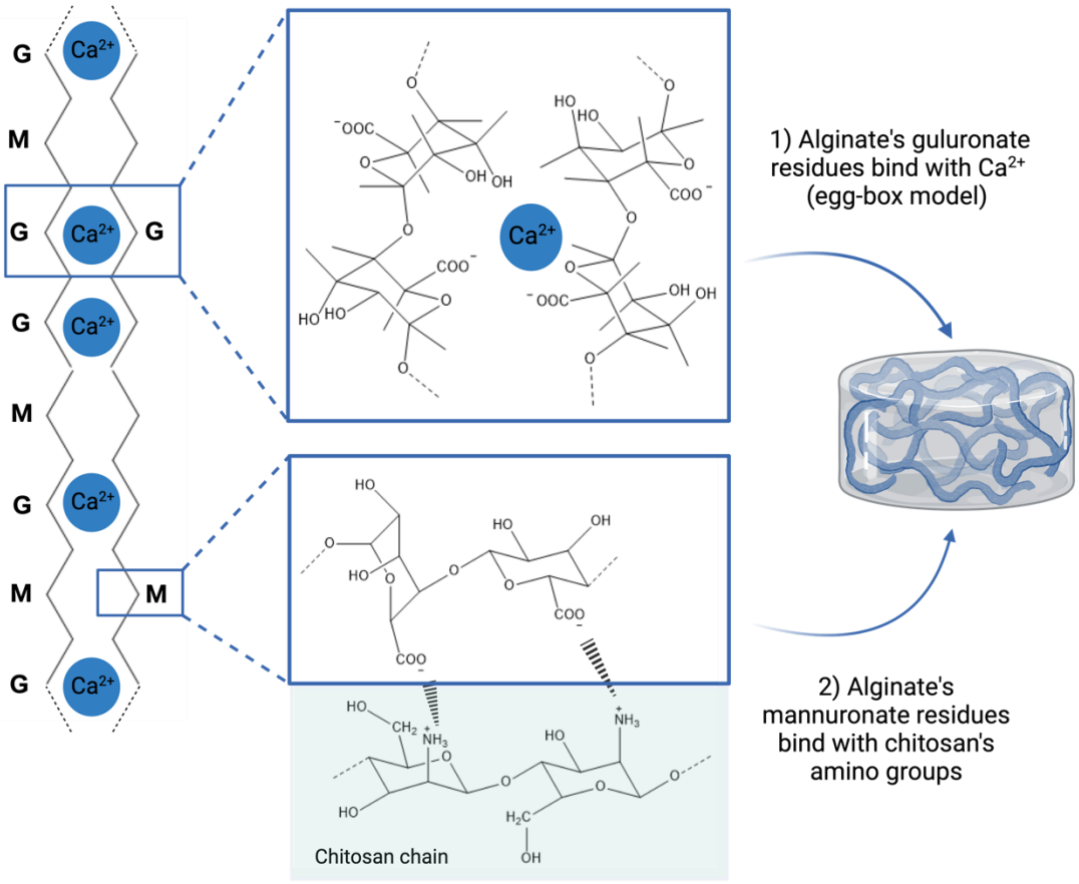


Figure 4.1 Ionic crosslinking of alginate to form a double network. Alginate polymers consist of two (1–4)-linked epimers, β-d-mannuronic acid (M units) and α-l-guluronic acid (G units). 1) Guluronate monomers bind with Ca^{2+} in the so-called "egg-box model"; 2) mannuronate residues bind with chitosan's residues. Adapted in part from Braccini et al.[442]. Created in BioRender.com.

4.1.2 Considerations over the interaction between alginate and chitosan

A number of parameters should be taken into consideration when developing polyelectrolyte complexes in order to modulate qualities like (but not limited to) porosity, permeability, absorptive behaviour in aqueous media, tensile strength, cytotoxicity, antibacterial activity[438]. For example, intrinsic factors such as viscosity, degree of deacetylation and molecular weight of the polymers can be evaluated based on the properties needed for the final product[438,460,461]. These characteristics also depend on the source from which the polymer has been extracted from[462]. Once the materials have been selected, the ratio between alginate and chitosan can be optimised, and further adjustment of the gel features can be obtained by adding excipients and surfactants[438,463]. Different strategies have been explored to obtain chitosan-alginate polymeric networks, and the most common are discussed below. Direct mixing has been described as a suitable method to obtain homogeneous amorphous scaffolds by simple dropwise addition of chitosan solution to alginate solution (same concentrations) under vigorous stirring[436,464]. However, macroscopically visible heterogeneity has been observed for polyelectrolyte gels of low-viscosity alginate and chitosan at a 3:1 ratio[438]. Furthermore, chitosan's molecular weight has been reported to affect the chances of interaction with the functional groups in the alginate chain, with chitosan's short chains showing facilitated conformational changes to bind to alginate. As a result, low-molecular-weight chitosan, when mixed with alginate, gives more homogeneous gels compared to chitosan with high molecular weight. However, the surface of these polyelectrolyte gels is clumpier than alginate-only formulations[464]. Homogenisation has been employed to improve the structural consistency of alginate/chitosan complexes after mixing different volume ratios[435]. The application of a chitosan coating has also been investigated as an alternative strategy to integrate alginate properties with chitosan functional features[449]. Chitosan coating has been shown to either increase or negatively affect the swelling capacity depending on the formulation, which has to be tailored based on the applications. This has been suggested to be dependent on the level of crosslinking of the alginate hydrogels, i.e., the level of carboxylic groups ionically linked to calcium ions or otherwise available to form a strong network with chitosan, and their pH affecting the level of protonation of alginate carboxylic groups[449].

Alginate and chitosan hydrogels, and the double-network gels obtained by the combination of the two polymers, have been largely employed not only to merely cover wounds but also to actively promote healing by means of their intrinsic beneficial properties or by delivering drugs, cells, and growth factors to the affected area[408]. The barrier function, aimed at protecting the wound from dangerous external agents, has been integrated with a scaffold and carrier function. As such, natural and synthetic medicinal products, phytochemical extracts, essential oils, etc., have been loaded into the polymer networks to deliver them to the wound area to aid their medical effect. Among the various natural agents, the incorporation of honey into hydrogels has been explored as an innovative strategy to enhance its biomedical potential on wounds and burn healing, as extensively described in Chapter 1[232]. Not only the honey variety and the polymer used but also the type of fabrication technique used to produce the scaffolds determines the technical features and, as a consequence, the suitability for different conditions and therapeutical indications[111,465]. Nevertheless, the inclusion of honey within a scaffold has the advantage of producing a standardised and repeatable administration of honey in terms of dosage delivered to the affected area, making it more suitable for commercialisation and use in clinical settings when compared to honey directly applied to the skin[192,194,207]. However, to the best of our knowledge, no research is available on honey-loaded medical devices (fabric dressings and hydrogels) containing heather honey, despite an increasing interest in the promising antibacterial properties of this honey variety[122,123,321], as also investigated in Chapter 3. Therefore, this chapter aims to design an alginate hydrogel loaded with Irish heather honey and investigate its potential for use as an antibacterial wound cover.

4.2 Chapter's aims

The aims of this chapter are:

- The formulation of alginate hydrogels that successfully incorporate honey;
- The evaluation of the hydrogels' swelling properties due to fluid uptake;
- The evaluation of the honey release from the hydrogel structures;
- The assessment of the antibacterial properties of honey-hydrogels *in vitro* against *S. aureus* and *E. coli*.

4.3 Materials and Methods

4.3.1 Materials

Alginic acid sodium salt (mannuronic to guluronic acid ratio, M/G ratio: 1.56, viscosity 15-25 cP, 1% in H_2O), chitosan (Low Molecular Weight, deacetylation $\geq$ 75%), calcium citrate tetrahydrate 99%, glycerol (BioXtra, $\geq$ 99% GC), D-(+)-Gluconic acid δ-lactone minimum 99.0% (GDL) and Phosphate Buffered Saline (PBS, tablets) were purchased from Merck Life Science Limited (Ireland). Glacial acetic acid and Spectra/POR3 Dialysis cellulose membrane tubing (3500 MWCO, Spectrum Laboratories Inc.) were purchased from Fisher Scientific (Ireland). Trypticase Soy Agar (TSA) and Trypticase Soy Broth (TSB) were purchased from Fisher Scientific (Ireland).

4.3.2 Instrumentation

An RCT basic IKAMAG™ magnetic stirrer (IKA Werke GmbH & Co. KG, Germany) was used to prepare the hydrogels. For the assessment of the hydrogels' antibacterial activity, sample handling and preparation were performed in an NU-425-300E Class II biosafety cabinet (NuAire Lab Equipment, Plymouth, USA). A MIR-153 incubator (Sanyo Electric Co., Ltd.) and an Excella E24 Incubator Shaker (New Brunswick Scientific, Eppendorf AG, Germany) were used to incubate samples at a controlled temperature of 37 °C. A UV-3100PC Spectrophotometer (VWR International LLC., Avantor) was used to measure the Optical Density at 600 nm (OD600) of liquid bacterial cultures.

4.3.3 Honey samples

A multifloral commercial honey purchased from a local supermarket was used for the preliminary tests to develop and optimise the hydrogel formulation. The same functional characterisation was then applied to the hydrogels loaded with the honey samples selected for investigation in this project, as outlined in Chapter 2, Section 2.2.2. (see Table 2.1). For the experiments carried out in this chapter, a new jar (same batch, and same production and purchase year, namely 2021) was used, which was opened exclusively in the Class II biosafety cabinet to avoid contamination.

4.3.4 Honey hydrogel formulation

A mixture of 0.6 g of sodium alginate (4% w/w on the final weight) was prepared with 4 g of glycerol (under stirring for approximately 10 mins), and then the calculated amount of deionised water was added under continuous stirring until alginate dissolved, forming a viscous solution. For the honey-loaded samples, honey was stirred into the mixture of glycerol and sodium alginate until homogeneously dispersed (as assessed by visual examination). A fine suspension of calcium citrate and GDL was then added, maintaining the mixture under stirring. Ultimately, the alginate formulations (summarised in Table 4.1) were poured into a petri dish (60 mm diameter) until full gelation occurred and hydrogels of thickness between 3 and 5 mm were obtained. The samples were characterised 24 h after their preparations. Figure 4.2 shows a diagram of the preparation method for the honey-hydrogels. A variety of honey hydrogels have been described in the literature, with honey concentrations ranging from as little as 2% (w/v)[216] up to 75%[213,466–468]. Two honey concentrations were evaluated here (AL2, with a honey concentration of 13.33%, and AL4, with a honey concentration of 26.66%), chosen on foot of multiple successful reports elsewhere of honey hydrogels.

Table 4.1 Formulation of alginate hydrogels with (sample codes: AL2 and AL4) or without (sample code: AL) honey incorporation. GDL = D-(+)-Gluconic acid delta-lactone.

Sample	Sodium Alginate (g)	Calcium Citrate (g)	Glycerol (g)	GDL (g)	Water (mL)	Honey (g)	Honey (w/w%)
AL	0.6	0.04	4	0.1	10.26	-	-
AL2	0.6	0.04	4	0.1	8.26	2	13.33
AL4	0.6	0.04	4	0.1	6.26	4	26.66

4.3.5 Chitosan coating

Chitosan powder was dissolved in 1% acetic acid (in deionised water) to obtain a 1% (w/w) chitosan solution – i.e., 1 g in total weight of 100 g. After 24h-equilibration at room temperature, samples of the alginate hydrogels were cut and soaked in the chitosan solutions (Figure 4.2). Three different immersion times were evaluated (30, 60 and 90 mins), and names were assigned to the samples by adding the letter C (as per "chitosan") and the numbers 30, 60 and 90 at the end of the names based on the coating

times. Once removed from the immersion vessel, the hydrogels were gently blotted with filter paper to remove the excess solution.

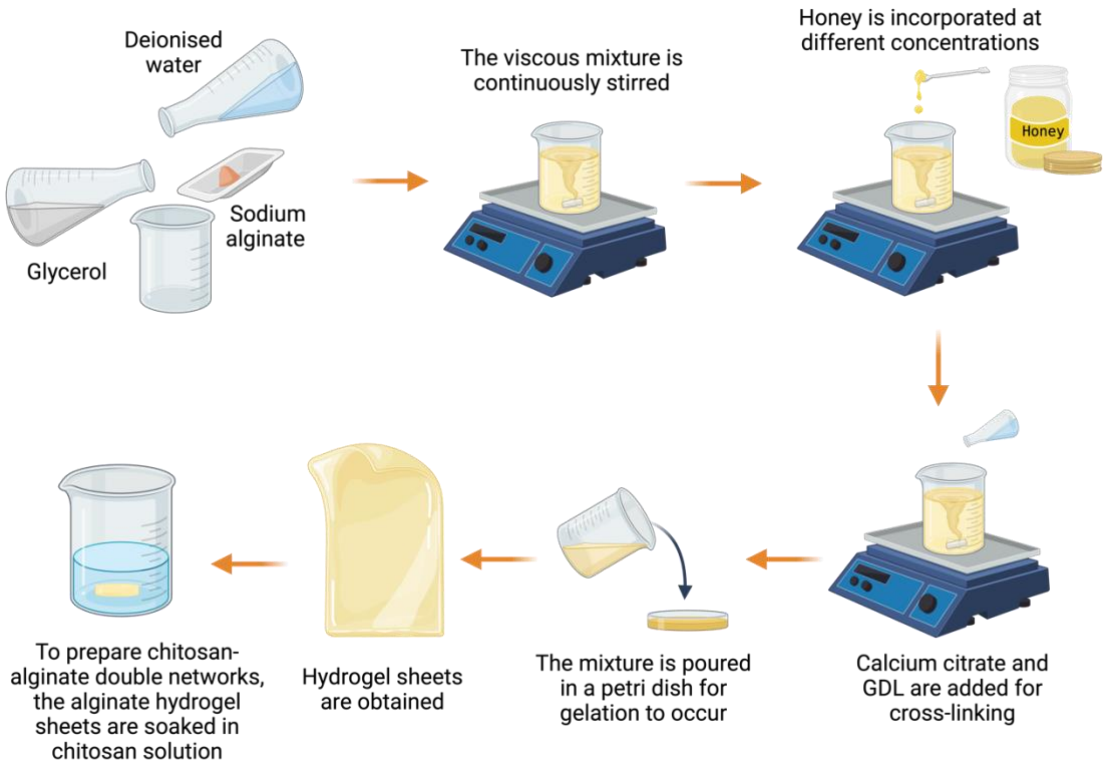


Figure 4.2 Hydrogels preparation method as outlined in Section 4.3.4 and 4.3.5. Briefly, sodium alginate was mixed with glycerol, and deionised water was added under continuous stirring until alginate dissolved, forming a viscous solution. For the honey-loaded samples, honey was stirred into the mixture. A fine suspension of calcium citrate and GDL was then added, maintaining the mixture under stirring. Ultimately, the alginate formulations were poured into a petri dish until full gelation occurred. To prepare coated double-networks, the previously prepared alginate hydrogels were soaked in a chitosan solution to induce cross-linking. Created with BioRender.com.

4.3.6 pH measurements

The pH values of the fabricated hydrogels were determined using the Mettler Toledo™ InLab™ pH Sensor - Surface Pro-ISM paired with the Mettler Toledo™ FiveEasy™ F20 pH/mV Meter. Data are shown as the average of three measurements ± standard deviations.

4.3.7 Gravimetric evaluation of the swelling capacity

The swelling degree of the hydrogels was determined according to the gravimetric method described by MacKenna et al.[469]. The hydrogels were dried for 24 h at 37 °C (UE400 Oven, Memmert GmbH + Co.KG, Germany). Specimens were cut in the shape of discs, their dry weight was recorded (W_d), and finally, they were soaked in 200 mL of

the PBS medium. Samples were then removed from the solution at predetermined time intervals, i.e., every 10 mins for the first 30 mins, then every 30 mins for 2.5 h; finally, one last measurement was carried out at 24 h. Before every measurement, the samples were gently blotted with filter paper to remove excess solution and weighed again (W_w). The swelling degree expressed as a percentage (%S) was then calculated by the following equation.

$$\%S = \frac{(Ww - Wd)}{Wd} 100 \quad (4.3)$$

where:

- %S is the percentage of water adsorption of hydrogels at equilibrium;
- W_d (dry weight) is the initial weight of the dry hydrogel;
- W_w (wet weight) is the weight after immersion.

4.3.8 *In-vitro* assessment of honey release from the hydrogel scaffolds

The *in-vitro* evaluation of honey release from the fabricated hydrogels was conducted according to El-Kased et al.[213] with some modifications. A hydrogel disk was cut, its weight was recorded, and it was inserted into a dialysis tubing which was sealed with suitable tubing closures. The samples prepared with this method were then left to float in a beaker containing 200 mL PBS solution (pH 7.4) at 37 °C with gentle magnetic stirring to simulate the extracellular body fluid. The volume of 200 mL was chosen for consistency with the swelling tests, as described in Section 4.3.7. A 1 mL volume of the immersion solution was withdrawn at 1-hour intervals over an 8-hour period to perform the measurement and replaced with the same volume of fresh PBS. The amount of honey released from the hydrogel scaffolds was then determined by quantification of the sugars naturally present in honey by enzymatic reaction using the RQflex® 20 Reflectometer and the Reflectoquant® Total Sugar Test strips (glucose and fructose, 65 – 650 mg/L) by Supelco® (Merck Life Science Limited, Ireland)[111]. Dialysis bags filled with honey solutions of concentrations equivalent to that of the hydrogel samples (indicated as 2H-Control and 4H-Control for AL2 and AL4 and their chitosan-coated analogues, respectively) were used to perform control tests with the same set-up, and regular measurements of the sugar concentration in the immersion medium were

carried out. For each formulation and control, the experiments were conducted in triplicate, and the average and standard deviation were calculated.

4.3.9 *In-vitro* evaluation of hydrogels' antibacterial properties

4.3.9.1 Bacterial strains

Two bacterial strains were studied: *S. aureus* (DSM799) and *E. coli* (DSM30083), both obtained from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH. Each strain was streaked individually on TSA plates and incubated at 37 °C overnight. Overnight liquid cultures were prepared by seeding a single colony in 5 mL of sterile TSB and kept overnight in a shaking incubator (37 °C, 180 rpm).

4.3.9.2 Assessment of hydrogels' antibacterial activity by dynamic contact method

The antibacterial effect of the alginate hydrogel was assessed by the dynamic contact method (modified ASTM E2149) as described by Sun et al.[470], with slight modifications. Circular-shaped samples (approximately 7 mm in diameter, with an average mass of 0.2024 ± 0.0286 g) were cut from the hydrogels and incubated with 4 mL of bacterial culture (10^6 CFU/mL, as determined by optical density) at 37 °C in a shaking incubator at 180 rpm for 24 h. The antibacterial activity of the hydrogel samples was tested against the Gram-negative bacterium *E. coli* and the Gram-positive bacterium *S. aureus*. After 24 h, 10 µL of the incubation media were plated in TSA (drop plate method) after adequate decimal dilutions and incubated for 24 h at 37 °C to test the viable cell counts. Results were expressed as the average value of the CFU/mL for three biological replicates (four technical replicates each) ± standard deviation. The percentage of bacterial growth inhibition was then calculated using the following equation:

$$Inhibition\% = \frac{(C_C - C_G)}{C_C} 100 \quad (4.4)$$

where

- C_c is the concentration of colonies (CFU/mL) in the control (untreated bacterial culture without the hydrogel);
- C_G is the concentration of colonies (CFU/mL) in the test sample (treated bacterial culture with the hydrogel).

4.3.10 Statistical analysis

The statistical analysis was carried out in Prism Version 10.0.0 (GraphPad Software, Inc.). Unpaired t-test (two-tailed) and one-way analysis of variance ANOVA were used for comparisons between two groups and three groups or more, respectively. The post-hoc analysis was performed using Tukey's, Bonferroni, and Dunnett's methods for multiple comparisons, with 0.05 as the alpha threshold (95% confidence interval). The level of significance in statistical analysis is coded as follows: nonsignificant $p > 0.05$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$. In the graphs used in this chapter, only statistically significant differences are displayed.

4.4 Results and discussion

This chapter outlines the development and characterisation of honey-hydrogels for wound dressings. Aspects of absorbency were examined, in particular the effect of honey incorporation and chitosan coating on the hydrogels' swelling properties, in order to estimate their exudate absorption properties. The rate at which honey was released from the hydrogels was estimated based on the concentration of sugars in the immersion solution. Finally, antibacterial properties *in vitro* against *S. aureus* and *E. coli* were investigated.

4.4.1 Hydrogel design

4.4.1.1 Visual evaluation

A preliminary visual inspection of the hydrogels was conducted. The samples showed an overall smooth surface with no lumps or other signs of macroscopic inhomogeneity; some bubbles could be observed after gelation. The hydrogels' colours varied from light yellow to golden, with increasingly more intense shades for the samples with higher honey concentrations. All the samples could be easily peeled off the petri dish.

All formulations produced transparent hydrogels, ideal for allowing regular monitoring of the wound area without the need to remove the hydrogel, which could otherwise cause discomfort to the patient and reduce compliance to frequent wound examinations[449]. Regardless of the frequency of dressing change, a balance between structural strength on one side and flexibility and softness on the other is of paramount

importance for wound coverings so that they can adapt to the irregular surface of wounds[471].

4.4.1.2 Considerations over the hydrogel formulation

In this research, the internal gelation method[447,449] was employed as a primary strategy to obtain alginate hydrogels loaded with increasing concentrations of honey. Different calcium salts were initially evaluated as calcium ion donors to induce the gelation process (Figure 4.3, a). Initial experiments focused on salt solubility, which could affect the polymer network's homogeneity.

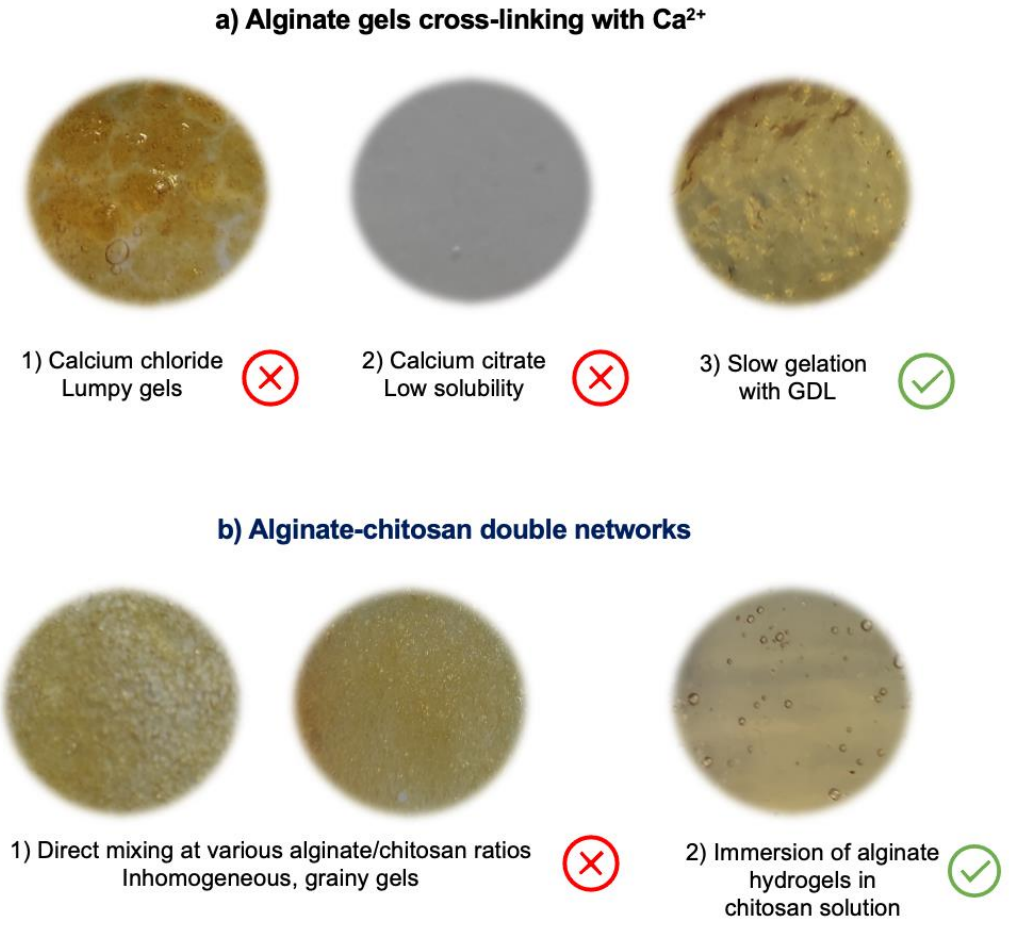


Figure 4.3 Parameters considered in the design and optimisation of alginate hydrogels to incorporate honey. a) Selection of the most suitable calcium salt to induce ionic crosslinking: 1) highly soluble $CaCl_2$ induced immediate, difficult-to-modulate gelation upon addition, which prevented suitable blending of the viscous mixture, resulting in lumpy, inhomogeneous gels. 2) Less soluble calcium salts (here citrate) can be employed but the solubility needs to be improved to release enough Ca^{2+} for ionic binding with alginate and avoid undissolved residues in the structure. 3) Slow gelation was induced with D-Glucono-δ-lactone (GDL) for controlled pH lowering. b) Formulation of alginate-chitosan double networks: 1) direct mixing of alginate and chitosan (various ratios) resulted in inhomogeneous, grainy gels. 2) Improved homogeneity was obtained by immersion of alginate hydrogels in chitosan solution (coating).

Calcium chloride ($CaCl_2$) is a highly soluble salt capable of inducing immediate crosslinking of alginate gels, which, however, often results in a poor distribution of Ca^{2+} within the hydrogel matrix (Figure 4.3, a1). This leads to both structural (inhomogeneous and lumpy gels) and functional (inconsistent and unpredictable degradation) defects[217]. Less soluble calcium salts have been assessed in the literature to overcome this issue. Calcium carbonate has been used to cause slow gelation of alginate solutions by adding D-Glucono-δ-lactone (GDL) to induce progressive availability of calcium as the crosslinking agent[217,449]. Calcium citrate was chosen as a calcium ions source for the hydrogels described in this thesis. GDL was added with the purpose of controlling the dissolution of calcium citrate, which presents low water solubility, and to obtain a progressively increased calcium availability to perform the internal gelation process (Figure 4.3, a2-3). Furthermore, the decelerated gelation rate achieved with GDL compared to rapid gelation-inducing $CaCl_2$ has been described to allow for slow-forming uniform hydrogels, from which a consistent mechanical and release performance may be expected[472]. Careful evaluation of the pH change achievable with GDL addition could be used to reach the optimal ratio of calcium salt and GDL based on the application that the polymers are being designed for. Partial protonation of alginate's carboxylate functional groups, due to excess GDL and consequent pH lowering, could allow for additional crosslinking via hydrogen bonding[449], leading to hydrogels with increased strength.

In the preparation of the alginate hydrogels, another method of crosslinking was adopted (Figure 4.3, b) by immersion of the previously prepared gels into 1% chitosan solutions (in 1% glacial acetic acid) for different lengths of time[449]. Shrinkage of alginate hydrogels has been described upon chitosan-coating, as its penetration in the alginate network increases the crosslinking[449]. However, it did not occur with the samples produced here, which may be attributed to the presence of GDL from the previous step. The controlled pH lowering due to GDL addition has been used in polyelectrolyte systems elsewhere in the literature (in the absence of calcium salts) as a strategy to regulate the charges of the amino residues in chitosan in order to induce their ionic interaction with alginate chain[441]. Straccia et al. developed wound dressings by soaking alginate hydrogels in a solution of water-soluble chitosan hydrochloride[449]. Thin chitosan-coating improved the tensile and knot strength of alginate filaments,

consequently tailoring essential properties for medical use, such as compressing and bending, which play a role in comfortable dressings application[460]. The fact that the knot properties' improvement was particularly pronounced on wet materials might also suggest its suitability for exudative wounds.

4.4.2 Surface pH of the hydrogels

Ideally, the pH of skin products should be compatible with the normal skin milieu and able to restore the physiological "acid mantle" covering the lipid barrier of the stratum corneum (the outermost epidermal layer) in those cases where the skin barrier function has been compromised[101,102]. As the end application of these hydrogels is that scenario, the pH of these hydrogels should be between 4.1 and 5.8, which is the typical range for normal skin[473].

Different techniques have been used to estimate the pH of hydrogels, e.g. direct measurement using penetration probes[449] or probes specifically designed for flat surfaces (as the one employed in this study) and measurements by the previous dissolution of a specimen of the hydrogel in purified water (applicable to those hydrogels for which the gelation process is reversible)[213]. The alginate hydrogels produced in this project and listed in Table 4.2 all showed a surface pH of approximately 4.5, regardless of the honey concentration. The pH value was instead lower for the samples where a chitosan coating was applied, with pH values typically under 4 and as low as 3.66. This was due to the need to solubilise chitosan in a slightly acidic solution, such as the acetic acid employed here. Acetic acid has been extensively used as a topical disinfectant throughout history, and it is currently used in burn hospital departments due to its antiseptic properties and efficacy against biofilm in regimens aimed at eradicating resistant *Pseudomonas* infections on burns, along with systemic antibiotics for synergistic activity[36]. As such, having pH values as low as 3.66, whilst lower than normal skin pH, is in line with the pH of current topical treatments and is acceptable for these hydrogels[474].

Table 4.2 Surface pH of the alginate hydrogels. The sample codes AL, AL2 and AL4 indicate hydrogels with no honey and with a honey concentration of 13.33 and 26.66%, respectively. C30, C60 and C90 in the name indicate the application of a chitosan coating and the immersion time in the chitosan solution (30, 60 or 90 mins). Values are expressed as the mean of three measurements ± standard deviation.

	pH		pH		pH
AL	4.43 ± 0.03	**AL2**	4.54 ± 0.02	**AL4**	4.40 ± 0.01
AL_C30	3.94 ± 0.04	**AL2_C30**	4.13 ± 0.09	**AL4_C30**	3.78 ± 0.03
AL_C60	3.84 ± 0.07	**AL2_C60**	3.92 ± 0.04	**AL4_C60**	3.69 ± 0.04
AL_C90	3.79 ± 0.03	**AL2_C90**	3.88 ± 0.06	**AL4_C90**	3.66 ± 0.06

Only a few research reports on the development of hydrogels for wound healing have reported the pH of their products. Despite the similarities in the fabrication, the pH values measured for this thesis are considerably lower than the pH of the alginate hydrogels obtained by Straccia et al.[449], whose pH ranges between 5.10 ± 0.40 and 8.34 ± 0.14. This difference in pH in the hydrogels explored in this thesis could be due to both the acidity of honey, which is incorporated as an active agent at concentrations up to 26.66% of the matrix in weight and the use of acetic acid to dissolve chitosan (whereas water-soluble chitosan is used by Straccia et al.[449]). A lower range of pH values, between 4.3 to 6.8, has been measured for six different Carbopol and chitosan honey-hydrogels specifically designed for burn healing[213].

4.4.3 Considerations over the liquid uptake capacity of hydrogels for wound healing

Investigating the fluid uptake capacity of hydrogels designed for applications on wounds is an extremely relevant parameter to assess the ability to absorb wound exudate while keeping a sufficiently moist environment in the periwound area, essential conditions to promote healing[41,220]. Intuitively, the experimental set-up for testing the swelling capacity of wound dressings should reflect the amount of fluid exuding from the wound to be treated. However, the clinical classification of the degree of exudation does not follow a standardised quantitative method, with most of the sources using descriptive and highly subjective parameters, such as "non-exudative" or "moderately/highly/severely" exudative[475,476]. Despite the limitations of the small group of patients examined, Dealey et al. suggested quantitative methods to measure the rate of exudation from wounds. The results based on the volumes collected via a gravimetric

method from wound dressings varied between 0.10 and 0.21 g/cm^2/24 h, while the mean volume measured during topical negative pressure therapy was 1.3 g/cm^2/24 h[477]. In the published *in-vitro* evaluations of the fluid handling capacity of different wound dressings, the volume of the immersion medium seems highly variable across the literature, as already highlighted in Chapter 1[232]. A 1.0 mL infusion of "artificial wound fluid" twice a day was used to reproduce the conditions of a highly exudative wound by Lutz et al. in the wound model developed to assess dressings and their exudate handling properties[478]. The British Pharmacopoeia method to test the aspects of absorbency for fibrous wound dressings indicates a 30-min incubation in a volume up to 40 times the sample's mass equivalent[41]. After much consideration, an excess volume (200 mL) was chosen for the experiments described in this chapter so that the medium volume would not represent a limiting factor to the absorptive capacity.

Many wound dressings with excellent exudate absorbency are commercially available, where the swelling properties seem to only depend on the intrinsic features of the material rather than on production aspects such as mass and thickness[41]. Other formulation components might play a role in determining the swelling performance. For example, the excipient glycerol is widely employed in pharmaceutical technology as a plasticiser for improved flexibility. However, glycerol incorporation into alginate formulations has been shown to negatively affect the fluid uptake of the hydrogels by a significant degree; in the same study, hydrogel thickness and alginate concentration were shown to have no effect on the equilibrium swelling rate[479].

The swelling equilibrium during immersion can be described as the balance between the liquid uptake and the mass loss due to the hydrogel's dissolution. The ions in the immersion media (such as Na^+ and K^+ from PBS and saline solution) can weaken the hydrogel structure by replacing the crosslinking ions, inducing an initially increased swellability but ultimately facilitating its disintegration[438,480]. For this reason, one of the critical factors determining the swelling degree of hydrogels is the ionic strength of the medium, i.e., the concentrations of ions in the solution, and this is particularly true for polymeric networks crosslinked by ionic forces. Nonetheless, a double crosslinking strategy, such as the one adopted in this chapter for alginate hydrogels with calcium ions and chitosan, has been reported to produce more stable networks[438].

4.4.3.1 Effect of honey incorporation on the swelling capacity of alginate hydrogels

All the alginate hydrogels (i.e., no chitosan) fabricated for this project showed a swelling trend characterised by a rapid increase within the first 30 mins of immersion in the medium, followed by a plateau, as can be seen in Figure 4.4, a). All the samples retained their shape and overall integrity during the entire observation time and could be removed from their swelling medium without major loss of fragments (Figure 4.4, c).

Honey incorporation in the alginate hydrogels progressively reduced the swelling degree with increasing honey concentration. The alginate hydrogel loaded with the maximum tested honey concentration (26.66% w/w), i.e., sample AL4, showed a significantly lower (p value < 0.05) swelling degree at each measurement point (3-hour measurement) when compared to the plain alginate hydrogel AL (no honey incorporation). The final swelling was recorded after a 24-hour soaking in the immersion medium (Figure 4.4, b). The plain alginate hydrogel AL showed the highest liquid uptake, with a swelling degree of 248 ± 37%. The maximum swelling degree for AL2 was 168 ± 18%, with a statistically significant difference of 80% when compared to AL (p value = 0.0228). For AL4, the gravimetric measures showed a steady absorption until the maximum swelling value was reached after 2 h of immersion (72 ± 5%), followed by a reduction in weight with a final swelling of 46 ± 25% (p value = 0.0002, AL vs AL4).

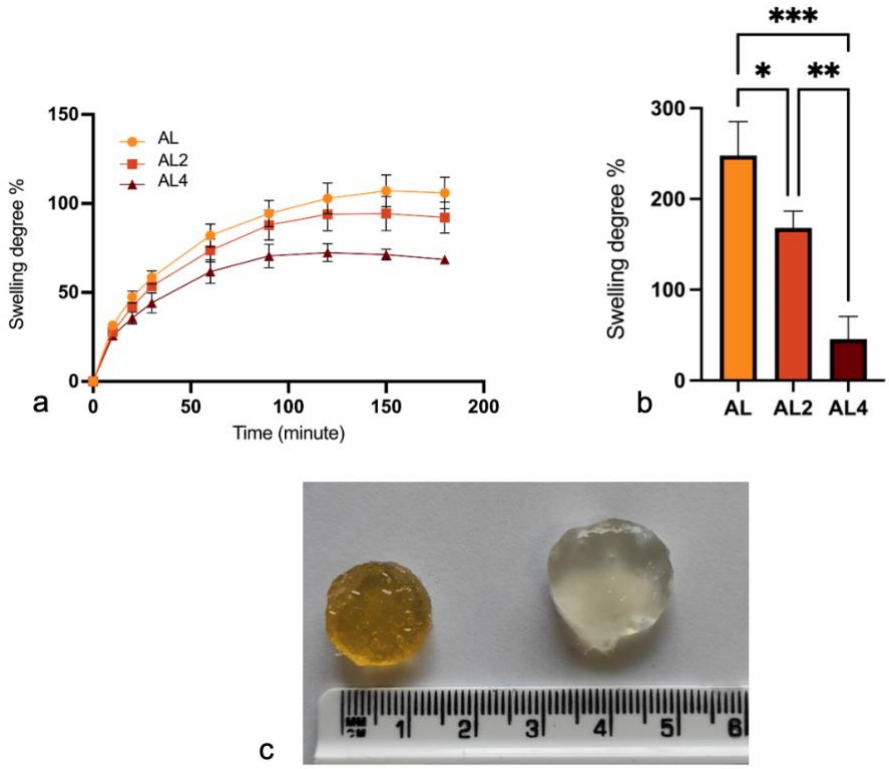


Figure 4.4 Effect of honey incorporation in the swelling degree (calculated as per Equation 4.1) of alginate-based hydrogels: evaluation by the gravimetric method after immersion in 200 mL of PBS. The sample codes AL, AL2 and AL4 indicate hydrogels with no honey and with a honey concentration of 13.33 and 26.66%, respectively. Each point represents the average of three replicates, with the error bars corresponding to the standard deviation. a) Representative graph of the swelling trend for the hydrogel samples over three-hour monitoring. b) Maximum swelling at equilibrium after 24 h. c) AL4 before and after (left and right side of the picture, respectively) the 24-hour immersion in PBS for liquid uptake evaluation. The pre-immersion specimen was prepared by oven-drying the hydrogel for 24 h at 37 °C.

A negative effect of honey addition on the fluid uptake capacity of alginate hydrogels has also been reported by Azam et al., with a swelling reduction from 512 ± 21% in plain alginate hydrogels to 430 ± 10% in 2% honey hydrogels, with further reductions for increasing honey concentrations to the lowest value of 197 ± 9% in hydrogels loaded with 10% manuka honey[221]. A swelling degree difference from 125 ± 0.50% to 73 ± 1.12% was observed for chitosan-based honey-hydrogels with honey concentrations of 25 and 75%, respectively, after 3 h of soaking in the test media. For the same increase in honey concentration, the fluid absorption of Carbopol 934 hydrogels halved (40 ± 0.18% to 20 ± 1.14%)[213].

4.4.3.2 Effect of chitosan coating on the swelling capacity of alginate hydrogels

Not only the choice of the crosslinking agent and its binding affinity but also its concentration and/or the immersion time in the crosslinker solution are parameters to keep in mind to modulate the strength of the hydrogels and their swelling degree. A chitosan concentration of 1% (w/v) was kept consistent for the preparation of coated hydrogels. However, three soaking times (30, 60 and 90 mins) in the chitosan solution were tested to coat the hydrogel samples. All the chitosan-coated alginate hydrogels showed significantly higher swelling capacity than their respective non-coated hydrogels (Figure 4.5). However, no linear trend emerged when the hydrogels coated for different times were compared to one another. In other words, the enhanced fluid absorption observed for increasing immersion times was not statistically significant (p value > 0.05).

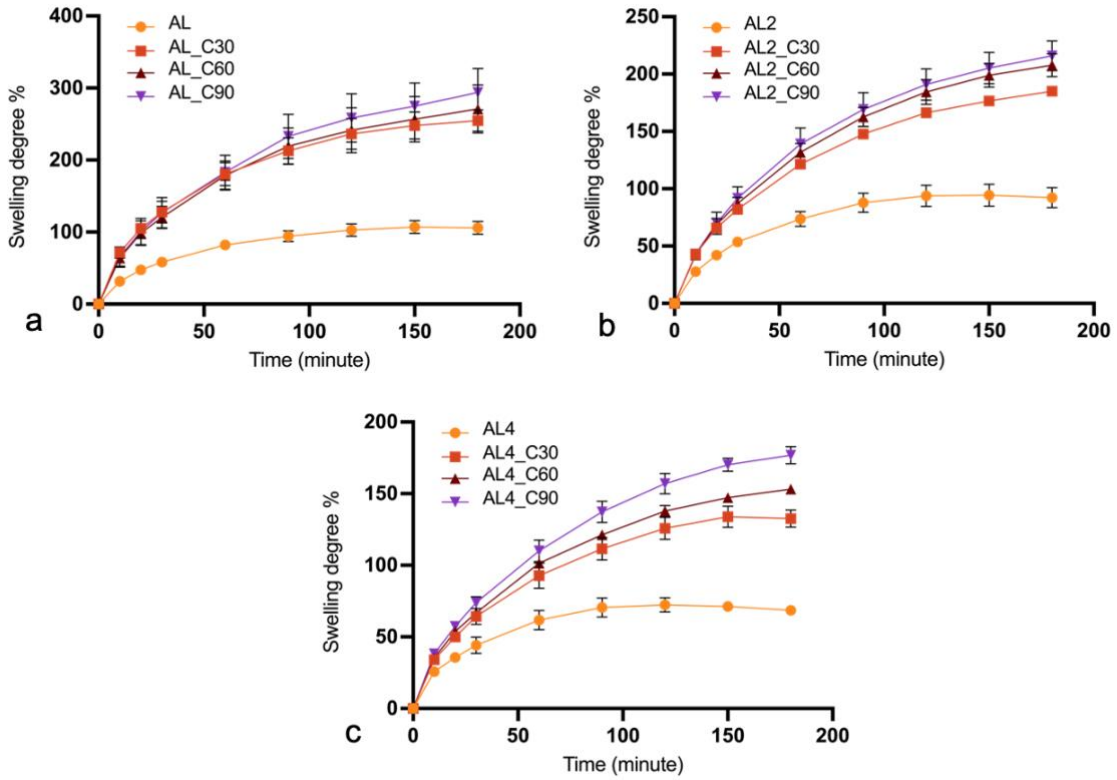


Figure 4.5 Effect of chitosan coating on the swelling degree (calculated as per Equation 4.1) of hydrogel samples during three-hour immersion in 200 mL of PBS. a) Plain alginate hydrogels AL, b) alginate hydrogels with 13.33% honey (AL2), and c) alginate hydrogels with 26.66% honey (AL4). The codes C30, C60 and C90 in the name indicate the application of a chitosan coating and the immersion time in the chitosan solution (30, 60 or 90 mins). Each point represents the average of three replicates, with the error bars corresponding to the standard deviation.

These swelling degree values shown here are in line with those reported by Verma et al. for polyelectrolyte complexes obtained by direct mixing of chitosan and alginate solutions (both 1% w/v), where the weight gain recorded varied between 111.0 ± 2.0% and 442.2 ± 24.8% based on the chitosan/alginate ratios, with the highest values measured for higher alginate concentrations[480]. A swelling between 49.27 ± 0.15% and 111.05 ± 0.5% was measured (after immersion in PBS at room temperature, volume not specified) for alginate/chitosan sponges loaded with curcumin and honey for tissue repair[481]. In their research, Momin et al. reported that for higher concentrations of chitosan, the swelling index increased, while high alginate content was shown to negatively affect absorption. Considering the concentration of honey was kept consistent for all the chitosan/alginate ratios, the effect of honey on the fluid-holding capacity in that study was not evaluated[481].

The double-crosslinking strategy adopted here provides hydrogels with good absorption properties, thanks to the three components alginate, calcium and chitosan contributing to the establishment of a solid network by different forces (as described in Section 4.4.1.2). Due to the improvement provided by higher coating times (despite it not being significant), the 90-min immersion time was selected to prepare chitosan-coated hydrogels for further testing. It is important to keep into account that any biomedical product is only effective as a function of the application needed, i.e., different applications require a product with a different set of features because a desirable effect for one disease could be detrimental to another condition that shows different symptoms. The alginate hydrogels (without chitosan coating) have been observed here to have moderate liquid uptake capacity; therefore, they could be more suitable for low-exudating wounds where high absorbency features are not required. On the other hand, the chitosan-coated formulations have shown potential for application on highly exudative wounds where removal of the excess liquid is critical not only to aid the wound healing process but also to avoid damage to the periwound area. For this reason, when further assessing the performance of the hydrogels, both chitosan-coated (90 mins) and non-coated systems will be tested.

4.4.4 Rate of honey release from the developed hydrogels

Upon honey incorporation into the hydrogels, it is necessary to monitor that it is retained in satisfactory concentration in order to prevent honey leakage from the scaffold. Nonetheless, the formulation should ensure that effective exposure to honey is maintained on the wound site throughout the treatment. The release pattern of an antibacterial agent from the hydrogel contributes, together with its intrinsic spectrum of antimicrobial activity and its concentration, to wound infection management. In other words, the active agent has to be released to actually impact the wound microbial environment[41].

Figure 4.6 shows that the release rate of honey from the tested hydrogels is independent of the concentrations of honey loaded, with 91.77 ± 8.34% and 95.32 ± 5.09% of honey released in 8 h from AL2 and AL4, respectively. Correspondingly, the honey release from AL2_C90 and AL4_C90 (chitosan-coated hydrogels) was not concentration-dependent, as can be seen from the overlapping in the release rate curves. In both cases, honey was continuously released from the scaffolds for 8 h, showing the potential for prolonged delivery of honey to an affected wound area that these hydrogels are designed to treat.

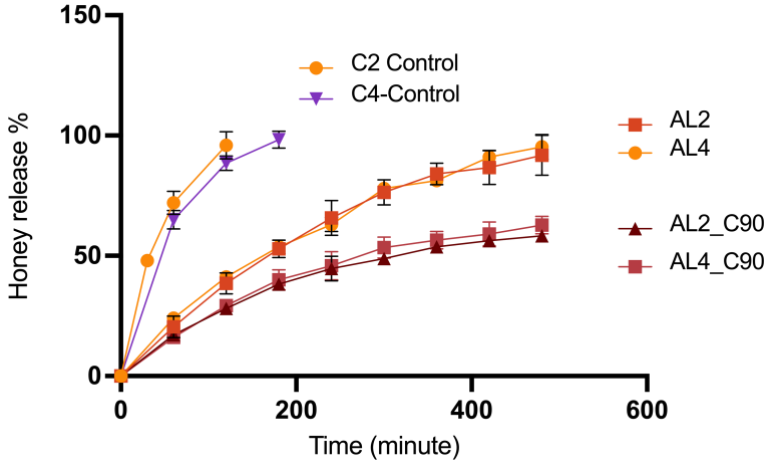


Figure 4.6 Honey release (percentage) from alginate hydrogels (chitosan-coated and not) with different honey concentrations over an 8h immersion in 200 mL PBS at 37 ±0.5 °C (as detailed in Section 4.3.8). The sample codes AL2 and AL4 indicate hydrogels with a honey concentration of 13.33 and 26.66%, respectively. The code C90 in the name indicate the application of a chitosan coating for an immersion time of 90 mins in the chitosan solution. Dialysis bags filled with honey solutions of concentrations equivalent to that of the hydrogel samples (indicated as 2H-Control and 4H-Control for AL2 and AL4 and their chitosan-coated analogues, respectively) were used to perform control tests. Each point represents the average of three replicates, with the error bars corresponding to the standard deviation.

The release of honey from amorphous hydrogels measured with a similar method (diffusion through dialysis bags) revealed a concentration-dependent release for both chitosan and Carbopol 934 hydrogels, in agreement with what was previously reported by El-Kased et al. More specifically, a complete release of honey was reached after three-hour monitoring from chitosan hydrogels containing 75% of honey, while only about 40% of honey was released from chitosan hydrogels containing 25% of honey[213]. When compared to the uncoated alginate hydrogels, chitosan treatment seemed to slow down the honey release, with a maximum cumulative release of 58.34 ± 2.66% for the lower honey concentration (13.33%) and 62.77 ± 3.57% for the maximum honey concentration tested (26.66%). This is similar to published work where the physical characterisation of honey-curcumin composite sponges revealed that chitosan concentration directly reduces drug-release speed. By contrast, in that study, the time required to obtain 80% curcumin diffusion from the hydrogels was inversely proportional to the alginate/chitosan ratio[481]. However, the release rate of honey from these scaffolds was not quantified, and it cannot be inferred that the kinetic release of honey could not differ. In the work by Straccia et al.[449], the maximum drug release reached during the observation time (8 h) was about 20% lower in chitosan-coated hydrogels than in the uncoated samples. This has been attributed to the coating layer hindering the drug diffusion from the alginate hydrogels. However, in the case of the honey-hydrogels investigated here, this reduced release rate cannot be entirely ascribed to the network modification due to the interaction between alginate and chitosan. In fact, the sugar content in the chitosan solution was measured before and after honey-hydrogel immersion to assess if part of the honey might have dissolved in the coating medium. Of the total quantity of honey incorporated in the hydrogels, 31 ± 6% and 26 ± 1% were detected in the chitosan solution where AL2 and AL4 had been immersed, respectively, whereas no sugar was detected in the fresh chitosan solution (pre-immersion). Therefore, upon chitosan coating, the final content of honey available within the scaffold to exert the biomedical effect was diminished. This indicates that the technological method of hydrogel fabrication plays a critical role in the drug release trend and ultimately can possibly affect the therapeutic effect. Other influential parameters are the method of honey incorporation, with hydrogels prepared with honey addition by direct mixing showing a typical burst release, and the intrinsic

structural features of different polymer networks, highly dependent on the degree of crosslinking[213].

4.4.4.1 Impact of dialysis tubing

Control tests were conducted to determine the extent to which the dialysis tubing prevented the passage of sugar and to evaluate by comparison the ability of the polymer network to retain honey. A cumulative release of 95.92 ± 5.70% for 2H-Control and 98.29 ± 3.47% for 4H-Control was reached in 2 and 3 h, respectively, indicating that the cellulose membrane used in the experimental set-up played a role in slowing the release of honey from the hydrogels. However, the percentage of honey released from all the fabricated hydrogels is significantly different (p value < 0.0001) from the release rate from the control experiments for both the honey concentrations evaluated here. This suggests that the method adopted here, when interpreted by considering the limitations of the set-up, can provide a useful estimation of the release performance of honey from the hydrogels developed in this study.

4.4.5 Final considerations on the optimised hydrogel formulation

In the previous sections of this chapter, the design and characterisation of alginate hydrogels were presented, with the evaluation of the effect produced by different modifications to the formulation. Some considerations were drawn, as follows:

- All hydrogels presented a superficial pH compatible with the requirements of topical application on damaged skin.
- Based on the results of the swelling tests, showing no statistical significance between the swelling degree of hydrogels coated with different immersion times, the 90-min immersion time in chitosan solution was chosen as the standard preparation procedure for chitosan-coated alginate hydrogels, to ensure consistency.
- Based on the results of the release tests, which showed a concentration-independent release rate, the highest honey concentration was selected for the next phases of this research. This decision was made keeping into consideration the loss of honey from the hydrogel during the coating in chitosan solution.

4.4.6 Incorporation of Irish heather honey and manuka honey into the designed and characterised hydrogels

In the previous sections, alginate hydrogels were developed and optimised using commercial multifloral honey. However, the focus of the project illustrated in this thesis is on Irish honey, specifically on samples marketed as unifloral heather honey. With the aim to evaluate the applicability of the previously developed hydrogel formulation to different varieties of honey, i.e. their reproducibility, the methods of characterisation described in Section 4.3 have been applied again, and the tests were repeated with the hydrogels containing the Irish honey samples under investigation in this project. This is particularly relevant because, to the best of our knowledge, no other research evaluating the incorporation of Irish honey in alginate hydrogels has been published. As discussed in Chapter 2 (Section 2.3.1), only one (sample IH2) of the honey samples, all marketed as heather honey, was confirmed as unifloral heather honey according to the melissopalynological analysis, which is internationally considered the only standard method to determine the floral origin of honey. Therefore, evaluating sample IH2 incorporation in the hydrogel was particularly important due to the peculiar rheology of heather honey. This honey variety presents a gel-like, highly viscous texture with non-Newtonian and thixotropic flow, possibly ascribable to a high protein content[482,483]. Consequently, the assessment of possible variations in the swelling and release rate, which could affect the process of incorporation into the hydrogels, has been deemed appropriate. This rheological behaviour is so distinctive that deviations from it have been suggested as a sign of adulteration by dilution with other types of honey[484,485].

4.4.6.1 Swelling behaviour of alginate hydrogels loaded with Irish honey

All the alginate hydrogels incorporated with Irish honey at a concentration of 26.66% showed a swelling behaviour that is highly similar to the trend reported in Section 4.4.3.1 for AL4 (hydrogel with commercial multifloral honey), as shown in Figure 4.7. Namely, the swelling values of hydrogels loaded with the confirmed unifloral Irish heather honey were 57.43 ± 4.57% for IH2-AL4 and 186.86 ± 6.73% for IH2-AL4_C90 after 3 h of immersion in the medium (PBS, 200 mL).

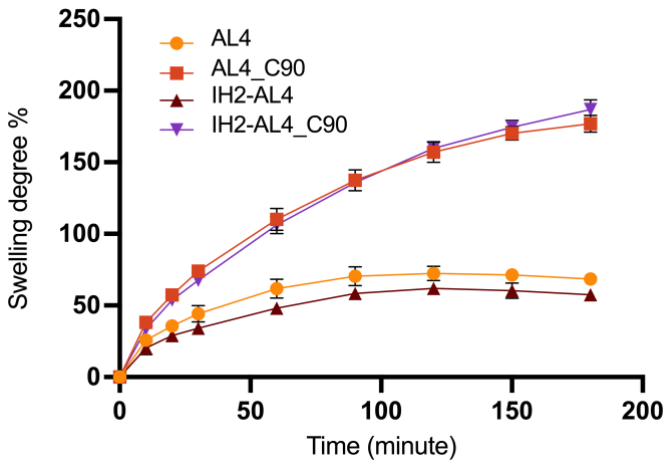


Figure 4.7 Representative graph of the swelling behaviour of alginate hydrogels (uncoated and chitosan-coated) loaded with Irish heather honey (marked with the prefix IH2) as compared to hydrogels containing commercial honey with no specified botanical origin. AL4 indicates alginate hydrogels with 26.66% honey, and the code C90 in the name indicates the application of a chitosan coating by immersion in the chitosan solution for 90 mins. The tests were performed by immersing the samples in 200 mL of PBS. Each point represents the average of three replicates, with the error bars corresponding to the standard deviation.

For each honey-hydrogel, the coating with chitosan solution caused a statistically significant increase (p value < 0.0001) in the swelling degree (Figure 4.8). The maximum values after 24 h in the swelling medium ranged between 69.59 ± 1.32% for IH2-AL4 and 99.01 ± 6.97% for IH4-AL4 for uncoated alginate hydrogels, while for chitosan-coated hydrogels the registered range was between 211.69 ± 9.13% for MH-AL_C90 and 258.87 ± 6.09% for IH2-AL_C90.

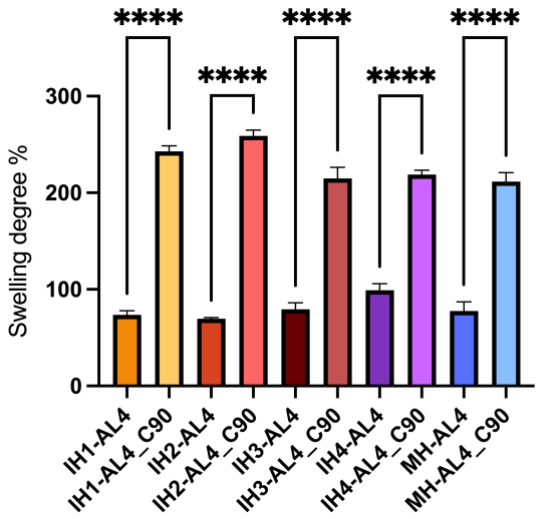


Figure 4.8 Effect of chitosan coating on the maximum swelling (calculated as per Equation 4.1) of alginate hydrogels loaded with different Irish honey samples (IH1, IH2, IH3 and IH4) and manuka honey (MH) after immersion for 24 h in 200 mL of PBS. AL4 indicates alginate hydrogels with 26.66% honey, and the code C90 in the name indicates the application of a chitosan coating by immersion in the chitosan solution for 90 mins. Each column represents the average of three replicates, with the error bars corresponding to the standard deviation.

4.4.6.2 Release kinetics of Irish honey samples from alginate hydrogels

The diffusion of the Irish honey samples through the alginate-based polymeric network was completed in 8 h (Figure 4.9) with the percentage of honey released between 87.70 ± 3.58 for IH1-AL4 and 102.02 ± 3.65 for MH-AL4. Chitosan-coated hydrogels showed a maximum cumulative release ranging between 70.11 ± 1.87% for IH2-AL_C90 and 78.73 ± 1.13% for IH4-AL_90. As previously discussed in Section 4.4.4, this reduced honey release when compared to the uncoated scaffolds with equal honey concentration can be explained by the fact that a percentage between 23.91 ± 3.31% and 28.16 ± 2.43% of the honey originally incorporated in the hydrogels was lost during the coating process through immersion in the chitosan solution. These release rates for both coated and uncoated hydrogels are comparable to what is shown for hydrogels containing commercial polyfloral honey and suggest that the release performance of honey depends on the structural properties of the polymer networks rather than on the botanical origin of honey.

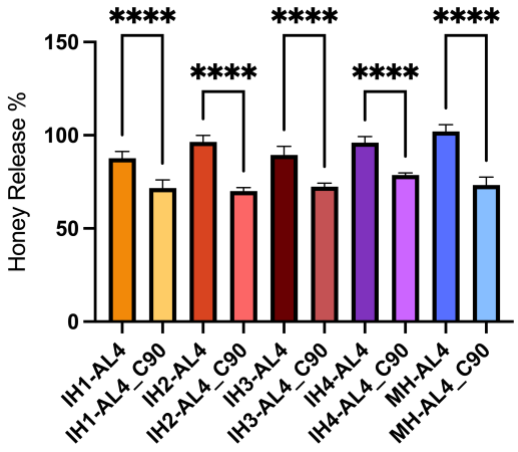


Figure 4.9 Cumulative honey release (percentage of the total incorporated quantity) after 8 h of immersion in 200 mL PBS at 37 °C. The hydrogels were loaded with different Irish honey samples (IH1, IH2, IH3 and IH4) and manuka honey (MH). AL4 indicates alginate hydrogels with 26.66% honey, and the code C90 in the name indicates the application of a chitosan coating by immersion in the chitosan solution for 90 mins. Each column represents the average of three replicates, with the error bars corresponding to the standard deviation.

4.4.7 Hydrogels' antibacterial activity

The ultimate goal of this study was to develop honey-based hydrogels for the treatment of infected wounds. Therefore, it was important to assess the antibacterial properties of these formulations, and for this aim, a dynamic contact assay was employed, which was followed by a viable cell counting method. Two honey samples were chosen to be

incorporated into the hydrogels for this assay, namely the confirmed unifloral Irish heather honey (sample IH2) and the manuka honey (MH) sample. The hydrogels developed in this study were tested against a Gram-positive and a Gram-negative bacterium, *S. aureus* and *E. coli*, respectively, which commonly infect chronic wounds[157]. When tested against *S. aureus,* all hydrogel samples induced a statistically significant (p value ≤ 0.0001) growth inhibition when compared to the control (untreated *S. aureus* cultured in TSB), measured as CFU/mL quantified in the viable cell counting method (Figure 4.10, a). The lowest percentage of reduction in bacterial growth was achieved by sample AL (85 ± 17%) and the highest by IH2-AL4_C90 (99.7 ± 0.2%), as can be seen in Figure 4.10, b). However, no statistical difference was observed between the inhibition achieved with AL (hydrogel without honey) and the honey-loaded hydrogels (IH2-AL4 and MH-AL4), regardless of the honey variety incorporated in the scaffold. This means that the alginate hydrogel formulated here without honey possesses an intrinsic antibacterial effect against *S. aureus* that is not significantly improved by the addition of either Irish heather honey or manuka honey. Likewise, the addition of a chitosan coating (samples AL_C90, IH2-AL4_C90 and MH-AL4_C90) did not significantly improve the antibacterial effect. Nonetheless, each of the chitosan-coated hydrogel samples achieved a reduction in bacterial growth of above 95%.

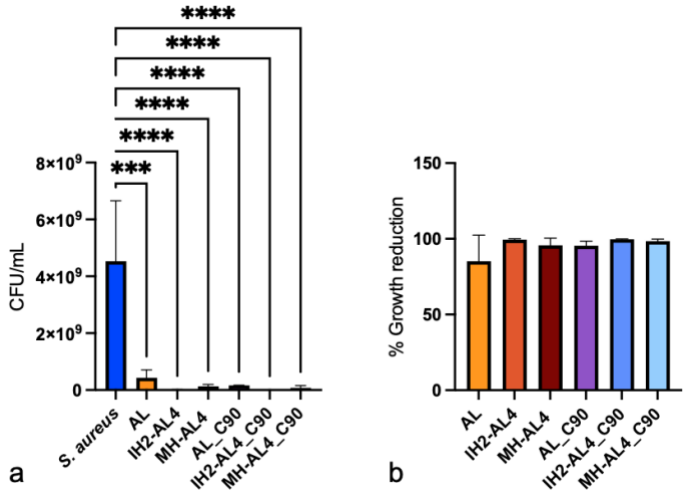


Figure 4.10 Hydrogels' antibacterial activity against S. aureus tested via dynamic contact method (as detailed in Section 4.3.9). a) CFU/mL of S. aureus in TSB (control) and in the presence of a specimen of the developed hydrogels after 24 h of incubation at 37 °C at 180 rpm. b) Percentage of growth reduction of S. aureus, calculated as per Equation 4.2 based on the CFU/mL shown in a). AL4 indicates alginate hydrogels with 26.66% honey, namely Irish heather honey (IH2) or manuka honey (MH). The code C90 in the name indicates the application of a chitosan coating by immersion in the chitosan solution for 90 mins. Results were expressed as the average value for three biological replicates (four technical replicates each) ± standard deviation.

When tested against *E. coli*, the hydrogels did not show antibacterial activity (Figure 4.11, a), apart from a percentage of growth reduction observed for AL_C90 and MH-AL4_C90, which was, however, not a statistically significant inhibition in growth. Furthermore, the bacterial growth was observed to be stimulated by the hydrogels, making them not suitable against *E. coli*, and it would be valuable to investigate possible mechanisms by which the growth stimulation occurs.

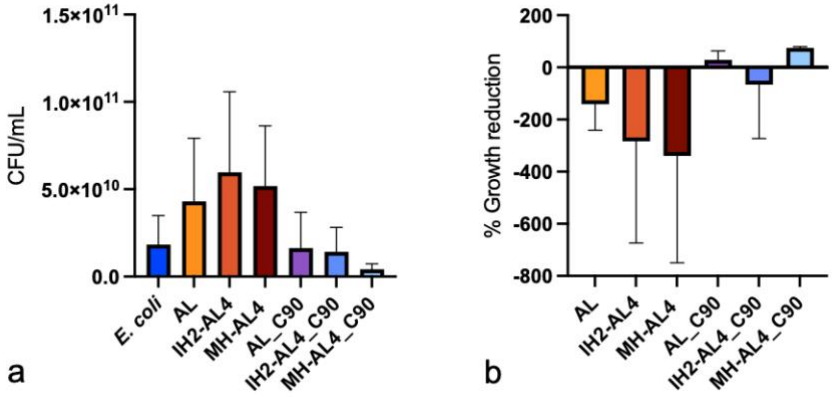


Figure 4.11 Hydrogels' antibacterial activity against E. coli tested via dynamic contact method (as detailed in Section 4.3.9). a) CFU/mL of E. coli in TSB (control) and in the presence of a specimen of the developed hydrogels after 24 h of incubation at 37 °C at 180 rpm. b) Percentage of growth reduction of E. coli, calculated as per Equation 4.2 based on the CFU/mL shown in a). AL4 indicates alginate hydrogels with 26.66% honey, namely Irish heather honey (IH2) or manuka honey (MH). The code C90 in the name indicates the application of a chitosan coating by immersion in the chitosan solution for 90 mins. Results were expressed as the average value for three biological replicates (four technical replicates each) ± standard deviation.

A higher inhibition degree against Gram-positive species than against Gram-negative is in line with what has been reported for chitosan-based hydrogels in previously published studies by Zhang et al.[486] (inhibition determined in viable cell-counting method) and Mohamed et al.[487] (by agar well diffusion test). However, Sun. et al.[470] observed a higher susceptibility of the Gram-negative *E. coli* than the Gram-positive *S. aureus* towards all the alginate-based hydrogel formulations in the dynamic contact assay. This different susceptibility of Gram-positive and Gram-negative to antibacterial treatments has been previously suggested in the literature to be due to the different cellular structures of these two classes of bacteria, in particular their outer enclosing layer[487]. Nonetheless, since the hydrogels developed in this study were only tested against *S. aureus* and *E. coli*, in order to confirm a different susceptibility of bacteria to the hydrogels' antibacterial effect due to their Gram-specificity, it would be necessary

to do a screening of several bacteria belonging to the two classes (Gram-positive and Gram-negative).

4.5 Conclusions

In this chapter, honey-hydrogels were successfully formulated, showing robust and reproducible performance with different honey varieties, and indicating good potential for absorption of wound exudate, with swelling degrees up to 258.87 ± 6.09%, and honey release completed at 8 h. The evaluation of functional parameters such as the swelling behaviour and the release rate suggested a promising potential to enhance wound healing, by keeping an optimum moist environment and releasing honey on the wound site at a time compatible with the frequency of dressing change.

The assessment of the antibacterial activity has shown, as previously reported in the literature for similar formulations, that the polymers selected for the development of the hydrogels in this study possess a remarkable antibacterial effect on *S. aureus*. However, the incorporation of honey has not been shown here to further improve the hydrogels' capacity to inhibit bacterial growth, despite honey's renowned intrinsic antibacterial effects and the encouraging preliminary results from Chapter 3. Nonetheless, the endpoint of a hydrogel for biomedical application would be to promote wound healing through an anti-inflammatory effect and by inducing re-epithelialisation and revascularisation, features extensively observed in honey, as extensively discussed in Chapter 1. This means that, despite honey incorporation not being critical to the antibacterial effect of these scaffolds, it could be of great relevance to investigate its contribution to other features critical to improving the course of wound healing. However, a wider range of honey concentrations should be tested to evaluate if the incorporation of higher honey concentrations within the hydrogels could improve the antibacterial effect or, potentially, a healing effect.

Chapter 5 Final conclusions and future perspectives

5.1 Significance of the work within the current state-of-the-art on honey research for biomedical application

Honey's potential for medicinal applications and its health benefits have been appreciated since antiquity. However, a shift in perspective is needed to reframe honey from a folkloristic practice of an ancient past to a valuable resource for the present and the future. In recent times, natural products such as honey have gained recognition for their potential to tackle modern-day medical challenges, such as antibacterial resistance (as discussed in Chapter 1). In the case of honey, New Zealand manuka honey gained huge international popularity in the past decades and is now renowned as the gold standard of medical-grade honey worldwide. Demand for manuka honey far outstrips its production, however, with counterfeit honey estimated to account for up to 80% of marketed manuka honey[488]. It is therefore worth considering that several other honey varieties, locally used in traditional ethnopharmacology for centuries, have been studied in the literature and are emerging as worthy of further investigation for the implementation of their use in medical settings and the development of commercial health products. The numerous biologically effective honey varieties should not be necessarily thought of as an alternative to manuka honey, but rather as complementary varieties in an expanding market of medical-grade honey, with the aim to offer greater therapeutic coverage. In the Irish context, a variety of honey types are produced, but Irish honey's characteristics have not been fully explored. Therefore, this thesis aimed to address this gap by investigating a rare artisanal variety of Irish honey, heather honey (as per commercial denomination), with an attempt to unlock its potential for biomedical applications and compare it with manuka honey. In particular, Chapter 2 characterised the composition of the honey samples investigated here, exploring aspects relevant to both their commercialisation as a food product and their biomedical use, namely compliance with international physicochemical standards, total phenolic content effect botanical origin determination to valorise honey's authenticity and protect consumers against adulteration and fraud, organoleptic features and aroma markers to confirm the botanical origin. Chapter 3 investigated the antibacterial activity of the honey samples, using both standard validated and new non-invasive analytical strategies to assess the ability of honey to inhibit the growth of bacteria common in

wound infections. Finally, in Chapter 4 a honey-loaded hydrogel formulation was designed and optimised under both functional (surface pH, fluid uptake capacity, and honey release rate) and biological (antibacterial) aspects. In this final chapter, future perspectives for the project are described.

5.2 Limitations of the project

This thesis used a multidisciplinary approach where analytical chemistry, microbiology and material chemistry were utilised to contribute to the current knowledge in apitherapy with a focus on Irish honey. However, given the fundamental nature of the project, some limitations are acknowledged. The research reported here would be strengthened with further work in the following aspects:

Measuring the antibacterial effect of honey samples is a fundamental preliminary step into understanding its potential for use to treat infections; to further comprehend this potential, the identification of the mechanism responsible for the observed antibacterial effect of a given honey type is a crucial step. Different techniques should be explored, such as the use of enzymatic assay, treatment with catalase and proteinase K, isolation of peptides of interest and GOx, to identify all the contributing factors[91]. The use of artificial honey as a control is important to determine any effect attributable simply to the high sugar content[318].

The assessment of the antibacterial activity of the honey samples in this study was carried out against only to bacterial strains, the Gram-positive *S. aureus* and the Gram-negative *E. coli.* However, to fully determine the spectrum of action of the Irish honey samples and honey-hydrogels, further testing should be carried out to evaluate the effectiveness in inhibiting the growth of a variety of bacterial species (both Gram-positive and Gram-negative). Moreover, to better appreciate their potential applicability as antibacterial products, multiple strains, including antimicrobial-resistant strains, should be included. Testing the samples against a variety of bacterial species would also allow to optimise and validate the proposed VOC-based diagnostic approach while keeping into account inter-and intra-species variability in terms of susceptibility to treatments and metabolite emission.

Among the quality parameters relevant for honey characterisation, aspects of freshness should also be investigated. Specifically, the Codex Alimentarius[42] and the Council directive 2001/110/EC[226] indicate the quantification of diastase activity and of hydroxymethylfurfural (HMF) as the parameters of reference. The diastase activity, expressed in Schade scale, should generally not be less than 8 (lower temperatures would indicate denaturation of the enzyme), while the HMF content is set to a maximum of 40 mg/kg (exceptions apply based on honey varieties or specific geographic regions). These parameters indicate if the samples underwent, before delivery to the laboratory, improper storage or processing conditions with exposure to high temperatures which are known to affect honey's antibacterial activity. Therefore, for example, their quantification could aid in the interpretation of higher-than-average MIC values.

As presented in Chapter 2, markers previously identified for the determination of heather honeys were also detected in the verified heather honey sample investigated here. However, the small sample size (which represented the totality of commercially available honey samples marketed as Irish heather honey at the time of study) did not allow for statistical analysis to investigate the relationship between the various parameters of composition and to establish if the abundance of markers of botanical origin correlate with the pollen content. Therefore, in order to evaluate if the results observed here are representative for this honey type, a bigger sample size would be needed. Moreover, this would allow for multivariate statistical analysis such as Principal Component Analysis (PCA) to investigate the correlation between relevant aspects of composition of the samples and observed biomedical effect of interest.

Tackling these limitations by investigating these aspects would further improve the present study and the deeper insight that would be gained through these additional experiments would represent an increase in the solid knowledge foundation for the future perspectives described below.

5.3 Future perspectives

5.3.1 VOC monitoring and multivariate analysis for a comprehensive understanding of complex systems

For honey to be of benefit in biomedical applications, a rapid, robust, and selective characterisation is required, to determine the extent to which the honey meets the biomedical requirements. Additionally, the requirements that sampling methods are non-invasive, and non-destructive are of emerging importance in providing reliable and quick results for the analysis of complex samples. A multifaceted approach to meet all these requirements was adopted in this thesis by exploring the potential of fingerprinting the volatile fraction of samples of honey and bacterial cultures as an identification and diagnostic tool with broad applicability, from the authenticity of food products and aroma features to biological diagnostic studies. The foundation of identification methods like the one explored in this thesis is the screening of the volatile fraction of a representative number of samples to define characteristic markers with diagnostic value. The identification of multiple signature compounds and value ranges is advisable, as opposed to the use of one single marker, to establish a reliable identification system with a higher discriminatory power. Therefore, such methods are based on the establishment of a comprehensive database of characteristic compounds. To further improve the significance of this study, a strategy such as diagnostic ratio analysis could be employed. Diagnostic ratio analysis, widely used in the environmental and forensic field, uses the characteristic concentration ratio of selected pollutants (such as polycyclic aromatic hydrocarbons, PAH) to identify their sources[489,490]. The two compounds of concern paired in each ratio are chosen based on their similar mass; consequently, they are assumed to show comparable physicochemical behaviour[491]. Nonetheless, caution has been suggested in the selection of compounds that undergo different fates in the system considered (e.g., different volatilisation rates or degradation processes) which would lead to the ratio at the emission source and in the sample not being constant (indicated as “receptor-to-source ratio” by Zhang et al.)[491,492]. This has considerable implications as different sampling times have been suggested to affect the distribution of compounds and lead to significantly altered ratios[490]. This limitation should be considered carefully when applying the method to

biological samples, such as the bacterial cultures examined in this thesis, where considerable shifts in emissions were observed at different stages of growth and metabolism, as observed in Chapter 3. Therefore, suitable validation should be conducted to evaluate its applicability in the specific case of this project considering the abovementioned limitations. Nonetheless, the use of diagnostic ratio analysis in this project is considered worth investigating to assess its potential to implement a systematic, repeatable and rigorous identification method for both botanical origin and pathogenic bacteria.

5.3.2 Further investigation of honey's medical properties

Two features are of paramount importance for honey destined for medical uses on wounds, namely antimicrobial activity, and effectiveness in inducing complete healing[183]. The antibacterial activity of Irish honey samples and the honey-loaded hydrogels against *S. aureus* and *E. coli* has been assessed in Chapter 3 and Chapter 4, respectively. Biofilms are a great global health concern and are frequently found in most chronic wounds contributing to the impaired healing process. Previous reports have already compared heather and manuka honey in their effect on inhibiting biofilm formation. The two varieties have been shown to possess comparable and complementary efficacy against numerous bacterial species in both single and mixed-biofilms[122,123]. Therefore, assessing the antibiofilm activity of the honey and hydrogel samples examined in this thesis would be an important measure of their applicability to a variety of infections.

The second area that should be investigated to further unlock honey's potential as a medical product is to assess honey's wound healing properties. Both the direct application of honey and its formulation (whose development was described in Chapter 4) should be evaluated to determine if the incorporation of honey in a scaffold would hinder the delivery of its beneficial compounds or improve its healing properties. To fulfil this aim, an *in vitro* wound model employing a full-thickness living skin equivalent would be developed as a viable alternative to current animal models. Compared to 2D wound models, 3D models mimic the complexity of the human skin and the multitude of cell types and extracellular components with their respective functions[493–495]. Engineered human skin equivalents can be functionalised not only to support the

physiological skin microbiota[496–498] and study its VOC emissions[499] but also to investigate various cutaneous conditions *in vitro*, allowing to test potential treatments, i.e. their efficacy, their absorption and the delivery of bioactive agents[500–504]. Furthermore, they can be artificially inoculated with microbial cultures to offer a more complex representation of the infection microenvironment[76,505,506], and can therefore be used to evaluate the antibacterial properties of honey in a more complex model.

5.3.3 Perspectives for honey-loaded wound dressings

In Chapter 4, alginate hydrogels for wound dressings incorporating honey were successfully formulated and crucial aspects were optimised (surface pH, swelling degree, and honey release rate). However, further optimisation in the applicability of the hydrogels to different types of wounds could be achieved by improving technical aspects of their design, such as aspects of permeability and mechanical properties.

When developing wound dressings, an optimal balance between permeability and breathability is essential to ensure that no occlusion or liquid accumulation occurs around the wound compromising successful healing and causing maceration of the periwound area[220]. The European Pharmacopoeia, with implemented test methods for Primary Wound Dressing (BS EN13726)[41], and the American Society for Testing and Materials[507] indicate critical parameters to assess the "aspect of absorbency" of wound covers. The free swell absorptive capacity has been evaluated in Chapter 4, but further tests could provide valuable insights into the suitability of the developed honey-based formulations for the treatment of exudative wounds. The Moisture Vapour Transmission Rate (MVTR) is measured at specific temperature and humidity values, by mounting a hydrogel specimen on the mouth of a vessel containing deionised water, saline solution, or PBS buffer, forming a closed system[41]. The hydrogel's lower surface is exposed to the vapour at equilibrium in the headspace above the liquid while the upper surface is in contact with the external environment. Alternatively, the Water Vapour Transmission Rate (WVTR) can be measured, with the hydrogel sample in direct contact with the liquid. The quantification is carried out by gravimetric method, by weighing the assembly at regular intervals[41,185,507,508]. The assessment of the MVTR is particularly critical for wound management products designed for the treatment of

burns, where a critical balance between breathability, evaporative water loss and moisture needs to be ensured since excessive dehydration can aggravate hypothermia and hypermetabolism[509,510].

Another relevant feature requiring evaluation would be the hydrogels' conformability, i.e., their ability to stretch and bend to adapt to and accommodate the shape and motion of the human body without causing skin damage[41]. Aspects such as tensile stress and strain, and Young's modulus for compressive strength have been widely investigated in honey-based dressings[413,468,511]. However, honey incorporation has been shown to influence the scaffold's properties (such as rheology, porosity, thermal properties, fluid uptake, degradation rate and more)[216,219,221,512,513]. Considering that honey varieties such as heather honey present a characteristic texture and rheological properties, it would be of interest to investigate to what extent honey's incorporation alters the rheology of the hydrogel formulation and, consequently, its mechanical performance[482,514,515].

While the characterisation of the hydrogels developed in this thesis was conducted *in vitro*, the endpoint of the development of any wound cover would be their use *in vivo*. The evaluation of permeability and mechanical performance would therefore be the next crucial step in investigating the applicability of these honey-formulations on a wound.

5.4 Contributions to the field and final conclusions

This project investigated samples of Irish honey, aiming to characterise their composition and their antibacterial potential, when used alone or incorporated in a hydrogel formulation. The use of VOC fingerprinting was proposed here not only to characterise honey's aroma, but also to assess honey's antibacterial activity. Despite being labelled and marketed as heather honey, the melissopalynological analysis only confirmed the botanical origin of one Irish honey sample, IH2. Volatile compounds, previously identified as markers of honey from the Ericaceae family, were identified at varying concentrations in the samples, and suggested as worthy of further investigation in a bigger sample group for their use in botanical origin determination. When testing the antibacterial activity of the honey samples, it was found that Irish heather honey

exerted a bacterial growth inhibition of interest when compared to that of manuka honey against *S. aureus* and, with higher susceptibility, against *E. coli*, as shown by the significant reduction in bacterial volatile metabolite emission. Finally, the hydrogels developed in Chapter 4 exerted a significant growth inhibition against *S. aureus* in the viable cell counting method, while they were shown to be ineffective against *E. coli*. However, this effect was observed regardless of the honey concentration, indicating that the intrinsic antibacterial properties of the polymer formulation, and not the honey, were to be considered responsible for it. Despite the limitations here acknowledged, an innovative approach was successfully proposed with the transversal use of VOC signatures, where the screening of the volatile fraction and the identification of characteristic compounds were used to assess honey's organoleptic features, evaluate the extent to which VOC and melissopalynology could be complementary to identify the floral origin of honey, to detect bacterial growth, to assess the efficacy of antibacterial treatment and potentially monitor the course of the recovery from infection. The combination of complementary techniques proposed here enabled us to add precious knowledge to the field. and advance honey research globally, while placing Irish honey in a more international context.

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Appendix A

Table A-1 Pollen composition (types and frequency) in four Irish honey samples marketed as heather honey (IH1, IH2, IH3 and IH4), identified by melissopalynological analysis. Results are expressed as a percentage (average ± standard deviation) of the total pollen count identified.

	IH1		IH2		IH3		IH4	
	Mean %	**SD**	**Mean %**	**SD**	**Mean %**	**SD**	**Mean %**	**SD**
Calluna	17	3	73	2	40	6	6	1
Vicia	40	2	-	-	4.7	0.7	4.7	0.5
Rosa	<1	-	10	4	-	-	23	3
Arabidopsis	-	-	-	-	13	4	14	4
Agrostis	-	-	-	-	<1	-	11	4
Geum	3.2	0.9	2	1	<1	-	10	4
Arum	<1	-	-	-	8	2	-	-
Oxalis	8	1	<1	-	-	-	<1	-
Hedera	2.2	0.5	1.6	0.8	<1	-	7	4
Angelica	<1	-	<1	-	6	2	-	-
Jasione	6	2	-	-	<1	-	-	-
Capsella	5	3	1.4	0.4	-	-	<1	-
Sambucus	5	2	<1	-	-	-	6	4
Potentilla	-	-	1.2	0.2	-	-	5	3
Castaneae	-	-	<1	-	-	-	5	2
Juncus	-	-	<1	-	4.8	0.2	3	2
Asteraceae	2.6	0.7	2.4	0.4	5	2	1.7	0.2
Molinia	-	-	4.7	0.3	-	-	-	-
Salix	-	-	-	-	4	1	-	-
Filipendula	-	-	-	-	4	1	-	-
Lolium	-	-	-	-	4	2	-	-
Dactylis	3	2	-	-	-	-	-	-
Festuca	2.5	0.4	-	-	-	-	-	-
Crataegus	<1	-	-	-	1.6	0.6	-	-
Betula	-	-	-	-	1.5	0.1	-	-
Rumex	<1	-	-	-	-	-	-	-
Sisyrinchium	-	-	-	-	<1	-	-	-
Centaurea	<1	-	-	-	-	-	-	-
Castanea	<1	-	-	-	-	-	-	-
Larix	<1	-	<1	-	-	-	-	-
Cirsium	<1	-		-	-	-	-	-
Hyperichum	<1	-	<1	-	-	-	-	-
Trifolium	-	-	-	-	<1	-	-	-
Alopecurus	-	-	-	-	<1	-	-	-
Galium	-	-	-	-	<1	-	<1	-
Ulex	-	-	-	-	<1	-	-	-

Continues on page A3

Table A-1 (continued)

Ilex	-	-	-	-	<1	-	-	-
Holucs	-	-	-	-	-	-	<1	-
Vaccinium	-	-	-	-	-	-	<1	-
Huperzia	-	-	-	-	-	-	<1	-
Conopodium	-	-	-	-	-	-	<1	-
Total	93.8		97.6		96.2		96.5	

Table A-2 Volatile Organic Compounds (VOCs) extracted from the headspace of four Irish honey samples marketed as heather honey (IH1, IH2, IH3 and IH4) by HS-SPME followed by GC-MS. RT: Retention time expressed in mins; RI: experimental Retention Index, calculated according to the Van den Dool and Kratz formula based on the RT of standard C7–C30 n-alkanes on an SLB®-5ms capillary column. Abundance is expressed as the average peak area; the total volatile fraction identified is reported at the bottom. Notes: [i] Exact isomer not identified; [ii] Tentatively identified.

				Average Peak Area			
#	**RT**	**RI**	**Compound ID**	**IH1**	**IH2**	**IH3**	**IH4**
1	3.88	831	Furfural	1.34E+07	5.06E+07	1.61E+07	3.06E+07
2	3.97	836	Butanoic acid, 3-methyl-	3.27E+06	4.40E+06	6.82E+06	4.11E+06
3	4.06	842	Butanoic acid, 2-methyl-, ethyl ester	-	-	-	3.01E+06
4	4.12	846	Butanoic acid, 3-methyl-, ethyl ester	-	2.09E+06	8.60E+05	6.30E+06
5	4.16	848	2-Furanmethanol	-	-	4.73E+05	-
6	4.37	861	Ethylbenzene	3.46E+05	5.48E+05	2.40E+05	-
7	4.4	863	1-Hexanol	4.53E+05	4.00E+05	1.54E+06	3.26E+05
8	4.53	871	p-Xylene	-	4.41E+05	-	-
9	4.78	886	2-Heptanone	4.73E+05	7.59E+05	5.28E+05	5.05E+05
10	4.93	896	Styrene	1.54E+06	2.07E+06	1.15E+06	5.95E+05
11	4.99	899	Nonane	1.75E+06	8.23E+05	1.58E+06	1.31E+06
12	5.02	901	5-Ethyl-2-methyl-2-vinyltetrahydrofuran	-	-	7.18E+05	-
13	5.03	901	Heptanal	-	1.71E+06	-	-
14	5.25	910	Ethanone, 1-(2-furanyl)-	1.23E+06	1.41E+06	8.86E+05	1.14E+06
15	5.42	917	Butyrolactone	2.53E+05	-	-	-
16	5.79	931	2-Heptanone, 4-methyl-	7.49E+05	-	4.10E+05	-
17	5.87	934	α-Pinene	-	3.17E+05	-	2.65E+05
18	6.28	950	Pentanoic acid, 3-methyl-, ethyl ester	-	-	-	3.63E+06
19	6.3	951	Isocumene	-	3.39E+05	-	-
20	6.45	957	2-Furancarboxaldehyde, 5-methyl-	-	-	-	1.19E+06
21	6.57	962	Benzaldehyde	1.86E+08	2.46E+08	2.03E+08	1.79E+08
22	6.62	964	1-Heptanol	-	-	2.77E+05	-
23	6.78	970	Dimethyl trisulfide	2.25E+06	-	-	-
24	6.86	973	1-Octen-3-ol	3.89E+06	7.07E+06	2.88E+06	9.64E+05
25	7.12	983	2-Octanone	-	7.42E+05	-	-
26	7.15	985	Furan, 2-pentyl-	3.15E+05	9.53E+05	2.37E+05	3.85E+05
27	7.3	991	Hexanoic acid, ethyl ester	-	7.00E+05	-	1.88E+06
28	7.48	998	Octanal	3.31E+06	8.24E+06	4.20E+06	6.38E+06
29	7.88	1011	Benzyl chloride	-	1.20E+06	2.43E+06	-
30	8.43	1029	Benzyl alcohol	9.73E+06	1.19E+07	1.01E+07	4.77E+06
31	8.64	1036	β-Isophorone	2.10E+06	4.35E+06	8.20E+05	6.09E+05
32	8.73	1039	Benzeneacetaldehyde	4.13E+07	4.22E+07	6.47E+07	2.96E+07
33	8.89	1044	Eucalyptol	-	-	1.63E+06	-
34	9.1	1051	2-Octenal	-	2.25E+06	-	-
35	9.23	1055	Benzenemethanol, α-methyl-	-	-	-	1.27E+06

Continues on page A5

Table A-2 (continued)

36	9.41	1061	Acetophenone	2.35E+07	3.92E+07	3.53E+07	1.60E+07
37	9.54	1065	cis-Linalool oxide	1.15E+07	3.54E+06	1.24E+07	2.10E+07
38	9.97	1079	Benzaldehyde, 2-methyl-	-	-	5.57E+05	-
39	10.03	1081	trans-Linalool oxide	2.10E+06	-	2.94E+06	8.46E+06
40	10.07	1082	2-Nonanone	-	1.28E+06	1.46E+06	-
41	10.23	1088	Heptanoic acid, ethyl ester	-	-	-	6.91E+05
42	10.28	1089	Benzoic acid, methyl ester	-	-	5.52E+05	-
43	10.38	1092	Linalool	3.21E+06	4.07E+06	3.85E+06	1.09E+07
44	10.52	1097	Hotrienol	-	-	-	2.00E+07
45	10.53	1097	Nonanal	2.03E+07	2.01E+07	1.83E+07	1.48E+07
46	10.94	1110	Phenylethyl Alcohol	8.73E+06	8.84E+06	7.69E+06	1.75E+07
47	11.24	1120	Isophorone	1.30E+08	1.47E+08	7.21E+07	6.24E+07
48	11.32	1122	Benzyl methyl ketone	1.37E+06	2.05E+06	9.27E+05	-
49	11.75	1135	Lilac aldehyde (isomer I)	3.66E+06	3.68E+06	6.39E+06	1.23E+07
50	11.93	1141	4-Oxoisophorone	3.59E+07	3.42E+07	2.19E+07	-
51	12.04	1144	Lilac aldehyde (isomer II)	-	-	1.46E+07	3.43E+07
52	12.06	1145	Lilac aldehyde (isomer III)	-	-	-	2.95E+07
53	12.11	1147	Ethanone, 1-(1,4-dimethyl-3-cyclohexen-1-yl)-	-	-	7.67E+05	-
54	12.12	1147	2-Hydroxyisophorone	9.70E+07	1.59E+08	3.90E+07	-
55	12.21	1150	p-Methoxystyrene	2.51E+05	3.38E+05	-	-
56	12.35	1154	Lanierone	1.44E+07	5.00E+07	5.25E+06	7.84E+06
57	12.49	1158	Lilac aldehyde A (isomer IV)	3.07E+06	2.50E+06	4.92E+06	1.07E+07
58	12.63	1163	Pyruvophenone	-	4.95E+06	1.78E+06	-
59	12.65	1163	1,4-Cyclohexanedione, 2,2,6-trimethyl-	-	-	1.71E+06	-
60	12.72	1166	1-Nonanol	-	4.45E+06	4.57E+06	-
61	12.76	1167	Benzoic acid, ethyl ester	2.13E+07	9.65E+07	1.56E+07	1.85E+08
62	12.85	1170	Benzeneacetic acid, methyl ester	2.88E+06	5.61E+06	1.54E+06	-
63	12.9	1171	endo-Borneol	5.32E+05	1.34E+06	3.84E+06	2.62E+05
64	13.12	1178	Terpinen-4-ol	-	2.59E+06	6.76E+05	-
65	13.28	1183	p-Cymen-8-ol	2.07E+06	1.89E+06	-	-
66	13.29	1183	Octanoic acid	-	8.04E+05	6.95E+06	4.67E+05
67	13.45	1188	Octanoic acid, ethyl ester	-	-	-	2.15E+06
68	13.57	1192	α-Terpineol	-	3.85E+06	1.59E+06	1.17E+06
69	13.61	1193	Myrtenal	-	-	-	1.15E+06
70	13.7	1196	Safranal	1.12E+06	2.10E+06	6.68E+05	1.12E+06
71	13.81	1199	Decanal	7.65E+06	1.21E+07	7.72E+06	7.08E+06
72	14.33	1217	p-Menth-1-en-9-al	6.65E+05	-	1.38E+06	4.98E+06
73	14.41	1219	4-Methyleneisophorone	-	2.76E+05	-	-
74	14.44	1220	Furan, 3-phenyl-	-	4.51E+06	6.19E+06	1.59E+06
75	14.7	1229	2-Hydroxy-4-oxoisophorone	5.31E+06	6.68E+06	4.18E+06	-
76	14.99	1239	Benzeneacetic acid, ethyl ester	4.25E+07	1.45E+08	3.75E+07	-

Continues on page A6

Table A-2 (continued)

77	15.25	1247	p-Menth-6-en-2-one, (S)-(+)-	-	-	1.07E+06	-
78	15.29	1249	Thymoquinone	-	-	-	2.29E+07
79	15.43	1254	Benzeneacetic acid	2.07E+07	-	1.66E+07	-
80	15.83	1267	Benzeneacetaldehyde, α-ethylidene-	-	-	-	3.34E+06
81	15.86	1268	Isopseudocumenol	1.08E+06	1.17E+06	1.23E+06	7.17E+05
82	15.96	1271	Cinnamaldehyde, (E)-	1.77E+06	4.97E+06	2.92E+06	4.02E+06
83	16.12	1277	Nonanoic acid	1.25E+07	2.35E+07	1.76E+07	-
84	16.46	1288	Thymol	5.51E+06	3.50E+06	-	9.22E+07
85	16.52	1290	Nonanoic acid, ethyl ester	-	4.00E+05	-	2.59E+06
86	16.68	1295	Tridecane	4.42E+05	-	-	-
87	16.69	1296	Carvacrol	-	-	1.66E+06	-
88	16.74	1297	2-Undecanol	-	7.53E+05	-	-
89	16.84	1301	Acetophenone, 2-amino-	8.42E+05	-	-	3.34E+06
90	17.23	1315	Phenol, 3,4,5-trimethyl-	4.13E+07	3.82E+07	4.32E+07	1.62E+07
91	18.09	1346	Benzenepropanoic acid, ethyl ester	-	-	-	3.23E+06
92	18.36	1356	Dehydro-ar-ionene	4.73E+05	1.57E+06	4.88E+05	5.51E+05
93	18.62	1365	n-Decanoic acid	-	-	3.73E+06	2.29E+06
94	19	1379	trans-β-Damascenone	2.82E+07	2.29E+07	4.40E+07	7.33E+07
95	19.18	1386	4-Hydroxy-2,6,6-trimethyl-3-oxocyclohexa-1,4-dienecarbaldehyde	-	-	-	4.11E+05
96	19.24	1388	Benzeneacetic acid, 2-methylpropyl ester	-	-	-	1.09E+06
97	19.33	1391	Decanoic acid, ethyl ester	-	-	-	7.87E+05
98	19.41	1394	cis-Jasmone	-	-	-	6.01E+05
99	19.75	1407	Dodecanal	-	1.48E+06	-	-
100	20.76	1446	trans-Geranylacetone	-	-	-	6.25E+05
101	21.32	1468	Ethyl cinnamate	-	1.19E+06	-	2.13E+06
102	22.11	1499	Pentadecane	-	4.79E+05	-	8.80E+05
103	22.31	1507	2,4-Di-tert-butylphenol	2.25E+06	8.69E+05	3.28E+06	-
104	23.61	1561	Dodecanoic acid	4.33E+05	8.68E+05	1.56E+06	-
105	23.63	1562	Nerolidol	-	-	-	4.13E+05
106	24.36	1592	Dodecanoic acid, ethyl ester	-	-	-	1.35E+06
107	25.47	1640	γ-Eudesmol	-	-	-	4.26E+05
108	26.03	1665	β-Eudesmol	-	-	-	5.78E+05
109	26.6	1690	α-Bisabolol	-	3.55E+05	-	9.65E+05
110	26.82	1700	Heptadecane	-	5.57E+05	-	1.17E+06
			Total	8.27E+08	1.26E+09	8.04E+08	9.85E+08

Table A-3 Volatile Organic Compounds (VOCs) extracted from the headspace of four Irish honey samples marketed as heather honey (IH1, IH2, IH3 and IH4) by HS-SPME followed by GC-MS. RT: Retention time expressed in mins; RI: experimental Retention Index, calculated according to the Van den Dool and Kratz formula based on the RT of standard C7–C30 n-alkanes on an SLB®-5ms capillary column. Abundance is expressed as a relative percentage (mean value ± standard deviation) of the total volatile fraction compounds identified for each sample. The letters indicate the classification based on the chemical structure: a) alkanes; b) alcohols; c) aldehydes; d) ketones; e) carboxylic acids; f) esters; g) others. Notes: [i] Exact isomer not identified; [ii] Tentatively identified.

					IH1		IH2		IH3		IH4	
#	**RT**	**RI**	**Compound ID**		**Mean %**	**SD**	**Mean %**	**SD**	**Mean %**	**SD**	**Mean %**	**SD**
1	3.88	831	Furfural	c	1.6	0.6	4.2	0.3	2.1	0.9	3.2	0.2
2	3.97	836	Butanoic acid, 3-methyl-	e	0.4	0.1	0.4	0.1	0.9	0.1	0.42	0.09
3	4.06	842	Butanoic acid, 2-methyl-, ethyl ester	f	-	-	-	-	-	-	0.31	0.08
4	4.12	846	Butanoic acid, 3-methyl-, ethyl ester	f	-	-	0.2	0.2	0.10	0.08	0.7	0.3
5	4.16	848	2-Furanmethanol	b	-	-	-	-	0.063	0.005	-	-
6	4.37	861	Ethylbenzene	g	0.041	0.006	0.05	0.01	0.031	0.007	-	-
7	4.40	863	1-Hexanol	b	0.05	0.01	0.03	0.01	0.20	0.02	0.035	0.006
8	4.53	871	p-Xylene	g	-	-	0.037	0.004	-	-	-	-
9	4.78	886	2-Heptanone	d	0.06	0.02	0.06	0.01	0.07	0.01	0.052	0.007
10	4.93	896	Styrene	g	0.19	0.04	0.17	0.04	0.15	0.02	0.061	0.007
11	4.99	899	Nonane	a	0.21	0.06	0.07	0.03	0.20	0.06	0.13	0.03
12	5.02	901	5-Ethyl-2-methyl-2-vinyltetrahydrofuran	g	-	-	-	-	0.10	0.05	-	-
13	5.03	901	Heptanal	c	-	-	0.14	0.03	-	-	-	-
14	5.25	910	Ethanone, 1-(2-furanyl)-	d	0.14	0.02	0.12	0.02	0.12	0.07	0.12	0.01
15	5.42	917	Butyrolactone	d	0.03	0.01	-	-	-	-	-	-
16	5.79	931	2-Heptanone, 4-methyl-	d	0.09	0.03	-	-	0.05	0.01	-	-
17	5.87	934	α-Pinene	g	-	-	0.028	0.002	-	-	0.027	0.004
18	6.28	950	Pentanoic acid, 3-methyl-, ethyl ester	f	-	-	-	-	-	-	0.38	0.07

Continues on page A8

Table A-3 (continued)

19	6.30	951	Isocumene	g	-	-	0.027	0.006	-	-	-	-
20	6.45	957	2-Furancarboxaldehyde, 5-methyl-	c	-	-	-	-	-	-	0.12	0.02
21	6.57	962	Benzaldehyde	c	22.8	0.8	21	1	25.9	0.3	18	1
22	6.62	964	1-Heptanol	b	-	-	-	-	0.04	0.01	-	-
23	6.78	970	Dimethyl trisulfide	g	0.26	0.02	-	-	-	-	-	-
24	6.86	973	1-Octen-3-ol	b	0.5	0.1	0.6	0.1	0.37	0.07	0.10	0.01
25	7.12	983	2-Octanone	d	-	-	0.062	0.007	-	-	-	-
26	7.15	985	Furan, 2-pentyl-	g	0.038	0.007	0.08	0.02	0.030	0.005	0.040	0.003
27	7.30	991	Hexanoic acid, ethyl ester	f	-	-	0.06	0.02	-	-	0.19	0.02
28	7.48	998	Octanal	c	0.4	0.1	0.7	0.2	0.5	0.1	0.7	0.3
29	7.88	1011	Benzyl chloride	g	-	-	0.10	0.02	0.31	0.02	-	-
30	8.43	1029	Benzyl alcohol	b	1.1	0.1	1.0	0.7	1.29	0.10	0.49	0.06
31	8.64	1036	β-Isophorone	d	0.27	0.07	0.37	0.09	0.10	0.02	0.063	0.006
32	8.73	1039	Benzeneacetaldehyde	c	5.1	0.8	3.5	0.6	8.2	0.3	3.0	0.2
33	8.89	1044	Eucalyptol	b	-	-	-	-	0.21	0.03	-	-
34	9.10	1051	2-Octenal, (E)-	c	-	-	0.19	0.04	-	-	-	-
35	9.23	1055	Benzenemethanol, α-methyl-	b	-	-	-	-	-	-	0.13	0.04
36	9.41	1061	Acetophenone	d	2.9	0.3	3.3	0.3	4.5	0.2	1.7	0.2
37	9.54	1065	cis-Linalool oxide	b	1.4	0.3	0.29	0.09	1.58	0.03	2.2	0.2
38	9.97	1079	Benzaldehyde, 2-methyl-	c	-	-	-	-	0.070	0.006	-	-
39	10.03	1081	trans-Linalool oxide	b	0.25	0.07	-	-	0.38	0.02	0.88	0.09
40	10.07	1082	2-Nonanone	d	-	-	0.11	0.02	0.19	0.02	-	-
41	10.23	1088	Heptanoic acid, ethyl ester	f	-	-	-	-	-	-	0.071	0.008
42	10.28	1089	Benzoic acid, methyl ester	f	-	-	-	-	0.07	0.02	-	-

Continues on page A9

Table A-3 (continued)

43	10.38	1092	Linalool	b	0.4	0.1	0.34	0.03	0.49	0.05	1.1	0.2
44	10.52	1097	Hotrienol	b	-	-	-	-	-	-	2.1	0.3
45	10.53	1097	Nonanal	c	2.5	0.3	1.7	0.4	2	2	1.5	0.8
46	10.94	1110	Phenylethyl Alcohol	b	1.1	0.1	0.7	0.2	0.97	0.09	1.8	0.2
47	11.24	1120	Isophorone	d	16	1	12.3	0.8	9.2	0.4	6.4	0.7
48	11.32	1122	Benzyl methyl ketone	d	0.17	0.02	0.17	0.03	0.12	0.01	-	-
49	11.75	1135	Lilac aldehyde (isomer I) [i, ii]	c	0.4	0.1	0.31	0.02	0.82	0.06	1.3	0.1
50	11.93	1141	4-Oxoisophorone	d	4.5	0.3	2.8	0.2	3	2	-	-
51	12.04	1144	Lilac aldehyde (isomer II) [i, ii]	c	-	-	-	-	5.0	0.7	3.46	0.05
52	12.06	1145	Lilac aldehyde (isomer III) [i, ii]	c	-	-	-	-	-	-	3	1
53	12.11	1147	Ethanone, 1-(1,4-dimethyl-3-cyclohexen-1-yl)-	d	-	-	-	-	0.10	0.01	-	-
54	12.12	1147	2-Hydroxyisophorone	d	11.9	0.7	12.9	0.8	1.8	0.4	-	-
55	12.21	1150	p-Methoxystyrene	g	0.029	0.004	0.027	0.006	-	-	-	-
56	12.35	1154	Lanierone	d	1.8	0.2	4	3	0.67	0.07	0.81	0.07
57	12.49	1158	Lilac aldehyde (isomer IV) [i, ii]	c	0.36	0.05	0.21	0.07	0.63	0.03	1.10	0.10
58	12.63	1163	Pyruvophenone	d	-	-	0.39	0.08	0.23	0.03	-	-
59	12.65	1163	1,4-Cyclohexanedione, 2,2,6-trimethyl-	d	-	-	-	-	0.22	0.03	-	-
60	12.72	1166	1-Nonanol	b	-	-	0.4	0.1	0.6	0.1	-	-
61	12.76	1167	Benzoic acid, ethyl ester	f	2.6	0.2	8.1	0.7	2.0	0.3	19	1
62	12.85	1170	Benzeneacetic acid, methyl ester	f	0.35	0.04	0.5	0.4	0.20	0.01	-	-
63	12.90	1171	endo-Borneol	b	0.07	0.02	0.12	0.01	0.5	0.2	0.027	0.002
64	13.12	1178	Terpinen-4-ol	b	-	-	0.2	0.1	0.09	0.04	-	-
65	13.28	1183	p-Cymen-8-ol	b	-	-	0.06	0.04	0.9	0.4	0.05	0.01
66	13.29	1183	Octanoic acid	e	0.26	0.07	0.16	0.07	-	-	-	-

Continues on page A10

Table A-3 (continued)

67	13.45	1188	Octanoic acid, ethyl ester	f	-	-	-	-	-	-	0.22	0.03
68	13.57	1192	α-Terpineol	b	-	-	0.32	0.01	0.20	0.02	0.12	0.03
69	13.61	1193	Myrtenal	c	-	-	-	-	-	-	0.12	0.02
70	13.70	1196	Safranal	c	0.14	0.03	0.18	0.01	0.085	0.009	0.12	0.02
71	13.81	1199	Decanal	c	1.0	0.1	1.0	0.2	1.0	0.2	0.7	0.2
72	14.33	1217	p-Menth-1-en-9-al	c	0.08	0.01	-	-	0.18	0.03	0.5	0.1
73	14.41	1219	4-Methyleneisophorone	d	-	-	0.023	0.002	-	-	-	-
74	14.44	1220	Furan, 3-phenyl-	g	-	-	0.37	0.03	0.79	0.10	0.17	0.03
75	14.70	1229	2-Hydroxy-4-oxoisophorone	d	0.66	0.07	0.5	0.2	0.54	0.09	-	-
76	14.99	1239	Benzeneacetic acid, ethyl ester	f	5.2	0.4	12.1	0.3	4.8	0.2	-	-
77	15.25	1247	p-Menth-6-en-2-one, (S)-(+)-	d	-	-	-	-	0.14	0.03	-	-
78	15.29	1249	Thymoquinone	d	-	-	-	-	-	-	2.4	0.2
79	15.43	1254	Benzeneacetic acid	e	2.6	0.5	-	-	2.1	0.8	-	-
80	15.83	1267	Benzeneacetaldehyde, α-ethylidene-	c	-	-	-	-	-	-	0.34	0.07
81	15.86	1268	Isopseudocumenol	b	0.13	0.02	0.10	0.02	0.156	0.007	0.074	0.009
82	15.96	1271	Cinnamaldehyde, (E)-	c	0.21	0.02	0.4	0.1	0.4	0.1	0.41	0.10
83	16.12	1277	Nonanoic acid	e	1.6	0.4	2.0	0.5	2.3	0.3	-	-
84	16.46	1288	Thymol	b	0.68	0.04	0.29	0.08	-	-	9.5	0.3
85	16.52	1290	Nonanoic acid, ethyl ester	f	-	-	0.03	0.01	-	-	0.27	0.05
86	16.68	1295	Tridecane	a	0.06	0.02	-	-	-	-	-	-
87	16.69	1296	Carvacrol	b	-	-	-	-	0.221	0.008	-	-
88	16.74	1297	2-Undecanol	b	-	-	0.07	0.04	-	-	-	-
89	16.84	1301	Acetophenone, 2-amino-	d	0.098	0.009	-	-	-	-	0.353	0.008
90	17.23	1315	Phenol, 3,4,5-trimethyl-	b	5.1	0.4	3	1	5.5	0.2	1.7	0.2

Continues on page A11

Table A-3 (continued)

91	18.09	1346	Benzenepropanoic acid, ethyl ester	f	-	-	-	-	-	-	0.33	0.05
92	18.36	1356	Dehydro-ar-ionene	g	0.056	0.006	0.13	0.05	0.062	0.006	0.06	0.02
93	18.62	1365	n-Decanoic acid	e	-	-	-	-	0.48	0.04	0.24	0.01
94	19.00	1379	trans-β-Damascenone	d	3.4	2.1	1.9	0.4	5.6	0.4	8	1
95	19.18	1386	4-Hydroxy-2,6,6-trimethyl-3-oxocyclohexa-1,4-dienecarbaldehyde	c	-	-	-	-	-	-	0.042	0.002
96	19.24	1388	Benzeneacetic acid, 2-methylpropyl ester	f	-	-	-	-	-	-	0.112	0.009
97	19.33	1391	Decanoic acid, ethyl ester	f	-	-	-	-	-	-	0.08	0.02
98	19.41	1394	cis-Jasmone	d	-	-	-	-	-	-	0.062	0.007
99	19.75	1407	Dodecanal	c	-	-	0.13	0.04	-	-	-	-
100	20.76	1446	trans-Geranylacetone	d	-	-	-	-	-	-	0.07	0.07
101	21.32	1468	Ethyl cinnamate	f	-	-	0.10	0.04	-	-	0.22	0.09
102	22.11	1499	Pentadecane	a	-	-	0.04	0.02	-	-	0.09	0.04
103	22.31	1507	2,4-Di-tert-butylphenol	b	0.3	0.3	0.07	0.03	0.41	0.08	-	-
104	23.61	1561	Dodecanoic acid	e	0.05	0.02	0.07	0.04	0.20	0.03	-	-
105	23.63	1562	Nerolidol	b	-	-	-	-	-	-	0.042	0.009
106	24.36	1592	Dodecanoic acid, ethyl ester	f	-	-	-	-	-	-	0.14	0.06
107	25.47	1640	γ-Eudesmol	b	-	-	-	-	-	-	0.043	0.005
108	26.03	1665	β-Eudesmol	b	-	-	-	-	-	-	0.06	0.05
109	26.60	1690	α-Bisabolol	b	-	-	0.028	0.005	-	-	0.10	0.02
110	26.82	1700	Heptadecane	a	-	-	0.046	0.009	-	-	0.12	0.04

Table A-4 Pollen composition (pollen types and frequency) of the Manuka honey (MH) sample, identified by melissopalynological analysis. Results are expressed as a percentage (average ± standard deviation) of the total pollen count identified.

	MH	
	Mean %	**SD**
Leptospermum	71.3	4.9
Lotus	13.0	1.1
Salix	4.3	1.1
Rosaceae	2.3	0.5
Trifolium	4.7	2.2
Eucalyptus	<1	-
Coriaria	<1	-
Vicia	<1	-
Taraxacum	<1	-
Calluna	<1	-
Asteraceae	<1	-
Ligustrum	<1	-
Ranunculus	<1	-
Angelica	<1	-
Cirsium	<1	-
Metrosideros	<1	-
Total	95.5	

Table A-5 Volatile Organic Compounds (VOCs) extracted from the headspace of one manuka honey sample (MH) by HS-SPME followed by GC-MS. RT: Retention time expressed in mins; RI: experimental Retention Index, calculated according to the Van den Dool and Kratz formula based on the RT of standard C7–C30 n-alkanes on an SLB®-5ms capillary column. Abundance is expressed as the average peak area; the total volatile fraction identified is reported at the bottom. Notes: [i] Exact isomer not identified; [ii] Tentatively identified.

#	RT	RI	Compound ID	Average Peak Area
1	3.88	831	Furfural	6.52E+07
2	4.78	886	2-Heptanone	6.62E+05
3	4.94	896	Styrene	8.84E+05
4	5	900	Nonane	6.29E+05
5	5.03	901	Heptanal	7.46E+05
6	5.24	909	Acetylfuran	1.07E+07
7	5.25	910	Tiglic acid	2.60E+05
8	5.35	914	Pyrazine, 2,5-dimethyl-	6.80E+05
9	5.42	917	Anisole	3.11E+05
10	5.87	934	α-Pinene	2.23E+06
11	6.45	957	Furfural, 5-methyl-	8.74E+05
12	6.56	961	Benzaldehyde	1.30E+08
13	6.72	968	Hexanoic acid	8.00E+05
14	6.86	973	1-Octen-3-ol	7.19E+05
15	7.19	986	trans-Anhydrolinalool oxide (furanoid)	8.68E+05
16	7.44	996	Benzofuran	1.45E+07
17	7.49	998	Octanal	7.84E+06
18	7.6	1002	cis-Anhydrolinalool oxide (furanoid)	1.34E+06
19	7.8	1008	2-Acetyl-5-methylfuran	2.93E+06
20	7.88	1011	Benzyl chloride	6.85E+05
21	8.05	1017	Anisole, p-methyl-	7.39E+05
22	8.32	1025	D-Limonene	7.66E+05
23	8.46	1030	Benzyl alcohol	4.72E+05
24	8.74	1039	Benzeneacetaldehyde	4.08E+07
25	9.43	1062	Acetophenone	4.63E+07
26	9.58	1066	cis-Linalool oxide (furanoid)	6.21E+07
27	10.05	1082	trans-Linalool oxide (furanoid)	2.10E+07
28	10.41	1094	Linalool	1.56E+07
29	10.62	1100	Nonanal	9.88E+07
30	10.82	1106	Benzofuran, 2-methyl-	4.95E+07
31	10.89	1109	Phenylethyl Alcohol	4.79E+06
32	11.19	1118	Isophorone	3.92E+06
33	11.3	1122	Benzyl methyl ketone	8.67E+05
34	11.55	1129	Acetophenone, 2'-methyl	6.59E+07
35	11.75	1135	Lilac aldehyde (isomer I)	1.14E+07
36	11.88	1139	4-Oxoisophorone	1.71E+07

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Table A-5 (continued)

37	12.04	1144	Lilac aldehyde (isomer II)	1.87E+07
38	12.12	1147	Ethanone, 1-(1,4-dimethyl-3-cyclohexen-1-yl)-	8.34E+06
39	12.19	1149	Anisole, p-vinyl-	7.54E+05
40	12.48	1158	Acetophenone, 2'-hydroxy-	5.31E+07
41	12.51	1159	Lilac aldehyde (isomer III)	8.48E+06
42	12.8	1168	p-Mentha-1,5-dien-8-ol	5.29E+05
43	13.04	1175	p-Mentha-6,8-dien-2-ol, cis-	9.69E+06
44	13.12	1178	Benzoic acid	2.19E+06
45	13.29	1183	p-Cymen-8-ol	1.12E+06
46	13.31	1184	Dill ether	1.38E+06
47	13.46	1189	Octanoic acid, ethyl ester	1.36E+06
48	13.5	1190	Methyl salicylate	1.35E+06
49	13.59	1192	α-Terpineol	4.43E+06
50	13.64	1194	Myrtenal	4.55E+07
51	13.71	1196	Safranal	2.67E+06
52	13.81	1199	Decanal	8.30E+06
54	14.4	1219	4-Methyleneisophorone	4.96E+06
55	14.95	1237	Benzeneacetic acid, ethyl ester	5.79E+06
56	15.05	1241	Cuminal	2.34E+06
57	15.76	1264	Myrtanol	1.20E+06
58	16.61	1293	Nonanoic acid, ethyl ester	7.98E+05
59	17.03	1307	o-Methoxyacetophenone	3.66E+08
60	17.03	1308	Cinnamaldehyde, α-methyl-	8.27E+05
61	17.15	1312	Acetophenone, 3'-methoxy-	9.55E+05
62	17.25	1316	trans-Edulan	1.01E+08
63	17.35	1319	Phenol, 3,4,5-trimethyl-	1.09E+07
64	18.35	1355	Acetophenone, 4'-methoxy-	5.69E+06
65	18.42	1358	Dehydro-ar-ionene	1.61E+06
66	18.67	1367	n-Decanoic acid	4.49E+06
67	19.06	1381	β-Damascenone	6.69E+06
68	19.52	1398	Acetophenone, 2'-hydroxy-6'-methoxy	9.01E+06
69	19.78	1408	Dodecanal	1.64E+06
70	20.23	1425	Caryophyllene	7.76E+05
71	20.32	1429	Naphthalene, 2,6-dimethyl-	9.46E+05
72	20.78	1447	trans-Geranylacetone	1.13E+06
73	20.98	1455	Cadina-3,5-diene	5.60E+05
74	21.34	1469	Cinnamic acid, ethyl ester	5.11E+05
75	21.62	1480	3,4-Dehydro-β-ionone	1.37E+06
76	21.95	1492	10,11-Epoxycalamenene	1.36E+06
77	22.11	1499	Pentadecane	4.34E+05
78	22.33	1508	2,4-Di-tert-butylphenol	1.55E+06

Continues on page A15

Table A-5 (continued)

79	22.72	1524	Cadina-1(10),4-diene	1.45E+06
80	22.81	1528	Cadina-1,3,5-triene	1.58E+07
81	22.85	1529	Epizonarene	1.07E+06
82	23.07	1539	Cubenene	2.29E+06
83	23.3	1548	α-Calacorene	1.05E+06
84	23.65	1563	Dodecanoic acid	9.35E+05
85	24.1	1581	Naphthalene, 2,3-dimethoxy	6.71E+07
86	24.15	1583	Benzoic acid, 3,5-dimethoxy-, methyl ester	8.91E+07
87	25.1	1624	α-Corocalene	2.50E+05
88	25.13	1626	Leptospermone	1.15E+06
89	25.39	1637	1,10-Diepicubenol	9.63E+05
90	25.49	1641	γ-Eudesmol	1.09E+06
91	25.74	1652	Epicubenol	1.50E+06
92	26.04	1665	β-Eudesmol	1.47E+06
93	26.41	1681	Cadalene	2.65E+06
94	26.82	1700	Heptadecane	1.05E+06
95	28.31	1769	Methyl syringate	3.66E+06
			Total	1.58E+09

Table A-6 Volatile Organic Compounds (VOCs) extracted from the headspace of one manuka honey sample (MH) by HS-SPME followed by GC-MS. RT: Retention time expressed in mins; RI: experimental Retention Index, calculated according to the Van den Dool and Kratz formula based on the RT of standard C7–C30 n-alkanes on an SLB®-5ms capillary column. Abundance is expressed as a relative percentage (mean value ± standard deviation) of the total volatile fraction compounds identified for each sample. The letters indicate the classification based on the chemical structure: a) alkanes; b) alcohols; c) aldehydes; d) ketones; e) carboxylic acids; f) esters; g) others. Notes: [i] Exact isomer not identified; [ii] Tentatively identified.

					MH	
#	**RT**	**RI**	**Compound ID**		**Mean %**	**SD**
1	3.88	831	Furfural	c	4.3	0.4
2	4.78	886	2-Heptanone	d	0.043	0.007
3	4.94	896	Styrene	g	0.06	0.01
4	5.00	900	Nonane	a	0.04	0.01
5	5.03	901	Heptanal	c	0.049	0.002
6	5.24	909	Acetylfuran	d	0.70	0.04
7	5.25	910	Tiglic acid	e	0.017	0.004
8	5.35	914	Pyrazine, 2,5-dimethyl-	g	0.04	0.02
9	5.42	917	Anisole	g	0.020	0.001
10	5.87	934	α-Pinene	g	0.15	0.03
11	6.45	957	Furfural, 5-methyl-	c	0.06	0.01
12	6.56	961	Benzaldehyde	c	8.5	0.4
13	6.72	968	Hexanoic acid	e	0.052	0.002
14	6.86	973	1-Octen-3-ol	b	0.047	0.004
15	7.19	986	trans-Anhydrolinalool oxide (furanoid)	g	0.06	0.02
16	7.44	996	Benzofuran	g	1.0	0.2
17	7.49	998	Octanal	c	0.52	0.04
18	7.60	1002	cis-Anhydrolinalool oxide (furanoid)	g	0.09	0.02
19	7.80	1008	2-Acetyl-5-methylfuran	d	0.19	0.01
20	7.88	1011	Benzyl chloride	g	0.045	0.003
21	8.05	1017	Anisole, p-methyl-	g	0.05	0.01
22	8.32	1025	D-Limonene	g	0.05	0.01
23	8.46	1030	Benzyl alcohol	b	0.031	0.003
24	8.74	1039	Benzeneacetaldehyde	c	3	2
25	9.43	1062	Acetophenone	d	3.0	0.4
26	9.58	1066	cis-Linalool oxide (furanoid)	b	4.1	0.4
27	10.05	1082	trans-Linalool oxide (furanoid)	b	1.4	0.3
28	10.41	1094	Linalool	b	1.03	0.07
29	10.62	1100	Nonanal	c	6.7	0.8
30	10.82	1106	Benzofuran, 2-methyl- [ii]	g	3.3	0.6
31	10.89	1109	Phenylethyl Alcohol	b	0.32	0.02
32	11.19	1118	Isophorone	d	0.26	0.02
33	11.30	1122	Benzyl methyl ketone	d	0.06	0.01
34	11.55	1129	Acetophenone, 2'-methyl	d	4.3	0.4

Continues on page A17

Table A-6 (continued)

35	11.75	1135	Lilac aldehyde (isomer I) [i]	c	0.75	0.06
36	11.88	1139	4-Oxoisophorone	d	1.12	0.09
37	12.04	1144	Lilac aldehyde (isomer II) [i]	c	1.2	0.1
38	12.12	1147	Ethanone, 1-(1,4-dimethyl-3-cyclohexen-1-yl)-	d	0.5	0.1
39	12.19	1149	Anisole, p-vinyl-	g	0.05	0.01
40	12.48	1158	2'-Hydroxyacetophenone, [ii]	d	3.5	0.4
41	12.51	1159	Lilac aldehyde (isomer III) [i]	c	0.56	0.09
42	12.80	1168	p-Mentha-1,5-dien-8-ol	b	0.03	0.03
43	13.04	1175	p-Mentha-6,8-dien-2-ol, cis-	b	0.66	0.07
44	13.12	1178	Benzoic acid	e	0.1	0.1
45	13.29	1183	p-Cymen-8-ol	b	0.074	0.009
46	13.31	1184	Dill ether	g	0.09	0.01
47	13.46	1189	Octanoic acid, ethyl ester	f	0.089	0.007
48	13.50	1190	Methyl salicylate	f	0.09	0.03
49	13.59	1192	α-Terpineol	b	0.29	0.06
50	13.64	1194	Myrtenal	c	3.0	0.2
51	13.71	1196	Safranal	c	0.17	0.02
52	13.81	1199	Decanal	c	0.55	0.04
53	14.40	1219	4-Methyleneisophorone	d	0.33	0.02
54	14.95	1237	Benzeneacetic acid, ethyl ester	f	0.4	0.1
55	15.05	1241	Cuminal	c	0.15	0.06
56	15.76	1264	Myrtanol	b	0.08	0.02
57	16.61	1293	Nonanoic acid, ethyl ester	f	0.05	0.01
58	17.03	1307	2'-Methoxyacetophenone [ii]	d	24	5
59	17.03	1308	Cinnamaldehyde, α-methyl-	c	0.05	0.02
60	17.15	1312	3'-Methoxyacetophenone	d	0.063	0.002
61	17.25	1316	trans-Edulan	g	6.6	0.9
62	17.35	1319	Phenol, 3,4,5-trimethyl-	b	0.72	0.02
63	18.35	1355	Acetophenone, 4'-methoxy-	d	0.38	0.08
64	18.42	1358	Dehydro-ar-ionene	g	0.105	0.007
65	18.67	1367	n-Decanoic acid	e	0.30	0.02
66	19.06	1381	β-Damascenone	d	0.44	0.08
67	19.52	1398	2'-Hydroxy-6'-methoxyacetophenone, [ii]	d	0.59	0.03
68	19.78	1408	Dodecanal	c	0.107	0.008
69	20.23	1425	Caryophyllene	g	0.051	0.004
70	20.32	1429	Naphthalene, 2,6-dimethyl-	g	0.06	0.02
71	20.78	1447	trans-Geranylacetone	d	0.07	0.02
72	20.98	1455	Cadina-3,5-diene	g	0.037	0.005
73	21.34	1469	Cinnamic acid, ethyl ester	f	0.034	0.009
74	21.62	1480	3,4-Dehydro-β-ionone	d	0.093	0.006
75	21.95	1492	10,11-Epoxycalamenene	g	0.09	0.03

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Table A-6 (continued)

76	22.11	1499	Pentadecane	a	0.028	0.007
77	22.33	1508	2,4-Di-tert-butylphenol	b	0.11	0.02
78	22.72	1524	Cadina-1(10),4-diene	g	0.094	0.009
79	22.81	1528	Cadina-1,3,5-triene	g	1.04	0.08
80	22.85	1529	Epizonarene	g	0.07	0.01
81	23.07	1539	Cubenene	g	0.151	0.005
82	23.30	1548	α-Calacorene	g	0.069	0.009
83	23.65	1563	Dodecanoic acid	e	0.062	0.006
84	24.10	1581	Naphthalene, 2,3-dimethoxy [ii]	g	4	2
85	24.15	1583	Benzoic acid, 3,5-dimethoxy-, methyl ester	f	5.9	0.4
86	25.10	1624	α-Corocalene	g	0.016	0.002
87	25.13	1626	Leptospermone	d	0.078	0.009
88	25.39	1637	1,10-Diepicubenol	b	0.063	0.008
89	25.49	1641	γ-Eudesmol	b	0.07	0.02
90	25.74	1652	Epicubenol [ii]	b	0.10	0.03
91	26.04	1665	β-Eudesmol	b	0.10	0.02
92	26.41	1681	Cadalene	g	0.17	0.03
93	26.82	1700	Heptadecane	a	0.07	0.02
94	28.31	1769	Methyl syringate [ii]	f	0.24	0.03

Appendix B

Table B-1 Volatile Organic Compounds (VOCs) extracted from the headspace of a liquid culture of E. coli in TSB. VOC sampling was performed by HS-SPME followed by GC-MS at 1, 2, 4, 6, 8, and 24 h of incubation (37 °C, 180 rpm). RT: Retention time expressed in mins; RI: experimental Retention Index, calculated according to the Van den Dool and Kratz formula based on the RT of standard C7–C30 n-alkanes on an SLB®-5ms capillary column. Abundance is expressed as the average peak area ± standard deviation.

			Time (h) 1		Time (h) 2		Time (h) 4		Time (h) 6		Time (h) 8		Time (h) 24	
RT	**RI**	**Compound ID**	**Peak area**	**SD**	**Peak area**	**SD**	**Peak area**	**SD**	**Peak area**	**SD**	**Peak area**	**SD**	**Peak area**	**SD**
2.72	601	2,3-Butanedione	-	-	8.33E+05	4.78E+05	1.36E+06	5.29E+05	1.06E+06	4.01E+05	-	-	-	-
2.76	605	2-Butanone	4.28E+06	3.93E+05	2.60E+06	1.66E+06	4.99E+06	4.13E+06	3.77E+06	8.53E+05	3.21E+06	2.02E+06	4.08E+06	1.87E+06
2.94	616	Acetic acid	-	-	-	-	-	-	3.54E+06	1.89E+06	-	-	-	-
2.93	616	Ethyl Acetate	-	-	-	-	-	-	1.53E+06	1.34E+05	-	-	-	-
3.11	628	2-Methylpropanol	-	-	-	-	-	-	8.73E+05	1.06E+05	-	-	-	-
3.47	652	3-Methylbutanal	5.05E+06	3.61E+05	-	-	-	-	-	-	-	-	-	-
3.62	663	2-Methylbutanal	1.73E+06	3.84E+05	-	-	-	-	-	-	-	-	-	-
3.61	662	1-Butanol	-	-	-	-	1.70E+06	1.19E+06	3.58E+06	8.63E+05	3.02E+06	1.35E+06	3.33E+06	5.43E+05
5.27	730	3-Methyl-1-butanol	4.89E+06	1.04E+06	7.22E+06	1.50E+06	7.76E+06	5.49E+06	1.24E+07	3.10E+06	1.18E+07	3.65E+06	1.31E+07	2.71E+06
5.50	736	Dimethyl disulfide	2.39E+06	4.84E+05	-	-	1.09E+06	6.20E+05	-	-	-	-	2.49E+06	1.22E+06
14.61	910	2,5-Dimethylpyrazine	6.29E+06	5.10E+06	8.66E+06	3.66E+06	6.59E+06	2.03E+06	6.24E+06	1.37E+06	5.51E+06	3.19E+06	6.41E+06	3.39E+06
14.68	911	2,6-Dimethylpyrazine	2.06E+06	1.65E+05	-	-	-	-	6.22E+06	3.08E+06	-	-	-	-
17.43	956	Benzaldehyde	2.18E+07	1.24E+07	1.33E+07	7.30E+06	1.17E+06	5.58E+05	-	-	-	-		
23.54	1069	1-Octanol	-	-	-	-	1.20E+06	6.39E+05	2.03E+06	5.99E+05	1.74E+06	4.86E+05	1.44E+06	2.21E+05
31.65	1274	1-Decanol	-	-	1.61E+06	8.14E+05	5.45E+06	2.23E+06	1.04E+07	3.20E+06	1.07E+07	2.50E+06	3.67E+06	2.54E+05
32.37	1295	Indole	6.13E+07	6.34E+07	4.57E+07	3.87E+07	4.33E+07	2.47E+07	5.23E+07	2.33E+07	8.20E+07	2.05E+07	1.06E+08	1.79E+07
36.88	1478	1-Dodecanol	-	-	4.20E+06	2.60E+06	6.52E+06	2.24E+06	1.02E+07	3.73E+06	1.08E+07	3.52E+06	4.24E+06	1.31E+06

Table B-2 Volatile Organic Compounds (VOCs) extracted from the headspace of TSB media control. VOC sampling was performed by HS-SPME followed by GC-MS at 1, 2, 4, 6, 8, and 24 h of incubation (37 °C, 180 rpm). RT: Retention time expressed in mins; RI: experimental Retention Index, calculated according to the Van den Dool and Kratz formula based on the RT of standard C7–C30 n-alkanes on an SLB®-5ms capillary column. Abundance is expressed as the average peak area ± standard deviation.

			Time (h) 1		Time (h) 2		Time (h) 4		Time (h) 6		Time (h) 8		Time (h) 24	
RT	**RI**	**Compound ID**	**Peak area**	**SD**	**Peak area**	**SD**	**Peak area**	**SD**	**Peak area**	**SD**	**Peak area**	**SD**	**Peak area**	**SD**
2.69	600	2,3-Butanedione	3.45E+05	8.31E+04	-	-	4.90E+05	2.08E+05	3.56E+05	3.92E+04	3.26E+05	1.24E+05	4.03E+05	8.20E+04
2.76	604	2-Butanone	1.79E+06	7.24E+05	1.56E+06	5.60E+05	1.48E+06	2.17E+05	1.88E+06	4.17E+05	1.37E+06	6.42E+05	2.09E+06	2.83E+05
3.42	649	3-Methylbutanal	5.60E+06	1.88E+06	6.40E+06	1.98E+06	6.24E+06	1.73E+06	7.61E+06	9.30E+05	6.83E+06	4.66E+05	9.55E+06	1.57E+06
3.59	661	2-Methylbutanal	3.67E+06	4.20E+06	1.58E+06	3.19E+05	1.60E+06	3.29E+05	1.59E+06	4.61E+05	1.53E+06	3.58E+05	2.57E+06	4.14E+05
5.32	731	Pyrazine	6.96E+05	1.27E+05	4.65E+05	2.36E+05	6.41E+05	1.47E+05	6.77E+05	3.35E+04	6.39E+05	7.76E+04	7.67E+05	2.01E+05
5.49	735	Dimethyl disulfide	1.53E+06	2.41E+05	1.24E+06	4.40E+05	1.14E+06	6.07E+05	1.35E+06	1.88E+05	8.51E+05	3.85E+05	4.56E+06	1.32E+06
14.51	908	2,5-Dimethylpyrazine	3.52E+06	1.26E+06	3.91E+06	1.67E+06	3.97E+06	1.83E+06	4.53E+06	1.39E+06	4.00E+06	2.03E+06	3.48E+06	1.91E+06
14.66	911	2,6-Dimethylpyrazine	6.41E+05	2.13E+05	8.27E+05	1.65E+05	-	-	6.20E+05	2.43E+05	9.40E+05	6.61E+05	7.25E+05	2.66E+05
17.34	955	Benzaldehyde	2.00E+07	8.90E+06	2.11E+07	7.89E+06	2.16E+07	3.68E+06	2.23E+07	3.82E+06	1.78E+07	6.81E+06	3.24E+07	3.04E+06
17.65	960	Dimethyl trisulfide	-	-	-	-	-	-	-	-	-	-	1.24E+06	7.91E+05
19.96	997	2-Ethyl-6-methylpyrazine	-	-	2.88E+05	4.29E+04	4.16E+05	7.41E+04	4.05E+05	5.19E+04	2.90E+05	3.34E+04	-	-

Table B-3 Volatile Organic Compounds (VOCs) extracted from the headspace of a E. coli cultured with Irish heather honey (IH2) - (honey solutions at 50% w/v in TSB). VOC sampling was performed by HS-SPME followed by GC-MS at 1, 2, 4, 6, 8, and 24 h of incubation (37 °C, 180 rpm). RT: Retention time expressed in mins; RI: experimental Retention Index, calculated according to the Van den Dool and Kratz formula based on the RT of standard C7–C30 n-alkanes on an SLB®-5ms capillary column. Abundance is expressed as the average peak area ± standard deviation.

			Time (h) 1		Time (h) 2		Time (h) 4		Time (h) 6		Time (h) 8		Time (h) 24	
RT	**RI**	**Compound ID**	**Peak area**	**SD**	**Peak area**	**SD**	**Peak area**	**SD**	**Peak area**	**SD**	**Peak area**	**SD**	**Peak area**	**SD**
2.70	600	2,3-Butanedione	-	-	1.21E+06	2.80E+05	1.37E+06	4.48E+05	1.34E+06	4.76E+05	-	-	-	-
2.75	603	2-Butanone	3.12E+06	6.07E+05	3.06E+06	6.13E+05	3.19E+06	7.78E+05	3.23E+06	8.50E+05	2.96E+06	5.39E+05	3.14E+06	1.34E+06
2.81	608	2-Methylfuran	7.26E+05	2.04E+05	-	-	-	-	-	-	-	-	-	-
2.93	616	Ethyl Acetate	9.02E+05	3.71E+05	1.05E+06	3.31E+05	1.09E+06	2.99E+05	1.39E+06	6.38E+05	1.45E+06	1.10E+05	2.13E+06	2.72E+05
3.12	629	2-Methylpropanol	-	-	-	-	-	-	-	-	-	-	1.17E+06	2.01E+05
3.04	624	Acetic acid	3.50E+06	1.00E+06	3.81E+06	1.46E+06	3.99E+06	1.59E+06	5.06E+06	1.63E+06	5.50E+06	1.77E+06	1.23E+07	2.50E+06
3.40	648	2-Butenal	-	-	-	-	7.56E+05	5.40E+04	7.35E+05	2.07E+05	7.31E+05	1.58E+05	6.70E+05	1.47E+05
3.46	652	3-Methylbutanal	1.43E+07	2.07E+06	1.74E+07	3.75E+06	1.94E+07	4.37E+06	2.06E+07	4.54E+06	2.01E+07	4.24E+06	2.23E+07	4.87E+06
3.61	662	2-Methylbutanal	3.77E+06	7.53E+05	3.98E+06	8.36E+05	4.37E+06	1.23E+06	4.55E+06	9.39E+05	4.40E+06	6.66E+05	5.03E+06	1.49E+06
4.24	702	Heptane	5.70E+05	1.22E+05	-	-	-	-	-	-	-	-	-	-
4.39	706	2,5-Dimethylfuran	3.89E+06	1.12E+06	1.79E+06	5.22E+05	7.89E+05	3.55E+05	-	-	-	-	-	-
5.29	730	3-Methylbutanol	1.17E+06	3.42E+05	1.28E+06	4.56E+05	1.24E+06	3.75E+05	1.18E+06	2.50E+05	1.33E+06	3.56E+05	1.02E+06	9.55E+04
5.49	736	Dimethyl disulfide	1.15E+06	1.48E+05	9.03E+05	1.09E+05	7.40E+05	2.22E+05	-	-	-	-	-	-
6.33	758	Toluene	1.25E+07	2.67E+06	6.72E+06	1.50E+06	3.50E+06	1.22E+06	1.47E+06	2.08E+05	-	-	-	-
7.85	799	Octane	1.82E+06	4.89E+05	9.25E+05	1.84E+05	1.48E+06	3.49E+05	-	-	-	-	-	-
9.65	829	Furfural	8.78E+06	1.00E+06	8.29E+06	3.48E+06	1.02E+07	2.09E+06	1.04E+07	2.61E+06	9.98E+06	2.31E+06	8.97E+06	1.43E+06
11.01	851	Ethyl 3-methylbutanoate	5.66E+05	1.13E+05	-	-	-	-	-	-	-	-	-	-
13.75	896	Nonane	4.85E+05	8.97E+04	-	-	-	-	-	-	-	-	-	-
14.53	909	2,5-Dimethylpyrazine	1.51E+06	2.34E+05	1.57E+06	2.10E+05	1.77E+06	1.10E+05	1.93E+06	3.85E+05	1.51E+06	6.64E+05	1.68E+06	3.09E+05

Continues on page B5

Table B-3 (continued)

14.68	911	2,6-Dimethylpyrazine	-	-	1.06E+06	3.54E+05	1.45E+06	8.82E+05	-	-	-	-	-	-
17.35	955	Benzaldehyde	4.70E+07	7.93E+06	5.12E+07	8.47E+06	5.69E+07	1.01E+07	5.75E+07	1.38E+07	5.87E+07	9.90E+06	5.45E+07	1.04E+07
19.91	997	Octanal	1.02E+06	6.91E+04	8.57E+05	2.69E+05	7.43E+05	2.31E+05	-	-	-	-	-	-
21.60	1030	Benzyl alcohol	1.36E+06	1.24E+05	1.43E+06	2.98E+05	1.63E+06	1.98E+05	1.95E+06	4.78E+05	1.57E+06	5.04E+05	1.26E+06	2.11E+05
21.98	1038	Benzeneacetaldehyde	1.01E+06	4.96E+05	1.08E+06	3.16E+05	1.24E+06	2.17E+05	1.40E+06	3.36E+05	1.47E+06	2.29E+05	1.24E+06	5.64E+05
23.09	1060	Acetophenone	2.86E+06	5.76E+05	3.51E+06	5.47E+05	3.91E+06	7.45E+05	4.09E+06	9.86E+05	3.95E+06	5.70E+05	2.67E+06	2.82E+05
24.99	1098	Nonanal	2.39E+06	2.03E+05	2.08E+06	3.19E+05	1.70E+06	4.51E+05	1.53E+06	3.35E+05	1.24E+06	2.78E+05	-	-
25.35	1106	Phenylethyl Alcohol	-	-	-	-	-	-	6.80E+05	2.47E+05	6.67E+05	2.92E+04	-	-
25.67	1114	Isophorone	1.49E+07	1.56E+06	1.48E+07	2.32E+06	1.57E+07	2.86E+06	1.61E+07	3.93E+06	1.55E+07	2.45E+06	1.49E+07	2.43E+06
26.68	1139	4-Oxoisophorone	4.17E+06	3.30E+05	4.26E+06	6.17E+05	4.53E+06	7.57E+05	3.96E+06	1.94E+06	4.45E+06	6.81E+05	4.44E+06	7.54E+05
26.80	1142	2-Hydroxyisophorone	1.17E+07	1.11E+06	1.17E+07	1.73E+06	1.25E+07	2.33E+06	1.30E+07	3.29E+06	1.25E+07	2.03E+06	1.14E+07	1.71E+06
27.32	1155	Lanierone	7.81E+05	3.84E+04	7.53E+05	7.74E+04	9.15E+05	1.95E+05	1.01E+06	3.40E+05	9.23E+05	1.61E+05	7.03E+05	9.86E+04
27.72	1164	2,6,6-Trimethyl-1,4-cyclohexanedione	6.61E+05	7.25E+04	7.41E+05	2.42E+05	7.35E+05	1.67E+05	7.00E+05	1.28E+05	7.39E+05	1.43E+05	8.26E+05	1.18E+05
27.81	1167	Ethyl benzoate	4.37E+06	4.50E+05	5.40E+06	8.12E+05	6.08E+06	1.29E+06	6.38E+06	1.51E+06	5.86E+06	8.23E+05	-	-
28.09	1173	Octanoic acid	-	-	-	-	-	-	6.49E+05	1.84E+05	7.63E+05	1.29E+05	8.45E+05	9.09E+04
29.22	1201	Decanal	1.17E+06	1.16E+05	1.16E+06	1.63E+05	1.14E+06	2.40E+05	8.67E+05	2.46E+05	8.47E+05	1.78E+05	-	-
30.53	1240	Ethyl phenylacetate	7.46E+06	1.26E+06	8.37E+06	1.10E+06	9.68E+06	1.63E+06	1.01E+07	2.12E+06	9.91E+06	1.44E+06	4.81E+06	4.46E+05
32.28	1293	Indole	2.18E+06	4.90E+05	3.19E+06	6.93E+05	3.90E+06	1.15E+06	5.36E+06	1.50E+06	5.26E+06	9.04E+05	1.21E+06	5.08E+05
32.97	1317	3,4,5-Trimethylphenol	1.47E+06	2.75E+05	1.56E+06	2.61E+05	1.87E+06	2.97E+05	1.99E+06	4.97E+05	1.77E+06	5.22E+05	1.22E+06	1.55E+05
34.71	1383	β-Damascenone	1.06E+06	1.10E+05	9.85E+05	1.67E+05	1.03E+06	2.03E+05	1.02E+06	2.50E+05	9.33E+05	1.63E+05	7.02E+05	1.14E+05

Table B-4 Volatile Organic Compounds (VOCs) extracted from the headspace of E. coli cultured with manuka honey (MH) - (honey solutions at 50% w/v in TSB). VOC sampling was performed by HS-SPME followed by GC-MS at 1, 2, 4, 6, 8, and 24 h of incubation (37 °C, 180 rpm). RT: Retention time expressed in mins; RI: experimental Retention Index, calculated according to the Van den Dool and Kratz formula based on the RT of standard C7–C30 n-alkanes on an SLB®-5ms capillary column. Abundance is expressed as the average peak area ± standard deviation.

			Time (h) 1		Time (h) 2		Time (h) 4		Time (h) 6		Time (h) 8		Time (h) 24	
RT	**RI**	**Compound ID**	**Peak area**	**SD**	**Peak area**	**SD**	**Peak area**	**SD**	**Peak area**	**SD**	**Peak area**	**SD**	**Peak area**	**SD**
2.70	600	2,3-Butanedione	3.54E+06	3.59E+05	3.69E+06	4.34E+05	4.94E+06	8.36E+05	4.41E+06	1.19E+06	5.91E+06	8.34E+05	7.80E+06	1.34E+06
2.76	605	2-Butanone	3.48E+06	6.46E+05	3.53E+06	8.00E+05	3.87E+06	9.27E+05	3.71E+06	8.15E+05	3.79E+06	1.24E+06	5.68E+06	1.57E+06
2.93	616	Ethyl Acetate	3.13E+06	7.51E+05	2.95E+06	1.08E+06	2.92E+06	1.04E+06	2.13E+06	1.03E+06	2.40E+06	7.35E+05	2.83E+06	9.70E+05
3.14	630	Acetic acid	5.38E+06	1.59E+06	5.86E+06	2.13E+06	7.63E+06	1.35E+06	6.78E+06	4.16E+06	8.40E+06	1.96E+06	2.10E+07	3.13E+06
3.46	652	3-Methylbutanal	1.41E+07	3.28E+06	1.72E+07	3.69E+06	2.05E+07	3.81E+06	1.76E+07	6.80E+06	2.09E+07	4.23E+06	2.28E+07	5.66E+06
3.61	662	2-Methylbutanal	3.57E+06	4.51E+05	4.03E+06	5.18E+05	4.32E+06	4.78E+05	3.30E+06	1.75E+06	4.50E+06	5.56E+05	5.25E+06	1.04E+06
3.76	672	1-Hydroxy-2-propanone	3.56E+06	6.36E+05	4.20E+06	4.31E+05	4.74E+06	3.61E+05	4.66E+06	2.33E+05	4.64E+06	9.56E+05	4.01E+06	1.07E+06
4.25	702	Heptane	8.19E+05	8.14E+04	-	-	-	-	-	-	-	-	-	-
5.11	725	3-methyl-3-butenol	1.14E+06	1.77E+05	1.19E+06	1.61E+05	1.33E+06	2.00E+05	1.25E+06	1.36E+05	1.29E+06	2.19E+05	1.26E+06	2.86E+05
5.28	730	3-methylbutanol	1.12E+06	2.60E+05	1.03E+06	2.01E+05	1.07E+06	1.97E+05	1.16E+06	3.50E+05	1.02E+06	1.84E+05	9.39E+05	2.27E+05
5.40	733	2-methylbutanol	7.93E+05	7.96E+04	7.76E+05	1.23E+05	8.35E+05	1.22E+05	7.55E+05	9.20E+04	-	-	-	-
5.49	736	Dimethyl disulfide	1.43E+06	1.94E+05	1.17E+06	1.31E+05	9.87E+05	7.42E+04	-	-	-	-	-	-
6.35	759	Toluene	8.86E+05	1.49E+05	-	-			-	-	-	-	-	-
7.45	789	2,3-Hexanedione	1.33E+06	3.91E+05	1.29E+06	4.15E+05	1.16E+06	7.36E+04	-	-	-	-	-	-
7.85	799	Octane	3.02E+07	7.82E+06	1.85E+07	5.82E+06	2.77E+07	8.26E+06	2.01E+07	7.34E+06	1.56E+07	4.61E+06	1.20E+07	3.78E+06
9.64	829	Furfural	7.93E+06	1.45E+06	8.38E+06	2.41E+06	9.54E+06	1.75E+06	8.91E+06	1.03E+06	9.44E+06	2.30E+06	-	-
14.25	904	2-Acetylfuran	-	-	1.13E+06	1.05E+05	1.33E+06	6.55E+04	1.15E+06	1.34E+05	-	-	-	-
14.48	908	2,5-Dimethyl pyrazine	1.66E+06	5.04E+05	1.63E+06	3.65E+05	2.02E+06	5.12E+05	2.30E+06	9.94E+05	2.08E+06	4.10E+05	1.59E+06	4.66E+05
14.57	909	2,6-Dimethyl pyrazine	-	-	-	-	-	-	-	-	1.90E+06	7.59E+05	1.64E+06	9.51E+05

Continues on page B7

Table B-4 (continued)

15.65	927	α-Pinene	3.99E+06	8.75E+05	1.99E+06	5.43E+05	2.75E+06	7.62E+05	1.52E+06	6.25E+05	1.13E+06	5.30E+04	-	-
17.35	955	Benzaldehyde	3.40E+07	8.66E+06	4.06E+07	1.86E+06	3.71E+07	1.28E+07	3.80E+07	1.06E+07	3.81E+07	1.14E+07	2.86E+07	1.17E+07
19.32	987	Benzofuran	1.85E+06	4.95E+05	2.37E+06	1.46E+05	1.63E+06	6.99E+05	1.75E+06	3.25E+05	1.40E+06	2.21E+05	-	-
19.42	989	Hexanoic acid	-	-	-	-	-	-	-	-	-	-	1.13E+06	2.39E+05
23.09	1060	Acetophenone	2.91E+06	5.78E+05	3.56E+06	4.79E+05	3.70E+06	9.14E+05	3.70E+06	5.34E+05	3.37E+06	1.87E+06	2.49E+06	4.01E+05
23.36	1065	cis-Linalool oxide	4.64E+06	3.53E+05	4.80E+06	8.37E+05	5.16E+06	9.75E+05	4.41E+06	8.04E+05	5.39E+06	9.09E+05	5.17E+06	9.45E+05
24.13	1081	trans-Linalool oxide	1.12E+06	1.25E+05	1.16E+06	1.04E+05	1.32E+06	2.29E+05	1.13E+06	1.37E+05	1.23E+06	9.91E+04	1.28E+06	2.29E+05
24.76	1093	Linalool	1.14E+06	1.42E+05	1.04E+06	1.87E+05	1.06E+06	2.10E+05	8.94E+05	1.60E+05	8.92E+05	1.88E+05	-	-
24.98	1098	Nonanal	2.10E+07	3.52E+06	1.85E+07	3.12E+06	1.58E+07	2.92E+06	1.14E+07	2.22E+06	8.93E+06	1.89E+06	1.68E+06	4.32E+05
25.09	1100	2-Methylbenzofuran	5.58E+06	1.63E+06	5.67E+06	1.42E+06	5.66E+06	1.80E+06	-	-	-	-	1.04E+06	1.60E+05
26.68	1139	4-Oxoisophorone	9.23E+05	4.57E+04	9.37E+05	1.68E+05	9.90E+05	1.98E+05	8.38E+05	6.40E+04	9.45E+05	1.38E+05	8.52E+05	1.70E+05
27.38	1156	2'-Hydroxyacetophenone	2.11E+06	7.22E+05	2.64E+06	4.58E+05	2.80E+06	5.11E+05	2.87E+06	3.16E+05	2.65E+06	8.05E+05	1.60E+06	4.62E+05
28.17	1175	Octanoic acid	-	-	-	-	1.58E+06	3.23E+05	1.60E+06	5.13E+05	2.01E+06	8.68E+05	1.90E+06	6.11E+05
28.83	1192	Myrtenal	3.80E+06	5.50E+05	3.69E+06	6.49E+05	3.68E+06	8.05E+05	3.03E+06	5.58E+05	3.25E+06	5.90E+05	2.41E+06	5.20E+05
29.22	1201	Decanal	7.02E+05	1.63E+05	-	-	-	-	-	-	-	-	-	-
31.06	1256	cis-Edulan	3.10E+06	5.52E+05	2.68E+06	4.98E+05	2.39E+06	4.78E+05	1.88E+06	4.33E+05	1.70E+06	2.96E+05	8.93E+05	2.12E+05
32.20	1290	ortho-Methoxyacetophenone	6.46E+07	1.11E+07	6.91E+07	9.12E+06	7.69E+07	1.35E+07	7.35E+07	6.78E+06	7.45E+07	1.26E+07	6.62E+07	1.07E+07
32.33	1294	Indole	2.07E+06	3.16E+05	2.62E+06	4.80E+05	2.82E+06	5.87E+05	2.67E+06	4.80E+05	2.09E+06	9.76E+05	-	-

Table B-5 Volatile Organic Compounds (VOCs) extracted from the headspace of Irish heather honey (IH2) control - (honey solutions at 50% w/v in TSB). VOC sampling was performed by HS-SPME followed by GC-MS at 1, 2, 4, 6, 8, and 24 h of incubation (37 °C, 180 rpm). RT: Retention time expressed in mins; RI: experimental Retention Index, calculated according to the Van den Dool and Kratz formula based on the RT of standard C7–C30 n-alkanes on an SLB®-5ms capillary column. Abundance is expressed as the average peak area ± standard deviation.

			Time (h) 1		Time (h) 2		Time (h) 4		Time (h) 6		Time (h) 8		Time (h) 24	
RT	**RI**	**Compound ID**	**Peak area**	**SD**	**Peak area**	**SD**	**Peak area**	**SD**	**Peak area**	**SD**	**Peak area**	**SD**	**Peak area**	**SD**
2.71	601	2,3-Butanedione	7.94E+05	1.53E+05	-	-	1.17E+06	1.17E+06	8.71E+05	2.06E+05	7.62E+05	2.95E+05	-	-
2.77	605	2-Butanone	2.26E+06	7.01E+05	1.84E+06	3.81E+05	1.77E+06	1.77E+06	1.43E+06	2.94E+05	1.93E+06	9.18E+05	1.92E+06	3.46E+05
2.94	617	Acetic acid	-	-	1.48E+06	2.98E+05	6.19E+06	6.19E+06	2.47E+06	1.50E+06	3.15E+06	1.02E+06	7.43E+06	1.34E+06
3.45	651	3-Methylbutanal	8.42E+06	6.55E+06	8.11E+06	5.87E+06	8.47E+06	8.47E+06	8.57E+06	2.03E+06	9.79E+06	2.46E+06	9.36E+06	6.31E+06
3.60	661	2-Methylbutanal	2.10E+06	1.49E+06	2.97E+06	1.67E+06	2.30E+06	2.30E+06	2.03E+06	4.68E+05	1.82E+06	4.77E+05	2.57E+06	8.74E+05
4.40	706	2,5-Dimethylfuran	1.80E+06	5.60E+05	6.47E+05	1.35E+05	4.35E+05	4.35E+05	-	-	-	-	-	-
5.50	736	Dimethyl disulfide	6.28E+05	2.12E+05	5.45E+05	8.46E+04	4.46E+05	4.46E+05	-	-	-	-	-	-
6.32	758	Toluene	6.46E+06	2.77E+06	3.64E+06	5.74E+05	2.73E+06	2.73E+06	8.85E+05	5.10E+05	5.99E+05	7.71E+04	-	-
7.72	796	Butanoic acid											5.45E+05	6.92E+04
7.83	799	Octane	1.03E+06	3.72E+05	-	-	5.74E+05	5.74E+05	-	-	-	-	-	-
9.65	829	Furfural	6.08E+06	2.43E+06	4.59E+06	1.05E+06	7.55E+06	7.55E+06	6.17E+06	1.93E+06	7.12E+06	4.53E+05	5.85E+06	2.46E+06
11.80	864	m-Xylene	2.05E+06	7.34E+05	9.99E+05	2.85E+05	-	-	-	-	-	-	-	-
12.98	883	p-Xylene	8.98E+05	2.71E+05	6.69E+05	4.05E+05	-	-	-	-	-	-	-	-
14.53	909	2,5-Dimethylpyrazine	1.89E+06	9.29E+05	2.01E+06	8.90E+05	3.07E+06	3.07E+06	3.09E+06	1.02E+06	2.88E+06	1.14E+06	2.81E+06	1.93E+06
17.34	955	Benzaldehyde	2.84E+07	1.09E+07	3.19E+07	1.21E+07	4.06E+07	4.06E+07	4.44E+07	7.20E+06	4.21E+07	2.43E+06	4.30E+07	8.01E+06
19.91	997	Octanal	6.33E+05	2.88E+05	-	-	-	-	-	-	-	-	-	-
21.47	1027	Benzyl alcohol	6.96E+05	4.19E+05	8.71E+05	3.32E+05	8.68E+05	8.68E+05	9.76E+05	3.90E+05	1.06E+06	2.34E+05	6.39E+05	1.16E+05
21.97	1037	Benzeneacetaldehyde	9.38E+05	4.96E+05	1.11E+06	6.24E+05	1.33E+06	1.33E+06	1.60E+06	4.02E+05	1.34E+06	1.73E+05	1.67E+06	6.86E+05
23.09	1060	Acetophenone	1.61E+06	8.33E+05	2.08E+06	7.93E+05	2.46E+06	2.46E+06	2.81E+06	5.00E+05	2.02E+06	7.42E+05	1.62E+06	6.72E+05

Continues on page B9

Table B-5 (continued)

25.10	1100	Nonanal	1.29E+06	7.10E+05	1.03E+06	5.60E+05	1.08E+06	1.08E+06	1.05E+06	2.64E+05	7.42E+05	1.70E+05	1.13E+07	1.20E+06
25.83	1118	Isophorone	9.79E+06	4.79E+06	9.00E+06	4.03E+06	9.99E+06	9.99E+06	1.04E+07	2.24E+06	9.21E+06	1.19E+06	3.43E+06	4.29E+05
26.69	1139	4-Oxoisophorone	2.67E+06	1.31E+06	2.46E+06	1.23E+06	2.87E+06	2.87E+06	2.98E+06	6.98E+05	2.66E+06	3.04E+05	8.52E+06	7.52E+05
26.88	1144	2-Hydroxyisophorone	7.90E+06	3.61E+06	7.08E+06	2.94E+06	7.95E+06	7.95E+06	8.17E+06	1.85E+06	7.35E+06	7.14E+05	5.48E+05	8.41E+04
27.38	1156	Lanierone	7.71E+05	3.69E+04	5.34E+05	4.81E+04	6.41E+05	6.41E+05	6.07E+05	4.47E+04	5.19E+05	5.77E+04	5.31E+05	6.20E+04
27.74	1165	2,6,6-Trimethyl-1,4-cyclohexanedione	-	-	-	-	5.04E+05	5.04E+05	5.16E+05	1.10E+05	-	-	1.22E+06	2.50E+05
27.80	1166	Ethyl benzoate	2.92E+06	1.14E+06	3.18E+06	1.40E+06	3.66E+06	3.66E+06	4.00E+06	1.15E+06	3.43E+06	3.33E+05	-	-
29.22	1201	Decanal	6.58E+05	3.51E+05	6.77E+05	1.87E+05	6.44E+05	6.44E+05	7.05E+05	1.48E+05	-	-	-	-
30.52	1240	Ethyl phenylacetate	4.48E+06	2.12E+06	4.65E+06	1.96E+06	5.83E+06	5.83E+06	6.65E+06	1.57E+06	5.89E+06	2.95E+05	3.45E+06	9.57E+05
32.97	1317	3,4,5-Trimethylphenol	8.08E+05	2.39E+05	7.88E+05	3.59E+05	1.17E+06	1.17E+06	1.31E+06	4.96E+05	1.31E+06	2.14E+05	1.00E+06	2.20E+05
34.71	1383	β-Damascenone	6.51E+05	3.06E+05	6.39E+05	2.00E+05	6.15E+05	6.15E+05	6.07E+05	1.54E+05	5.23E+05	7.22E+04	5.22E+05	9.32E+04

Table B-6 Volatile Organic Compounds (VOCs) extracted from the headspace of manuka honey (MH) control - (honey solutions at 50% w/v in TSB). VOC sampling was performed by HS-SPME followed by GC-MS at 1, 2, 4, 6, 8, and 24 h of incubation (37 °C, 180 rpm). RT: Retention time expressed in mins; RI: experimental Retention Index, calculated according to the Van den Dool and Kratz formula based on the RT of standard C7–C30 n-alkanes on an SLB®-5ms capillary column. Abundance is expressed as the average peak area ± standard deviation.

			Time (h) 1		Time (h) 2		Time (h) 4		Time (h) 6		Time (h) 8		Time (h) 24	
RT	**RI**	**Compound ID**	**Peak area**	**SD**	**Peak area**	**SD**	**Peak area**	**SD**	**Peak area**	**SD**	**Peak area**	**SD**	**Peak area**	**SD**
2.70	600	2,3-Butanedione	2.23E+06	5.12E+05	-	-	-	-	3.45E+06	4.26E+05	-	-	-	-
2.75	604	2-Butanone	2.35E+06	1.03E+06	2.50E+06	9.57E+05	2.57E+06	5.51E+05	2.48E+06	1.27E+05	2.77E+06	1.26E+06	3.03E+06	5.76E+05
2.91	615	Ethyl Acetate	-	-	1.47E+06	8.56E+05	1.60E+06	5.53E+05	1.79E+06	9.69E+05	1.45E+06	8.02E+05	1.14E+06	6.49E+04
3.10	627	Acetic acid	-	-	4.84E+06	2.39E+06	5.71E+06	1.30E+06	6.06E+06	3.92E+05	6.55E+06	2.19E+06	1.39E+07	2.04E+06
3.45	651	3-Methylbutanal	9.38E+06	1.44E+06	1.24E+07	3.57E+06	1.47E+07	2.62E+06	1.41E+07	7.17E+05	1.39E+07	3.06E+06	1.61E+07	3.83E+06
3.60	661	2-Methylbutanal	2.23E+06	4.45E+05	2.71E+06	1.06E+06	3.01E+06	7.94E+05	2.83E+06	3.25E+04	2.92E+06	8.59E+05	3.65E+06	8.82E+05
3.74	671	1-Hydroxy-2-propanone	2.60E+06	5.43E+05	-	-	3.44E+06	1.43E+06	3.51E+06	1.00E+06	2.76E+06	5.31E+05	2.85E+06	4.65E+05
5.07	724	3-methyl-3-butenol	6.71E+05	9.66E+04	2.72E+06	4.13E+06	8.61E+05	2.12E+05	8.49E+05	1.02E+05	7.66E+05	2.18E+05	7.78E+05	1.53E+05
5.38	733	2-Methylbutanol	-	-	-	-	6.16E+05	2.45E+05	-	-	-	-	-	-
5.47	735	Dimethyl disulfide	1.04E+06	5.35E+05	6.76E+05	1.94E+05	8.34E+05	2.22E+05	-	-	-	-	-	-
7.43	788	2,3-Hexanedione	6.96E+05	3.14E+05	6.99E+05	3.15E+05	-	-	-	-	-	-	-	-
7.83	799	Octane	1.66E+07	4.74E+06	9.77E+06	2.74E+06	1.39E+07	3.17E+06	1.02E+07	1.96E+06	6.99E+06	2.03E+06	5.00E+06	1.55E+06
9.62	829	Furfural	-	-	6.87E+06	2.63E+06	6.64E+06	1.83E+06	5.48E+06	1.46E+06	6.47E+06	2.27E+06	5.33E+06	9.25E+05
11.77	864	m-Xylene	2.21E+06	5.64E+05	1.04E+06	3.32E+05	7.12E+05	2.43E+05	-	-	-	-	-	-
13.12	886	p-Xylene	9.66E+05	4.18E+05	6.58E+05	2.36E+05	-	-	-	-	-	-	-	-
14.26	904	2-Acetylfuran	8.05E+05	1.44E+05	-	-	9.15E+05	1.74E+05	9.66E+05	2.69E+05	9.42E+05	2.50E+05	7.63E+05	2.56E+05
14.46	908	2,5-Dimethyl pyrazine	2.43E+06	1.43E+06	2.40E+06	1.42E+06	-	-	3.68E+06	8.73E+05	3.18E+06	1.03E+06	2.29E+06	4.60E+05
14.68	911	2,6-Dimethyl pyrazine	-	-	6.43E+05	7.21E+04	-	-	-	-	-	-	-	-
15.62	927	α-Pinene	2.30E+06	6.00E+05	1.20E+06	4.88E+05	1.59E+06	5.37E+05	8.78E+05	2.38E+05	-	-	-	-

Continues on page B11

Table B-6 (continued)

17.33	954	Benzaldehyde	1.70E+07	7.78E+06	2.34E+07	1.37E+06	2.36E+07	3.01E+06	1.72E+07	8.34E+06	2.66E+07	1.56E+06	1.59E+07	2.69E+06
19.30	987	Benzofuran	1.10E+06	2.42E+05	1.32E+06	7.15E+05	1.28E+06	3.20E+05	9.76E+05	1.83E+05	9.24E+05	3.97E+05	-	-
21.97	1037	Benzeneacetaldehyde	-	-	-	-	-	-	-	-	-	-	6.27E+05	1.62E+05
23.07	1059	Acetophenone	1.93E+06	2.77E+05	2.65E+06	2.67E+05	2.59E+06	3.59E+05	3.01E+06	4.06E+05	2.93E+06	5.94E+05	1.78E+06	1.91E+05
23.34	1065	cis-Linalool oxide	3.36E+06	9.66E+05	3.61E+06	1.34E+06	3.40E+06	8.20E+05	3.79E+06	1.00E+06	3.37E+06	1.08E+06	3.28E+06	9.75E+05
24.11	1080	trans-Linalool oxide	8.09E+05	2.49E+05	8.84E+05	3.37E+05	8.56E+05	2.28E+05	8.84E+05	2.10E+05	7.78E+05	2.29E+05	7.70E+05	3.08E+05
24.74	1093	Linalool	7.16E+05	2.64E+05	7.02E+05	2.71E+05	6.72E+05	1.49E+05	-	-	-	-	-	-
24.96	1097	Nonanal	1.25E+07	9.15E+05	1.16E+07	1.13E+06	1.02E+07	1.21E+06	8.06E+06	1.03E+06	5.93E+06	9.89E+05	9.52E+05	1.93E+05
25.00	1098	2-Methylbenzofuran	3.36E+06	6.49E+05	3.85E+06	9.58E+05	-	-	2.93E+06	7.94E+05	2.63E+06	1.11E+06	6.31E+05	1.82E+05
26.66	1138	4-Oxoisophorone	6.86E+05	2.03E+05	7.01E+05	2.50E+05	6.83E+05	1.58E+05	7.55E+05	1.23E+05	5.42E+05	3.58E+04	5.80E+05	1.18E+05
27.36	1156	2'-Hydroxyacetophenone	1.42E+06	3.05E+05	1.76E+06	2.11E+05	2.11E+06	2.96E+05	2.10E+06	3.97E+05	2.20E+06	2.35E+05	1.15E+06	2.16E+05
28.14	1175	Octanoic acid	-	-	7.08E+05	1.82E+05	1.02E+06	2.25E+05	-	-	1.52E+06	5.50E+05	1.55E+06	5.04E+05
28.81	1191	Myrtenal	2.67E+06	7.87E+05	2.61E+06	8.34E+05	2.44E+06	5.52E+05	2.32E+06	6.21E+05	1.95E+06	6.04E+05	1.63E+06	4.67E+05
31.04	1256	cis-Edulan	2.08E+06	7.29E+05	1.87E+06	5.58E+05	1.66E+06	3.85E+05	1.45E+06	3.01E+05	1.19E+06	2.91E+05	6.38E+05	1.82E+05
31.51	1270	Nonanoic acid	-	-	-	-	-	-	-	-	5.71E+05	7.77E+04	-	-
32.18	1290	ortho-Methoxyacetophenone	4.53E+07	4.80E+06	5.17E+07	8.47E+06	5.49E+07	8.94E+06	6.03E+07	8.36E+06	5.65E+07	1.01E+07	4.49E+07	5.22E+06
34.32	1368	n-Decanoic acid	-	-	-	-	8.50E+05	4.13E+05	-	-	-	-	-	-