An investigation into the role of the skin odorants and microbiota in the attraction of malaria mosquitoes to human beings.

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Abstract

Skin microbiota plays an important role in human body odour production, and mosquitoes primarily use olfaction to locate their hosts, therefore, an understanding of the mosquito important compounds released by bacteria could be exploited for a novel intervention. This project investigated the role of skin bacteria on mosquito attractiveness to human beings. In order to investigate this, 30 volunteers were recruited and asked to comply with a washing regime. Bacteria and odour samples were taken from the feet, back, forearm and axilla of 30 volunteers. Odour was collected using nylon stocking that were worn overnight and by carrying out headspace entrainment for each body site. The nylon stockings were tested behaviourally to *An. stephensi*. Volunteers' feet increased in attractiveness to *An. stephensi* between day 1 and day 4, however the other sites (axilla, forearm and upper back) did not. The heaspace entrainment samples were pooled together according to the body site and visit and tested with coupled Gas chromatography-electroantennography (GC-EAG) to test which compounds were detected by mosquitoes. 52 compounds were found to be EAG active across all sites. The samples were individually analysed with GC, and bacteria samples were sequenced with 16S rRna, and a correlation was done for each body site. Over 60 bacteria significantly changed between day 1 and day 5 for feet, however fewer bacteria significantly changed for the other sites (1 bacteria for axilla, 3 for forearm and 10 for the upper back). Furthermore, the correlations for feet revealed that the following compounds: Ethyl-cyclohexane (RI 841), 2-nonanal (RI 1130), menthol (RI 1172) and RI 1232, RI 1711 and RI 1817 (unidentified) were associated with *Phascolarctobacterium, Tyzzerella, Sutterella, Turicella, Schlegelella, Oryzihumus, Parabacteroides, Megasphaera, Shingopxis, Paludibacter, Ralstonia, Tuberibacillus* and *Peptococcus.* This study demonstrated that the interactions between bacteria and compounds are highly complex and further research is needed to explore a causual relationship between the two.

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Contents

1 Literature review....12
1.1 Mosquitoes as vectors of disease....12
1.1.1 *Anopheles* mosquitoes....12
1.1.2 Life cycle of *Anopheles* mosquitoes....14
1.2 Malaria burden....16
1.3 Malaria lifecycle and transmission....17
1.4 Malaria vector control....18
1.5 Host seeking behaviour in mosquitoes....21
1.5.1 Visual, chemical and physical cues....21
1.5.2 Olfactory stimuli from hosts....22
1.6 Differential attractiveness in humans....22
1.6.1 Body odour....22
1.6.2 Age....23
1.6.3 Diet....24
1.6.4 Blood group....24
1.6.5 Pregnancy status....24
1.6.6 Genetics....25
1.7 Source of human derived compounds....26
1.7.1 Skin structure....26
1.7.2 Human skin microbiota....28
1.8 Molecular tools for skin microbiota identification....28
1.9 VOCs emitted from skin microbiota....30
1.10 Production of VOCs by skin microbiota in relation to mosquito host seeking behaviour....31
1.11 Chemical ecology studies of mosquito behaviour and human odour....32
1.12 Conclusion....41
1.12.1 Aims and objectives:....41
2 Behavioural responses of *Anopheles stephensi* mosquitoes to odour from different body sites....42
2.1 Introduction....42
2.1.1 Body odour collection methods....44
2.1.2 Behaviour experiments....44
2.2 Aims and objectives....45

2.3 Materials and methods 46
2.3.1 Mosquito colony 46
2.3.2 Recruitment of 30 volunteers 46
2.3.3 Volatile collection 47
2.3.4 Behavioural experiments 48
2.3.5 Statistical analysis 51
2.4 Results 52
2.4.1 Preliminary tests 52
2.4.2 Response to stockings worn by 29 volunteers 54
2.4.3 Differential attractiveness of the volunteers to *An. stephensi* 56
2.5 Discussion 62
2.6 Conclusion 66
3 The identification of electrophysiologically-active compounds from human odours 67
3.1 Introduction 67
3.2 Aims and Objectives 70
3.3 Materials and methods 71
3.3.1 Mosquitoes 71
3.3.2 Volatile collection 71
3.4 Sample analysis 74
3.4.1 GC-EAG 74
3.4.2 GC-MS 75
3.5 Results 76
3.6 Discussion 84
3.6.1 Human derived compounds 84
3.6.2 Non-human derived compounds 88
3.7 Conclusion 91
4 Method development for bacteria and compound analysis 92
4.1 Introduction 92
4.2 Aims and objectives 93
4.3 Methods 94
4.3.1 Visualisation parallel coordinates 94
4.3.2 Beta diversity plots 94
4.3.3 Presence or absence of bacteria 94

4.3.4 Comparative testing....95
4.4 Results....96
4.4.1 Parallel coordinates....96
4.4.2 Beta diversity....96
4.4.3 Presence or absence of bacteria....96
4.4.4 Comparative testing....97
4.5 Discussion....103
4.6 Conclusion....104
5 Associations of bacteria and compounds for foot....105
5.1 Introduction....105
5.1.1 Bacteria on different body sites....106
5.1.2 Association of bacteria and volatiles in relation to mosquitoes....107
5.2 Aims and objectives....109
5.3 Methods....110
5.3.1 Bacteria analysis....110
5.4 Gas chromatography analysis....113
5.4.1 Gas chromatography....113
5.4.2 Quantitative analysis of volatiles in air entrainment samples....114
5.5 Statistical analysis....114
5.6 Results....116
5.6.1 Bacteria found on feet....116
5.6.2 Volatiles found on feet....116
5.6.3 Comparison of bacteria between day 1 and day 4....116
5.6.4 Comparisons of volatiles between day 1 and day 4....117
5.6.5 Correlation between bacteria and volatiles....117
5.6.6 Strong positive correlations....118
5.6.7 Positive correlations....118
5.6.8 Strong negative correlations....119
5.6.9 Negative correlation....119
5.7 Discussion....149
5.7.1 Differences in bacteria between washed and unwashed feet....150
5.7.2 Correlation with bacteria and compounds....151
5.7.3 EAG active compounds....152

5.8 Conclusion 155
6 Associations of bacteria and compounds for axilla, upper back and forearm 156
6.1 Introduction 156
6.1.1 Compounds found on axilla, forearm and back 157
6.1.2 Associations of bacteria and volatile organic compounds 158
6.2 Aims and objectives 159
6.3 Methods 160
6.4 Results 160
6.4.1 Bacteria found on axilla, arm and back 160
6.4.2 Volatiles found on axilla, arm and back 160
6.4.3 Comparison of bacteria between day 1 and day 4 160
6.4.4 Comparison of volatiles between day 1 and day 4: 161
6.4.5 Correlation between bacteria and volatiles for axilla 161
6.4.6 Strong positive correlation for axilla 161
6.4.7 Positive correlation for axilla: 161
6.4.8 Strong negative correlation for axilla 162
6.4.9 Negative correlation for axilla 162
6.4.10 Correlation between bacteria and volatiles for forearm 163
6.4.11 Correlation betwe en bacteria and volatile for upper back 164
6.5 Discussion 183
6.5.1 Differences in bacteria between day 1 and day 4 184
6.5.2 Correlation with bacteria and compounds 184
6.6 Conclusion 187
7 Discussion 188
7.1 Study caveats 192
7.1.1 Volunteer participation 192
7.1.2 Volatile collection 192
7.1.3 GC-EAG 193
7.2 Future research 194
7.3 Conclusion 196
8 References 197
Appendix 1 211
Appendix 2 212

List of figures

Figure 1-1. The distribution of malaria vectors worldwide (Sinka et al., 2012). 14
Figure 1-2. The mosquito lifecycle (Source: adult mosquito picture: *arctec*, LSHTM. Egg, larvae and pupae pictures: Harry Weinburgh, CDC). 16
Figure 1-3. Malaria distribution by country, categorised as malaria free, eliminating malaria or controlling malaria (Cotter *et al.* 2013). 17
Figure 1-4 Lifecycle of *Plasmodium*parasite (Source: Su *et al.,* 2007) 18
Figure 1-5 Diagram showing a cross-section of the skin (Grice & Segre 2011). 27
Figure 1-6. The distribution of aprocrine, eccrine and sebaceous glands (Image modified from: Verhulst *et al.* 2010). 27
Figure 2-1 Timeline for collection of volatiles from volunteers. 47
Figure 2-2. Body sites where odour was collected from volunteers. 48
Figure 2-3. The dual-choice windtunnel olfactometer used to test measure behavioural responses of *An. stephensi* to odour stimuli. 49
Figure 2-4. Diagram of the cage bioassay to observe the attractiveness of odour stimuli to *An. stephensi*. 50
Figure 2-5. Mean number of mosquitoes in the dual-choice olfactometer, separated by area: the release chamber, wind tunnel, in the trapping chambers with the worn stocking and the control. Different letters indicate significant differences at 0.05. 53
Figure 2-6. Mean number of mosquitoes probing on the worn stocking and the negative control. *P*<0.001 54
Figure 2-7. Relative attractiveness of the different body sites of volunteers by visit to *An. stephensi.* Different letter indicate differences at 0.05 significance. 56
Figure 2-8. Relative attractiveness of volunteers to *An. stephensi* compared to the negative and positive control (sock worn by researcher) using the total mean of all body sites and visits combined. Significant differences of relative attractiveness between volunteers is marked with a letter. Levels not connected by the same letter are significantly different. 57
Figure 2-9. Relative attractiveness of volunteers to *An. stephensi* for the foot. Data is ranked according to overall mean and separated by day 1 and day 4. 59
Figure 2-10. Relative attractiveness of volunteers to *An. stephensi* for the axilla. Data is ranked according to overall mean and separated by day 1 and day 4. 59
Figure 2-11. Relative attractiveness of volunteers to *An. stephensi* for the arm. Data is ranked according to overall mean and separated by day 1 and day 4. 60
Figure 2-12. Relative attractiveness of volunteers to *An. stephensi* for the back. Data is ranked according to overall mean and separated by day 1 and day 4. 60
Figure 3-1. Gas chromatography coupled with electroanntenography (GC-EAG). 69
Figure 3-2. Timeline of headspace entrainment 71

Figure 3-3. Headspace entrainment set up of feet and forearms. The airflow leaving the pump was purified with a charcoal filter, the airflow leaving the bag passed through a Porapak q filter where the volatiles were captured. 73
Figure 3-4. Headspace entrainment set up for the upper back and the axilla. The airflow leaving the pump was purified with a charcoal filter, the airflow leaving the bag passed through a Porapak Q filter where the volatiles were captured. 73
Figure 3-5. Examples of coupled GC-EAG traces of *An. stephensi* responses to human air entrainment extracts from foot day 1 (top figure) and foot day 4 after washing (bottom figure). 80
Figure 3-6. Examples of coupled GC-EAG traces of *An. stephensi* responses to human air entrainment extracts from axilla day 1 (top figure) and axilla day 4 after washing (bottom figure). 81
Figure 3-7. Examples of coupled GC-EAG traces of *An. stephensi* responses to human air entrainment extracts from forearm day 1 (top figure) and axilla day 4 after washing (bottom figure). 82
Figure 3-8. Examples of coupled GC-EAG traces of *An. stephensi* responses to human air entrainment extracts from upper back day 1 (top figure) and axilla day 4 after washing (bottom figure). 83
Figure 4-2. Parallel coordinate for compound RI 966 at genus level. The compound was split into 5 categories (1 representing the lowest amount and 5 the highest); orange= 5, purple= 4, green= 3; red=2, blue=1 98
Figure 4-4. Beta diversity plot for all samples for visit 1 and visit 4 with compound RI 966 overlaid. Red dots represent day 1, blue dots represent day 4. 99
Figure 4-5. Beta diversity plot for per site for day 1 and day 4 with compound RI 966 overlaid as a contour. The darker the contour the higher amount of compound present. Upper left: foot; upper right: axilla; lower left: upper back; lower right: forearm. Red dots represent day 1, blue dots represent day 4. 100
Figure 4-6. Presence and absence of *Flavobacterium* and *Dermabacter* against the presence or absence of compound RI 966. Compound RI 966 0=absent; 1=present. Bacteria 10=present; -9999990= absent. Dots represent individual samples. 101

List of tables

Table 1-1. List of compounds tested electro-physiologically and behaviourally in laboratory settings with *Anopheles* mosquitoes 36
Table 2-1. Tukey analysis for the comparison of body sites and visits. 55
Table 2-2. Significant differences of relative attractiveness between volunteers at 0.05 significance. Levels not connected by the same letter are significantly different. 58
Table 2-3 Spearman's rank correlation of different body sites between day 1 and day 4 61
Table 2-4 Spearman's rank correlation between different body sites for day 1. 61
Table 2-5 Spearman's rank correlation between different body sites for day 4 61
Table 3-1. GC-EAG active compounds for *An. stephensi* from the foot, axilla, arm and back from 30 volunteers and the number of responses for each sample. 78
Table 4-1. Mean relative abundance predicted for the 'High' and 'Low' categories for compound RI 966. 102
Table 5-1. Ingredients for the lysis 111
Table 5-2. Components used in bacteria qPCR assay 111
Table 5-3. Components in end-point PCR assay for illumina sequencing 112
Table 5-4. Mean amount of bacteria counts collected from the foot on day 1 and day 4. P values indicates significant difference between day 1 and day 4. Bacteria with significant changes between days are in bold. 120
Table 5-5. Mean amount of volatile compound collected from each body site on day 1 and day 4. P values indicates significant difference between day 1 and day 4. 125
Table 5-6. Correlation between bacteria and compounds between day 1 and day 4 at 0.001% significance. 127
Table 6-1. Mean amount of bacteria collected from the axilla, arm and upper back (P-values indicate significant differences between day 1 and day 4). Bacteria in bold had significant changes between day 1 and day 4. 165
Table 6-2. Mean amount of volatile compounds expressed as percetages collected from each body site on day 1 and day 4. Compounds in bold indicate significant differences between day 1 and day 4. 175
Table 6-3. Correlation between bacteria and compounds between day 1 and day 4 for axilla at 0.0001% significance. 178

Abbreviations

VOCs: Volatile organic compounds

LSHTM: London School of Hygiene and Tropical Medicine

GC: Gas chromatography

GC-MS: Gas chromatography-mass spectrometry

GC-EAG: Gas chromatography- electroantennography

OTUs: Operational taxonomic unit

SPME: Solid Phase Microextraction

CHAPTER 1

1 Literature review

1.1 Mosquitoes as vectors of disease

Mosquitoes (Diptera: Culicidae) are haematophagous insects that are found worldwide. They are found on every continent except Antarctica. In tropical regions of the world, mosquitoes are found all year round. In more temperate climates they are seasonal as they are active during warmer months. There are approximately 3500 known mosquito species. All mosquitoes belong to the family Culicidae which can be divided into three different subgroups: *Toxorhynchitinae, Anophelinae* and *Culicinae* (Clements, 1992). As well as being nuisance biters, mosquitoes are vectors of disease, including malaria, dengue fever, yellow fever, lymphatic filariasis, Rift Valley fever, West Nile virus, Japanese encephalitis, chikungunya and Zika (WHO 2017b). The economic and social impact of mosquito-borne disease is significant; globally, over 1 million people die from mosquito-borne diseases, and many more suffer illness which impacts livelihoods. Poverty can be exacerbated as people may be unable to return to work due to illness and disability (WHO 2014). Dengue fever is a widespread viral disease across tropical regions that is primarily vectored by *Aedes aegypti*, it can be fatal and can affect over 50-100 million people every year (Bhatt *et al.* 2012). Yellow fever is another viral disease and there are 200,000 estimated cases every year occurring in Africa and Latin America (WHO 2018b). However, the deadliest mosquito-borne disease is malaria, caused by the parasite *Plasmodium*, with an estimated 212 million cases of malaria occurring in 2015 and 490 000 deaths (WHO 2018a).

1.1.1 *Anopheles* mosquitoes

Malaria is transmitted by female *Anopheles* mosquitoes. There are 430 *Anopheles* species, however, only 30-40 species are medically important (Clements 1992). Different regions of the world have different *Anopheles* mosquitoes that can transmit malaria. The majority of malaria occurs in sub-Saharan Africa where 80% of worldwide cases are seen, and 90% of the mortalities. *Anopheles gambiae* sensu lato a species complex and *An. arabiensis* are the principal vectors of malaria in sub-Saharan Africa (Bass *et al.* 2007). The *An. gambiae* species complex was discovered in the 1960s and

each sub species is morphologically indistinguishable (Bass *et al.* 2007). *Anopheles gambiae* mosquitoes are highly anthropophilic and are found in association with human communities and dwellings (White, 1974), whereas *An. arabiensis* has been described as both anthropophilic and zoophilic (Sinka *et al.* 2010). In the Americas, *An. quadrimaculatus, An. albimanus* and *An. darling* are the major vectors of malaria. In Asia, *An. stephensi*, *An. dirus* and *An. minimus* complexes dominate Southeast Asia (Figure 1-1) (Sinka *et al.* 2012).

Anopheles stephensi, which is the insect studied in this thesis, is an important malaria vector that has a large geographical distribution ranging from southern Asia, the Indian subcontinent, the Middle East and the Arabian Peninsula (Figure 1-1) (Rowland *et al.* 2002; Sinka *et al*. 2011). This species was chosen for this study as it is a competent malaria vector, as they transmit *Plasmodium falciparum* and *Plasmodium vivax*, and in some areas it is the sole vector of malaria. Furthermore, there have been very few studies that have investigated *An. stephensi* behaviour as the majority of studies have investigated behaviour in *An. gambiae.*

Three phenotypes have been identified, *An. Stephensi stephensi* (type form), *An. stephensi mysorensis* and *An. stephensi* intermediate form (Alam *et al.* 2008). The type form is an important malaria vector in urban areas, and it prefers to bite humans rather than cattle, while the *Mysorensis* form is restricted to rural and mountainous areas and has poor vectoral capacity as it exhibits considerable zoophilic behaviour. The type form is prevalent in rural and peri urban areas (Sinka *et al.* 2011; Oshaghi *et al.* 2006). The different type forms can be identidied by the number of ridges on the egg float. In this study, the Sind-Kasur Nijmengen strain (type form) was used (Feldmann and Ponnudurai, 1989).

They are active at dusk and in short bursts throughout the whole night (Rowland, 1989). Transmission of malaria parasites from different species is often dependant on seasonal patterns such as rainy seasons (WHO, 2017a).

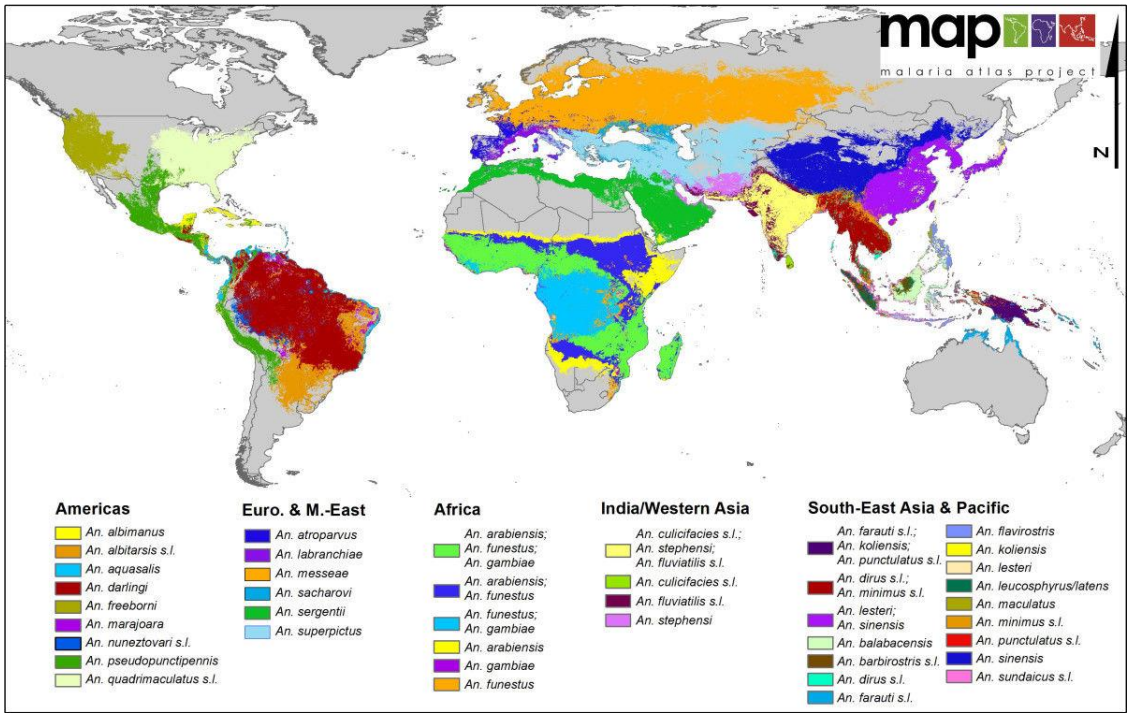


Figure 1-1. The distribution of malaria vectors worldwide (Sinka et al., 2012).

1.1.2 Life cycle of *Anopheles* mosquitoes

Mosquitoes have 4 life stages in their life cycle: egg, larva, pupa and adult (Figure 1-2). An adult female can lay between 50-500 eggs per oviposition in water (Sumba et al. 2004). Eggs can take between 2 days to 3 weeks to hatch, depending on the temperature, the warmer the temperature, the faster they hatch. The four larval instar stages live in water and spend most of their time feeding on algae and other microorganisms found in their environment. In urban areas, *An. stephensi* larvae are typically found in artificial containers in homes and in industrial locations. In rural areas, larvae are found in fresh-water pools, stream margins and beds as well as domestic wells and water-storage containers (Sinka *et al.* 2011). Larvae have a head for feeding, a segmented abdomen and a large thorax. They remain flat at the surface of the water line to breathe through spiracles on the side of their abdomen as they lack a respiratory siphon, present in other mosquitoes such as *Aedes*. After four instars, the larva turn into pupae. Pupae are shaped like a comma, and do not feed. They remain as pupa between 5 days to 14 days before developing into adults (Clements, 1992).

Adults have a head, thorax and an abdomen. The head consists of olfactory organs including antennae and maxillary palps. Olfactory cues are extremely important for mosquitoes, as they use olfaction to seek nectar, ovipositon sites and blood hosts. In the first 24 hours of adult life, male and female mosquitoes seek nectar which provides an energy source for metabolic processes such as flight (Clements, 1992; Takken & Knols 1999). Female mosquitoes also feed on blood from hosts, the protein in the blood meal is used for egg development. In the first 3-5 days after emergence, females mate with males. Male *Anopheles* mosquitoes form swarms, and females mate by encountering a male once they have entered the swarm. Females need only mate once in their lifetime as the spermatozoa is retained. Once mated, females seek hosts for blood feeding. After blood feeding, the female will rest, typically indoors and on walls, until the eggs are fully developed. *An. stephensi* generally bite and rest indoors, however, they have been shown to bite outdoors in warmer months. Resting sites for *An. stephensi* include poorly constructed human and animal dwellings.

Blood meal analysis indicate that *An. stephensi* tend to feed on humans rather than cattle. *An. stephensi* are most active with host seeking behaviour during the night (Sinka *et al.* 2011). Two to three days after the blood meal is taken, the female mosquito is gravid and will locate ovipositioning sites using olfactory and visual cues (Githeko *et al.* 1993; Takken & Knols 1999).

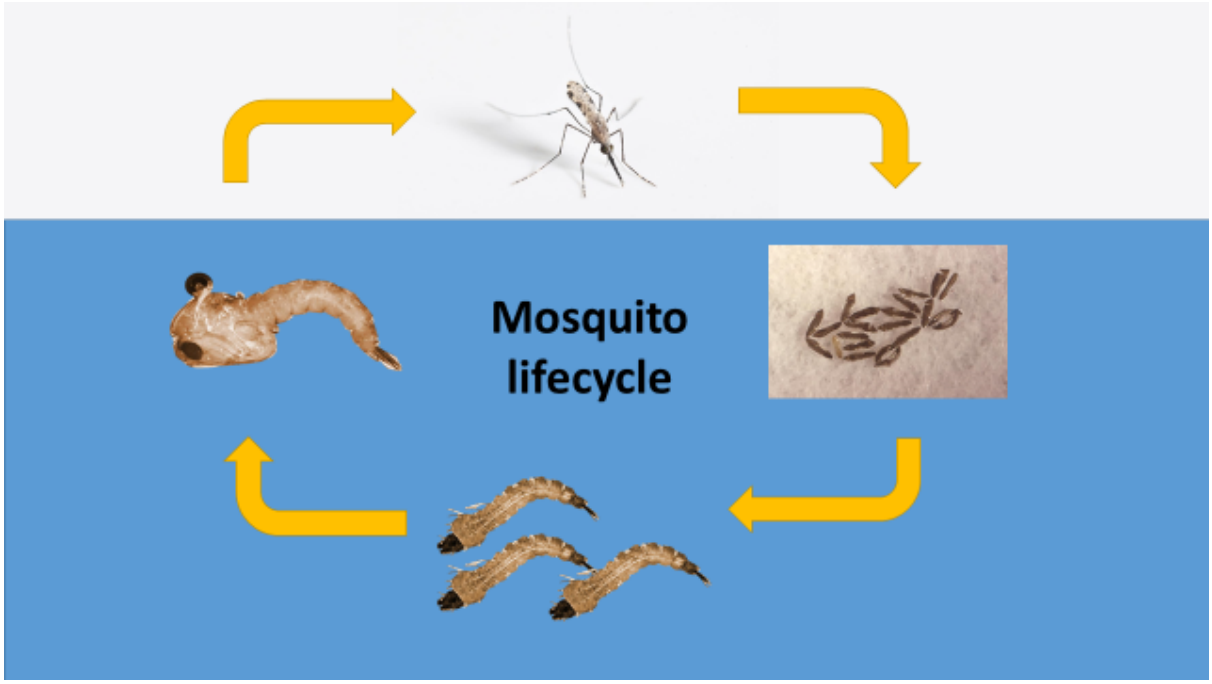


Figure 1-2. The mosquito lifecycle (Source: adult mosquito picture: *arctec*, LSHTM. Egg, larvae and pupae pictures: Harry Weinburgh, CDC).

1.2 Malaria burden

Malaria is caused by *Plasmodium* parasites which are transmitted by infected *Anopheles* mosquitoes. There are four parasite species that cause malaria in humans, *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae* and *Plasmodium ovale*. The former two are the most common and a large proportion of all malaria mortality and morbidity is caused by *P. falciparum*. Malaria is endemic in a band around the equator (Figure 1-3). In 2015, 212 million cases and 429 000 deaths were estimated globally. An estimated 70% of deaths occur in children, and children under 5 are particularly at risk of infection, illness and death. Incidence rates decreased by 21% between 2010 and 2015, and malaria death rates in under 5's decreased by 29% between 2010 and 2015 due to the increased use of long lasting insecticide treated nets (LLINs). However, a 2017 report stated that the progress in malaria control has stalled as there were approximately 5 million more malaria cases in 2016 than 2015, furthermore, malaria death rates have not decreased between 2015 and 2016 (WHO, 2018a).

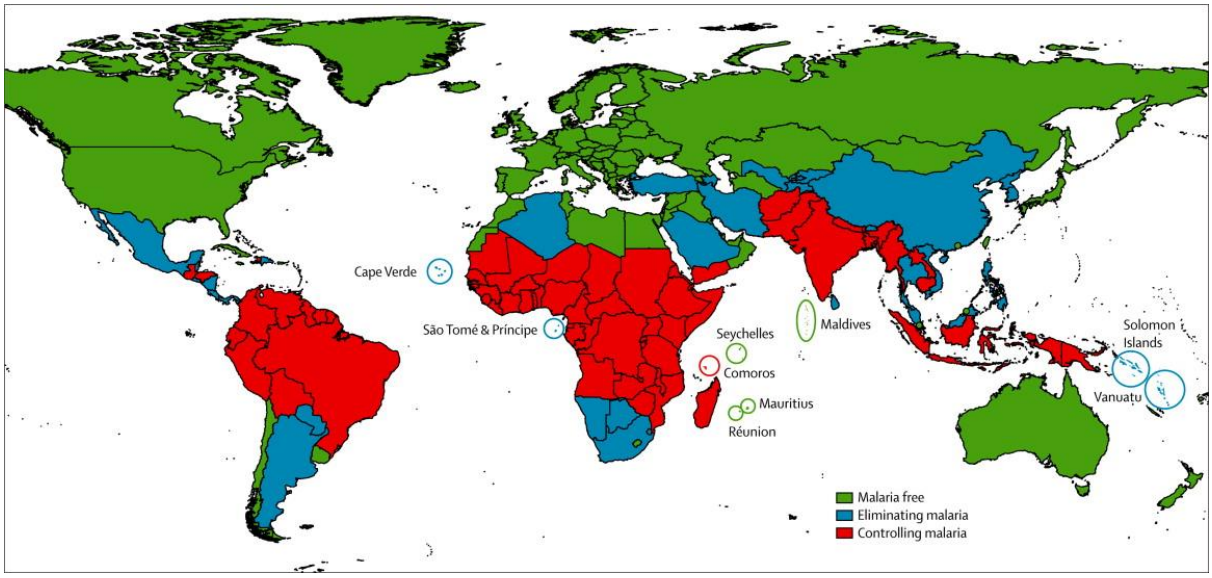


Figure 1-3. Malaria distribution by country, categorised as malaria free, eliminating malaria or controlling malaria (Cotter *et al.* 2013).

1.3 Malaria lifecycle and transmission

Human beings must be bitten by infected female mosquitoes for malaria transmission to take place. Malaria parasites are found in the salivary glands at the sporozoite stage (Figure 1-4). When mosquitoes take a blood meal, the sporozoites are injected into the bloodstream, then travel to the human liver and invade liver cells. They multiply inside liver cells and become schizonts which rupture and release merozoites into the blood stream. In the bloodstream, the parasites invade red blood cells and undergo asexual reproduction which consists of immature trophozoite (ring stage) maturing into schizonts, which then rupture releasing merozoites. The asexual cycle continues by merozoites invading new red blood cells and developing further. However, some immature trophozoites develop into gametocytes (sexual stage) due to environmental stressors including high parasitaemia or drug treatment. The parasites at this stage become elongated and develop into a distinctive 'banana' shape, the female gametocytes are more elongated than the males (Josling & Llinás 2015). When the parasite has reached sexual maturity, they can be transmitted to mosquitoes via a blood meal. Inside the mosquito, the gametocytes exflagellate and multiply in the mosquito's gut. Male gametes then fertilize female gametes to form ookinetes. They

invade the midgut wall where they mature into oocysts. The oocysts mature further, burst and release sporozoites which travel to the salivary gland (CDC, 2012).

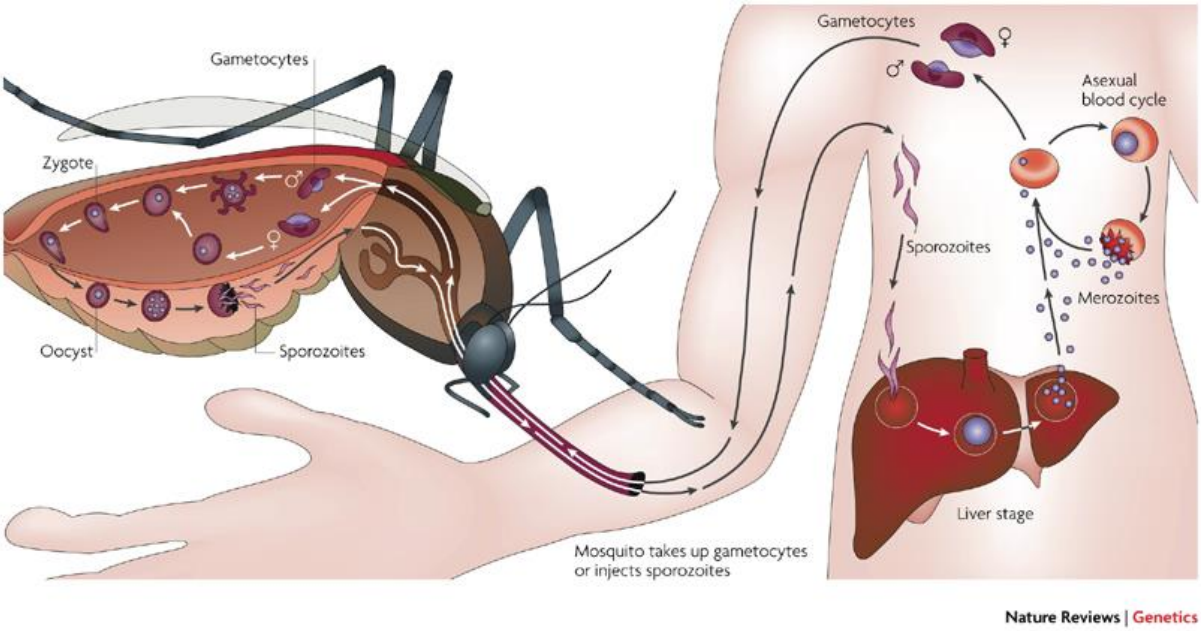


Figure 1-4 Lifecycle of *Plasmodium*parasite (Source: Su *et al.,* 2007)

1.4 Malaria vector control

Current control methods for malaria primarily focus on chemical control with the use of LLINs and indoor residual spraying (IRS). The World Health Organisation (WHO) pesticide evaluation scheme (WHOPES) recommends pyrethroids, organochlorines, organophosphates and carbamates, however, only pyrethroids are recommended to be used for LLINs by the WHO (WHO, 2015). Widespread distribution of these methods can have a dramatic decrease in malaria transmission if used correctly. It is estimated that over 220 000 deaths every year, mainly among children under the age of 5, are prevented by the application of these methods. No new insecticides have been developed for vector control in the last 30 years, therefore, pyrethroids have been used during this period for malaria control (Cameron & Lorenz 2013). This has led to resistance in mosquito populations, consequently reducing the effectiveness of control methods. Furthermore, resistance has increased and spread in recent years (Ranson *et al.* 2011). Since 2010, up to 60 countries have reported insecticide resistance to one class of insecticide, and up to 50 countries have reported resistance to two or more

classes of insecticide. Furthermore, the complete extent of resistance is poorly understand due to lack of routine monitoring (WHO, 2017).

Mosquito larval source management is a further environmental management approach, where mosquito breeding sites are targeted to prevent the development of larvae into adults. This can be done via irrigation and drainage, and could be effective for malaria when used in conjunction with LLINs or IRs (Tusting *et al.* 2013). Other environmental approaches include the usage of natural predators such as copepods, *Toxorhynchites*, fish and fungi. However, the use of *Toxorhynchites* and copepods are generally used for the control of *Aedes* species (Cameron & Lorenz 2013). The limitation of these methods, is that breeding sites have to be found, which can be logistically difficult.

Genetic control is a further control method being explored which is based around Sterile Insect Technique (SIT). Specifically in mosquitoes, a control method known as Release of Insects carrying a Dominant Lethal (RIDL) is used. Males are released into the wild, and after mating, the female lays sterile eggs (Carvalho *et al*. 2014). It is very effective at reducing mosquito populations, however, it works best in closed environments such as islands, as in a non-enclosed environments, migration could provide females with more opportunities to mate with wild-type males. The use of RIDL is very effective when one species of mosquitoes is the dominant vector for a particular disease, such as *A. aegypti* with dengue fever. However, as various mosquito species are capable of transmitting malaria, genetic control would be expensive and impractical (Alphey *et al.* 2010).

Personal outdoor protection against mosquito bites can be achieved through the use of repellents such as N, N-Diethyl-meta-tuluamude (DEET). Several studies have been done, some of which demonstrate that repellents can protect against malaria transmission, and some of which don't (for example, Hill *et al.* 2007; Chen-Hussey *et al.* 2013). A meta-analysis found a reduction in malaria transmission of around 30% through the use of repellents, but this was not significant (Wilson *et al.* 2015). The authors conclude that there are too few studies to determine the true benefit of repellent for malaria control and further, well designed, studies are needed.

A push pull method using attractants and repellents has also been investigated for mosquito control. A semi-field experiment placed different repellents (para-menthane, 3,8-diol (PMD), catnip oil and delta-undecalactone) at four corners of an experimental hut, attractive baited traps were simoultenaously used. A significant reduction of mosquito house entry was observed, and this was amplified using an attractive bait outside the experimental hut (Menger *et al.* 2014). A follow up field study by the same researchers showed that fabric impregnanted with delta-undecalactone placed in the eaves of houses reduced mosquito entry by 50%. Furthermore, attractive baited traps placed outside the houses caught high numbers of mosquitoes. Model simulations predict that this control method could reduce entomological inoculation rate 20 fold (Menger *et al.* 2015). Another field experiment that placed screens on eaves observed a reduced house entry of mosquitoes by 61% to 99%, however the addition of repellent to the screen had little impact as the screens were efficient on their own. Furthermore, the use of an attractive baited trap did not impact mosquito house entry (Menger *et al.* 2016).

With increasing and spreading resistance, and with limitations to other control methods, novel control methods need to be investigated and used in conjunction with existing methods. Research that has aimed to understand vector-host interaction have aimed to exploit semiochemicals for the development of attractants and lures. Semiochemicals are behaviour and developmental modifying chemicals that can be detected through olfaction and they can be divided into two groups: pheromones and allelochemicals. Pheromones are intraspecific and are stimuli that are emitted and received by the same species. Allelochemicals are interspecific and can be divided into two further groups: kairomones and allomones. Kairomones induce a response by the receiver which benefits the receiver but not the emitter, and allomones induce a response by the receiver which benefits the emitter but not the receiver (Norlund *et al.* 1981). With mosquitoes, kairomones are compounds that are attractive to mosquitoes, whereas allomones acts as repellent compounds (Norlund *et al.* 1981).

1.5 Host seeking behaviour in mosquitoes

1.5.1 Visual, chemical and physical cues

Mosquitoes use visual, physical and chemical cues for host seeking. These cues can act at long-range, mid-range or short-range. Long-range attraction is initiated by carbon dioxide (CO_2) and other breath and skin emanations emitted from the host. Mosquitoes can detect plumes of odours that are over 20 meters away, and follow it upwind. (Nordlund & Lewis 1976). Medium range attraction takes place between 2 to 20 meters away from the host, body odour and CO_2 are detected at this range. Short-range attraction involves visual cues, CO_2, body heat and odour. Different gradients of humidity and temperature are also detected and used to approach the host. Nocturnal mosquitoes such as *An. gambiae* and *An. stephensi* respond to noticeable objects and vertical barriers (Gibson & Torr 1999).

Of these cues, olfaction is the most important for host seeking behaviour. Mosquitoes also use olfaction for other behaviour including searching for a mate, nectar and ovipositioning sites. Olfactory organs include the antennae, maxillary palps and proboscis. These organs are covered in highly sensitive hairs called sensilla, which are found in abundance in the antennae, however, some are also located in the maxillary palps and proboscis. Sensilla have different purposes and they can be classified as olfactory, gustatory, or mechanosensory. Mosquitoes also have hygro and thermoreceptors which detect moisture and temperature respectively (Guidobaldi *et al.* 2014). There are different types of sensilla on mosquitoes including: sensilla trichodea, grooved peg sensilla, sensilla coelonica and sensilla ampullacea. Sensilla trichodea are found in large quantities on the antennae and there have been five types identified in *An. stephensi*. Each sensilla can house more than one bipolar Olfactory Sensory Neurons (OSN) which contain molecular receptors for specific odours. Odour molecules are often non-polar and must permeate the sensillum wall pores before reaching the olfactory receptor (Steinbrecht, 1997).

1.5.2 Olfactory stimuli from hosts

1.5.2.1 Breath

Carbon dioxide has been identified as an important kairomone therefore, breath can be attractive to mosquitoes. Carbon dioxide is primarily present in breath, however it is also present in small amounts on the skin. A small amount of CO_2 (0.01-0.03%) is needed to activate host seeking behaviour, furthermore, the plume structure of CO_2 influences upwind host-seeking behaviour, turbulent and filamentous plumes have been found to be more attractive than homogenous plumes (Healy & Copland, 1995; Geier *et al.* 1999; Gibson and Torr, 1999). A total of 317 compounds have been identified in human breath, and the compounds in breath are dependent on diet and composition of saliva (de Lacy Costello *et al.* 2014), although there is little evidence that diet affects mosquito behaviour. Of these compounds, alkanes, alcohols, ketones, aldehydes, amines and sulphur have been identified (Phillips 1997), a few of which have been shown to elicit a behavioural response in mosquitoes (Table 1-1).

1.5.2.2 Skin

Human body odour is made up of a complex blend of volatile organic compounds that are emitted from the skin. More than 300 volatile compounds emanating from human skin (Bernier *et al.* 2000) can be divided into different categories including carboxylic acids, alcohols, aldehydes, amides/amines, esters halides, heterocyclics, aliphatic/aromatics, sulphides, thioesters, unsaturated carboxylic acids, and ketones. (Bernier *et al.* 2000). Many of these contribute to human body odour generally, however, not all of the skin compounds act as kairomones, as mosquitoes can detect only some of these (Table 1-1).

1.6 Differential attractiveness in humans

1.6.1 Body odour

It is well documented, both in the literature and anecdotally, that some people get bitten more than others, therefore, humans have varying degrees of attractiveness to mosquitoes (Logan *et al.* 2008). Body odour plays a major role in how attractive humans are to mosquitoes, along with factors such as age, body size, genetics, pregnancy status and parasite infection status, which can all be associated with

physiological changes in the body, altering the way we smell. Some of these factors have been investigated in association with mosquito behaviour.

A previous laboratory study collected skin emanations from 27 participants using glass beads. The glass beads were tested in a dual-choice olfactometer using *An. gambiae* mosquitoes against ammonia, a known kairomone. Different levels of attractiveness between participants were seen in this study (Qiu *et al.* 2006). A similar semi-field study ranked the attractiveness of a group of individuals against each other by collecting odours on nylon and cotton socks and tested them with *An. gambiae*. The individuals were put in groups of 4 and their worn socks were placed in counter-flow geometry traps in a screened house where 200 mosquitoes were released and given the choice between the 4 worn socks. This study also collected volatiles from participant's feet and analysed them with gas chromatography (GC). The most attractive participant was 8 times more attractive than the least attractive individual, and the authors concluded that differential attractiveness was due to the different volatile compounds and total amount of compounds that each participant emitted from their feet (Omolo *et al.* 2013). Another semi-field study investigated this further, using total body emanations. Participants were placed in a three-port olfactometer and *An. gambiae* were released at the centre. This study showed that participants had different levels of attractiveness, which was due to differences in complete body odour composition (Mukabana *et al.* 2002). A study identified 33 compounds that could play a role in unattractiveness by assessing body emanations from a group of individuals using gas chromatography coupled with electroanntenography (GC-EAG). When the compounds were tested with behavioural experiments, five compounds decreased upwind flight behaviour when added to an attractive human hand. However, this study used *A. aegypti* mosquitoes, therefore the compounds that reduced attractiveness could be specific to that *A. aegypti* and it is possible that the same compounds may not reduce attractiveness with *Anopheles* mosquitoes (Logan *et al.* 2008).

1.6.2 Age

A study investigated the effect of age and gender on mosquito attractiveness, and found that *An. gambiae* mosquitoes prefer to blood-feed from adults compared to children and teenagers. This is correlated with an increased body surface area and

weight, whereby more body odour, heat and CO_2 are being produced by the host. This difference could also be explained by the development of the human body as it ages. The apocrine and sebaceous glands do not develop fully until puberty, which affects body odour. Furthermore, children generally sweat less than adults, which could explain further this difference in attractiveness (Port *et al.* 1980).

1.6.3 Diet

Two studies have investigated the effects of drinking alcohol in relation to mosquito attractiveness with *Aedes albopictus* and *An. gambiae*. Both studies found that consuming alcohol increased mosquito attractiveness (Shirai *et al.* 2002; Lefèvre *et al.* 2010). Other studies have investigated the effect of the ingestion of vitamin B on mosquito attractiveness as it is often recommended as a mosquito repellent in popular media. Vitamin B has been found to have no effect on mosquito attractiveness with *An. stephensi* and *A. aegypti* (Ives & Paskewitz 2005).

1.6.4 Blood group

It was believed that *An. gambiae* preferred hosts that had O blood group out of the ABO blood group status (Wood *et al.* 1972; Wood, 1974). However, a later study showed that *An. gambiae* could not differentiate between different blood groups and therefore had no preference (Thornton *et al.* 1976). One study found that individuals with O blood group had a higher prevalence of malaria in a malaria-endemic area in south Iran, however this could be linked to immunity and not necessarily with mosquito attractiveness. Blood-fed mosquitoes were collected and tested to identify the blood type, however, the results were inconclusive as the number of mosquitoes caught was low (Anjomruz *et al.* 2014). Another study found that *Aedes albopictus* were significantly more attracted to blood group O than blood group A (Shirai *et al.* 2004). There is little consistency on blood-group preference.

1.6.5 Pregnancy status

Pregnancy status has been found to influence mosquito attractiveness to humans. Lindsay *et al.* (2000) conducted a semi-field study in The Gambia using experimental huts and found that *An. gambiae* mosquitoes were significantly more attracted to pregnant women compared to non-pregnant women. Increased attraction in this study was over a distance of 15 meters. A follow up study observed the same effect over a

short-range setting (Ansell *et al.* 2002). This could be because pregnant women have a larger surface area than non-pregnant women, furthermore, the skin temperature also increases. During pregnancy, there is a change in hormones which could affect body odour and this may also play a role in increased attractiveness.

1.6.6 Genetics

Body odour in humans has a genetic component. Studies have investigated this using twin pairs, where monozygotic twin body odour is found to be more similar than non-identical twin by independent human and dog sniffers (Roberts *et al.* 2005). This suggests that body odour is influenced by specific genes. Specifically, the genes that influence body odour are strongly correlated with the Major Histocompatibility Complex (MHC), which are thought to also have a role in mate selection in human beings (Roberts et al. 2008; Wedekind & Füri 1997). MHC molecules bind to peptides and volatile components such as carboxylic acids, however there could be many other genes involved (Wedekind & Penn 2000; Penn *et al.* 2007).

Studies have investigated whether mosquito attractiveness could have a genetic component. Kirk *et al.*, (2000) sent questionnaires to monozygotic and dizygotic adolescent twins investigating their perceived susceptibility to mosquito bites and concluded that there was a strong genetic influence as there was more similarity between monozygotic twins compared to dizygotic twins. However, this study could potentially have a confounding effect of the reaction to bites as larger reactions to bites may be perceived as being more attractive to mosquitoes, or having no reactions to bites may be perceived as not being attractive to mosquitoes. Furthermore, the results may be objective it is based on a questionnaire.

A further laboratory study ranked the attractiveness of 48 individuals by collecting their skin emanations using glass beads, the beads where then tested in an olfactometer and the differential attractiveness was assessed. Participant's blood was taken to profile the Human leukocyte antigen. In this study, people carrying the Cw*07 HLA gene were found to be more attractive to *An. gambiae* mosquitoes, however this was not significant (Verhulst *et al.* 2013). Fernández-Grandon *et al.* (2015) tested the heritability of attractiveness to *A. aegypti* mosquitoes from monozygotic and dizygotic

twins. Monozygotic twins showed a high correlation in attractiveness, whereas dizygotic twins had a significantly lower correlation.

1.7 Source of human derived compounds

1.7.1 Skin structure

The skin is one of the largest organs in the body. It is composed of two main layers, the epidermis and the dermis (Figure 1-5). The epidermis consists of 5 layers. The top layer is the stratum corneum which consist of squames (dead keratinized cells) and lipids. The dermis is thicker and consists of millions of hair follicles, sebaceous glands and sudoriferous glands. Sebaceous glands secrete sebum which consists of triglycerides, wax monoesters, fatty acids and squalene. They are regularly found in conjunction with hair follicles, which make up a pilosebaceous unit (Verhulst et al. 2010). Higher numbers of sebaceous glands are found in the upper body, however they are not found on the underside of hands and feet. Sudoriferous glands are sweat producing glands and two types are found on the human body: eccrine and apocrine glands (Figure 1-6). Eccrine glands are found everywhere on the body, with higher concentrations on feet, hands, armpits and head. Their primary role is to thermoregulate via the excretion and evaporation of sweat. Fresh eccrine sweat is odourless and has a complex composition. Apocrine glands are found primarily in the axilla. Smaller numbers are also found on the nipple and in the genitoanal regions (Figure 1-6). It is hypothesized that apocrine glands play a role in the production of pheromones (Smallegange *et al.* 2011).

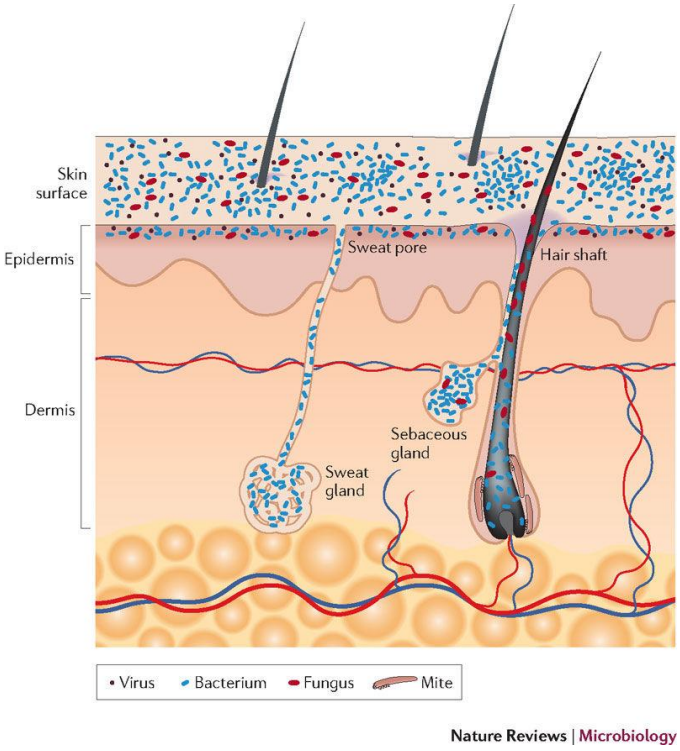


Figure 1-5 Diagram showing a cross-section of the skin (Grice & Segre 2011).

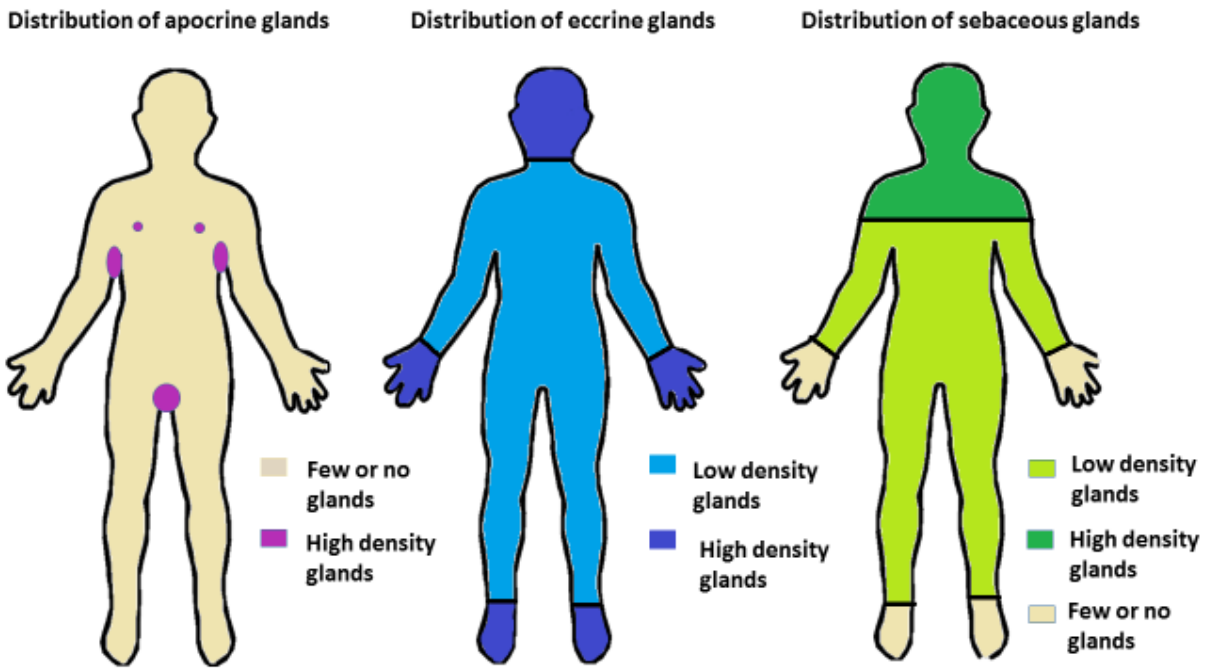


Figure 1-6. The distribution of aprocrine, eccrine and sebaceous glands (Image modified from: Verhulst *et al.* 2010).

1.7.2 Human skin microbiota

The human skin is generally a dry and acidic environment. Glands can affect the nutrient content, moisture content, the pH and osmolarity of the skin. Skin temperature can range from 25-35°C depending on glands present, hair follicles, body location and external environmental factors. All of these factors affect the composition and the abundance of the skin microbial community and create different skin habitats. The skin microbiota is mostly composed of gram-positive aerobic bacteria, as they are better adapted to drier environments. The main factors that influence the skin's microbiota survival include temperature, low moisture content, high osmolarity, low pH, oxygen concentration, nutrient availability, interactions with other microbes (may be advantageous or disadvantageous) and host defence systems (Wilson, 2008). A body site with high sweat gland numbers tend to have a higher moisture content which has an effect on the nutrient availability, osmolarity and pH of the skin site, and therefore affecting the microbiota composition. Similarly, a body site that is contained (such as the axilla or the toe web) has higher temperatures and increased sweat production compared to other body areas. They, therefore, have a higher population density and a different composition of skin microbiota to other, drier areas. Skin microbiota consist of long-term, short-term and transient microorganisms (Wilson, 2008).Other factors that influence the skin microbiota composition include the host's age, type of clothing worn, cosmetic use, antibiotic use, and gender (Wilson, 2008). During puberty, the sebaceous and apocrine glands mature which influence the skin microbiota present. The UV exposure the skin receives will also affect the skin microbiota (Wilson, 2008; Grice & Segre 2011).

1.8 Molecular tools for skin microbiota identification

Information on skin microbiota has been traditionally collected using culturing techniques (Wilson, 2008). This has disadvantages as it is labour-intensive and is dependent on the use of a non-selective growth media that is capable of supporting all the microorganisms in the sample. The greatest disadvantage of using this method is that organisms grow at different rates, which may result in overgrowth of plates of certain microorganisms and fails to identify slower growing organisms (Gao *et al.* 2007). Furthermore, a non-selective growth media cannot providethe optimal

environmental conditions such as the correct pH, oxygen content or CO_2 content necessary for all of the organisms. Therefore, the use of a non-selective media can severely underestimate the number of microorganisms present and relative abundance of each taxa (Wilson, 2008). Culture independent molecular techniques have been used recently to analyse complex microbial communities. One method involves using the 16S rRNA gene sequencing. This new tool amplifies the small ribosomal subunit genes (16S rRNA) using universal primers, and the amplified sequences are cloned and sequenced. The gene encoding 16S rRNA has highly variable regions that are species-specific and they are found on all bacteria which allows taxonomic classification. It also has conserved regions which bind to PCR primers. The sequences are identified by comparing the amplified sequences to an existing database. This method can be tailored to identify microorganisms to the genus or the species level. If a sequence has 98% similarity to a sequence in the database it can be assumed that it is the same species (Gao *et al.* 2007).

A study that used this method to examine the diversity of microbiota on the left and right arm identified10 phyla, 119 genus-level taxonomic units and 247 species-level operational taxonomic units. Half of the organisms identified were from the genera *Propinonibacteria, Corynebacteria, Staphylococcus* and *Streptococcus*. Furthermore, the authors detected 7 out of the 8 genera that have been previously associated with skin microbiota, however they also found a further 100 genera that had not been cultured previously (Gao *et al.* 2007).

Similarly, another study used 16S rRNA gene sequences to analyse 20 skin sites from 10 healthy volunteers. A total of 19 phyla were detected, however, most sequences belonged to the four phyla: *Actinobacteria* (51.8%), *Firmicutes* (24.4%), *Proteobacteria* (16.5%) and *Bacteroidetes* (6.3%). Specific bacteria were correlated with certain habitats, for example, *Propionibacteria* species and *Staphylococci* species were in abundance in sebaceous sites. *Corynebacteria* and *Staphylococc*i species were found in abundance in moist areas. Sebaceous areas had less diversity, whereas dry and moist areas had a higher diversity. Samples were taken from the same individuals 4 to 6 months after the initial sampling and concluded that the skin's microbiota composition remained relatively stable over time (Grice *et al.* 2009). Another similar study sampled

27 body sites and analysed the microbiota from different habitats using 16S rRNA gene sequences. Twenty-two phyla were detected, however, most sequences corresponded to *Actinobacteria* (36.6%), *Firmicutes* (34.3%), *Proteobacteria* (11.9%) and *Bacterioidetes* (9.5%). This study also sampled its volunteers over time and found that the microbiota composition was stable overtime. Both studies show that there is a higher variation between individuals than within an individual (Costello *et al.* 2009).

1.9 VOCs emitted from skin microbiota

Fresh sweat is odourless and only when it is incubated, do its characteristic volatile organic compounds (VOCs) emit from the skin, although some compounds may be released by skin cells themselves. Microbial communities biotransform odourless secretions (apocrine, eccrine and sebaceous) into VOCs, such as short and medium chained volatile fatty acids. Microorganisms thrive on body sites that have large numbers of glands, and the number of microorganisms present and the intensity of body odour are strongly correlated (Wilson, 2008). Skin microbiota therefore play an important role in the production of body odour and many VOCs produced by humans are a result of the skin's microflora metabolic activity. Some species produce specific VOCs, however, other species have been shown to produce up to 60 VOCs (Kai *et al.* 2009).

Human malodour from the axillary vault and feet have received a lot of focus from the cosmetic industry, however, few studies have investigated other body areas. It has been shown that genera *Propionibacterium, cornybacterium* subgroup A and *Staphylococcus* bacteria break down the lipids secreted by sebaceous glands and transform it into carboxylic acids (Smallegange *et al.* 2011). In the axilla, sebaceous, eccrine and apocrine glands are present, high numbers of *Corynebacteria* and *Staphylococci* transform sweat into volatile fatty acids (VFAs), thioalcohols and a few 16-androstene steroids which contribute to malodour (James *et al.* 2013). The feet are dominated by *Staphylococci* and eccrine glands, which are found in very high densities on the sole. Volatile Fatty Acids, particularly isovaleric acid, L-leucine are associated with foot malodour (James *et al.* 2013). *Staphylococci* bacteria transform sweat into odorous short chain carboxylic acids. Specifically, acetic, propanoic, 2-

methylpropanoic, 3-methylbutanoic and decanoic acid have been detected (Smallegange *et al.* 2011).

1.10 Production of VOCs by skin microbiota in relation to mosquito host seeking behaviour

Some compounds from skin have been identified in relation to characteristic malodour, however, what humans perceive as malodour may not act as an olfactory cue for mosquitoes. A study compared fresh and incubated human sweat in a dual-choice olfactometer and found that *An. gambiae* responded to incubated sweat only compared to fresh sweat, signifying that bacteria play a role in mosquito attractiveness. The authors then tested ammonia, as it is a component of sweat, in a dual-choice olfactometer and found it was a kairomone (Braks & Takken 1999). Furthermore, incubated sweat harbours higher ammonia concentrations, therefore, making it more attractive (Smallegange *et al.* 2005). One study incubated bacteria and added skin squames to the culture and found that *Bacillus thuringiensis, S. haemolyticus, S. paucimobilis, S. epidermidis, M. leutus* and *M. organophilum* all produced ammonia that was significantly higher than the control (Ng *et al.* 2017). Various studies have identified and tested lactic acid (another sweat component) and also found it to be attractive to mosquitoes (Acree *et al*. 1968; Smith *et al.* 1970; Geier *et al.* 1996; Smallegange *et al.* 2005). A study by Cork & Park, (1996) analysed components of sweat, and found 20 aliphatic and three aromatic carboxylic acids elicited electroanntenography reactions. Subsequent experiments in the laboratory and in the field have tested ammonia and lactic acid and demonstrated them to be attractants (Table 1-1).

A laboratory study incubated feet bacteria on agar and tested it in a dual-choice olfactometer and showed that *An. gambiae* were attracted to the agar. The incubated agar plate was analysed by GC-MS and a synthetic blend was made from the compounds identified (Table 1-1), The synthetic blend was attractive when tested in a dual-choice olfactometer (Verhulst *et al.* 2009). A follow up study ranked participants based on their attractiveness to *An. gambiae*, and bacterial samples were taken from the soles of their feet. Bacterial plate counts and 16S rRNA sequencing showed highly attractive participants had a higher abundance but lower diversity of bacteria

compared to unattractive participants (Qiu, *et al.* 2011). This study was unable to correlate volatiles to bacteria however.

Research suggests that the composition and abundance of certain bacteria may affect how attractive an individual is. Furthermore, different body sites house diverse environments that affect the skin microbiota composition across the body. Therefore, within an individual, certain body sites may be more attractive to mosquitoes. One study that investigated this, found that *Anopheles* mosquitoes preferred the head and foot regions. When the authors washed the feet with antibacterial soap and removed the exhaled breath, the bites were uniform across the body. The authors concluded that the attraction to feet was due to the high number of eccrine glands, and the attraction to the face was due CO_2 being emitted from exhaled breath (De Jong & Knols 1995). Similarly, two studies tested the biting preference sites of various *Anopheles* species. In both experiments, *Anopheles* mosquitoes preferred biting feet. However, when both experiments were repeated with the volunteers lying on the floor, the bites were uniform across the body. Both papers concluded that biting preference sites are influenced by convection currents, rather than specific body sites and bacteria (Dekker *et al*. 1998; Braack *et al.* 2015).

Furthermore, a study tested the attractiveness of the axilla, hands and feet with *An. coluzzi* and found the axilla less attractive than the hands and feet. When the experiment was repeated after 5 days of not washing, there was no difference between the body sites. Deodorant residues were found in the first experiment, therefore, the authors concluded that this caused the difference in attractiveness between body sites (Verhulst *et al.* 2016). In the literature, there is a lack of consistency on whether different body sites are more or less attractive than other body sites, therefore more research is needed to investigate this effect.

1.11 Chemical ecology studies of mosquito behaviour and human odour

Body odour must be collected from individuals in order to identify semiochemicals. This can be done using various techniques such as headspace entrainment or solid phase microextraction (SPME). Once the volatiles have been captured on a filter, they can be eluted to obtain a sample that can then be used for gas-chromatography coupled with electroanntenography (GC-EAG). This method can simultaneously

identify compounds with gas chromatography and which compounds induce a physiological response from mosquitoes. To establish if the identified semiochemical acts as a kairomone (attractant) or allomone (repellent), further behavioural experiments with mosquitoes are needed (Qiu *et al.* 2006; Logan *et al.* 2008).

In the laboratory, host-seeking behaviour can be assessed using various bioassays. Y-tube and dual-choice olfactometers allow the mosquitoes to choose between two odour sources. *A. aegypti* respond well to Y-tube olfactometers, however, *An. gambiae* does not (Smallegange & Takken, 2010). Dual-choice olfactometer wind tunnel are more suitable for *Anopheles* as they have a larger space for them to fly (Knols et al. 1994). Mosquitoes are placed in a release chamber and are allowed to fly upwind to either trapping chamber. Purified heated humidified air is passed through the trapping chambers. The use of these bioassays only explores the behavioural response for medium-ranged cues, semi-field and field studies are needed for long-range host-seeking behaviour. Alternatively, cage assays can be used to measure mosquito behaviour. In this situation, mosquitoes are placed into a cage, without any airflow and their landing and probing behaviour in response to a volatile stimulus can be recorded. These assays have the advantage that they are rapid and repeatable (Stanczyk *et al.* 2013).

There are many studies in the literature that have investigated mosquito behavioural responses to human-derived VOCs. Although the origin of those compounds are often unknown, many of them are likely to be produced by bacteria. These studies are summarised in Table 1-1 and described in detail here.

A study investigating which compounds from sweat samples elicited a physiological response from *An. gambiae* antennae using EAG found that 1-octen-3-ol and 4-methylphenol produced a dose response. Additionally, methanoic, ethanoic, propanoic, butanoic, pentanoic, hexanoic acids also had high EAG responses (Cork & Park 1996). Similarly, volatiles collected from human foot on nylon stockings were analysed with GC-EAG to identify which compounds acted as kairomones. Fourteen compounds were identified as potential kairomones, however, 6 compounds detected were believed to be from the nylon stocking rather than the host (Qiu *et al.* 2004)

Lactic acid is a carboxylic acid and it is found on the skin. A study found that it was not attractive on its own, however, when it was combined with CO_2, skin odour and human skin emanations, attraction increased significantly (Dekker *et al.* 2002). Furthermore, a laboratory study tested a range of oxocarboxylic acids using a wind tunnel against water. They found six of the nine compounds to be attractive to *An. gambiae*, with 2-oxopentanoic acid beingespecially highly attractive (Knols *et al.* 1997; Healy & Copland 2000; Braks *et al.* 2001; Healy *et al.* 2002). A different study observed that *An. gambiae* mosquitoes did not respond to lactic acid or carboxylic acids on their own, but a synergistic blend of lactic acid, ammonia and 12 carboxylic acids was attractive to mosquitoes tested in the laboratory (Smallegange *et al.* 2005). A follow up study compared the attractiveness of single carboxylic acids added to a basic blend of ammonia and lactic acid. Seven compounds were tested, however, tetradecanoic acid was attractive at all flow rates in a dual choice olfactometer (Smallegange *et al.* 2009). The addition of 3 methyl-1-butanol, butan-1-amine and CO_2 to the 3-component blend (ammonia, lactic acid and tetradecanoic acid) increased attractiveness in *An. gambiae* mosquitoes (van Loon *et al.* 2015). Qiu *et al.* (2011) added components of human breath, sweat and urine to the basic blend of ammonia and lactic acid. The odour blend with 7-octenoic acid increased the attraction of the blend, however, 4-ethylphenol, indole, 3-methyl-1-butanol and two ketones reduced the attraction of the basic blend.

Verhulst *et al.* (2013) showed that attractive individals to *An. gambiae* were associated with lactic acid, 2-methylbutanoic acid, tetradecanoic acid and octanal, whereas unattractive individuals were associated with limonene, 2-phenlethanol and 2-ethyl-1-hexanol. Lactic acid did not attract *An. stephensi* when tested alone, however, when combined with CO_2 resulted in attraction (Omrani *et al.* 2012). Mukabana *et al.* (2004) found that, although breath is known to elicit host-seeking behaviour in mosquitoes, it also has components that have repellent properties, and concluded that semiochemicals found in breath could play a major role in differential attractiveness between individuals with *An. gambiae*.

The majority of studies that investigate semiochemicals have been done with *An. gambiae*, few studies have been carried out with *An. stephensi*. Differences between

the species have been investigated, for example, *An. stephensi* responded well to CO_2 alone, whereas *An. gambiae* did not. *An. gambiae* responded to CO_2 and acetone, whereas *An. stephensi* did not respond to this. Furthermore, *An. stephensi* responded particularly well to CO_2 and 1-octen-3-ol. The differences in host-seeking behaviour between the two species could be attributed to host preference. *An. gambiae* are highly anthropophilic and *An. stephensi* tend to feed on humans and sometimes cattle (Takken *et al.* 1997).

Table 1-1. List of compounds tested electro-physiologically and behaviourally in laboratory settings with *Anopheles* mosquitoes

COMPOUND	RESPONSE	SOURCE	ASSAY	SPECIES	REFERENCE
Carbon Dioxide				*An. gambiae*	
	Little attraction when tested alone	Breath	Dual-choice olfactometer	*An. gambiae*	Knols *et al.* 1994; Takken *et al*. 1997
	Activation in host-seeking behaviour	Breath	Dual-choice olfactometer	*An. gambiae*	Healy & Copland, 2000
	Attractive	Breath	Dual-choice olfactometer	*An. stephensi*	Takken *et al.* 1997
Alcohols					
1-octen-3-ol	Dose-dependent response	Breath & sweat	EAG	*An. gambiae*	Cork & Park, 1996
	Attractive when tested with CO2	Breath	Dual-choice olfactometer	*An. stephensi*	Takken *et al.* 1997
2-phenylethanol	Unattractive	Foot	Dual-choice olfactometer	*An. gambiae*	Verhulst *et al.* 2013
2-ethyl-1-hexanol	Unattractive	Foot	Dual-choice olfactometer	*An. gambiae*	Verhulst *et al.* 2013
1-Dodecanol	No effect when added to NH3 + LA	Sweat	Dual-choice olfactometer	*An. gambiae*	Qiu *et al.* 2011

COMPOUND	RESPONSE	SOURCE	ASSAY	SPECIES	REFERENCE
Ammonia					
	Attractive when tested alone	Sweat	Dual-choice olfactometer	*An. gambiae*	Smallegange *et al.* 2005
Aldehydes					
Octanal	Strong response	Foot odour	GC-EAG	*An. gambiae*	Qiu *et al.* 2004
	Attractive	Foot	Dual-choice olfactometer	*An. gambiae*	Verhulst *et al.* 2013
Decanal	Strong reponse	Foot odour	GC-EAG	*An. gambiae*	Qui *et al.* 2004
Carboxylic acids					
Lactic acid	No landing response when tested alone	Sweat	Y-tube olfactometer	*An. gambiae*	Healy & Copland, 2000; Dekker *et al.* 2002
	Attractive when combined with CO2, skin odour and human skin extracts	Sweat	Dual-choice olfactometer	*An. gambiae*	Dekker *et al.* 2002
	Attractive	Foot	Dual-choice olfactometer	*An. gambiae*	Verhulst *et al.* 2011
	Attractive when combined with CO2, not attractive when tested alone	Sweat	Dual-choice olfactometer	*An. stephensi*	Omrani *et al.* 2012

COMPOUND	RESPONSE	SOURCE	ASSAY	SPECIES	REFERENCE
Tetradecanoic acid	Attractive	Foot	Dual-choice olfactometer	*An. gambiae*	Verhulst *et al.* 2011
2-oxobutanoic, 2-oxo-3-methylbutanoic, 2-oxo-3-methylpentanoic, 2-oxo-4-methylpentanoic, 2-oxohexanoic acids	Attractive compared to water	Sweat	Dual-choice olfactometer	*An. gambiae*	Healy *et al.* 2002
2-oxopentanoic acid	Very attractive compared to water	Sweat	Dual-choice olfactometer	*An. gambiae*	Healy & Copland, 2000; Healy *et al.* 2002
2-oxopropanoic, 2-oxooctanoic and 2-hydroxypentanoic acids	No landing response compared to water	Sweat	Dual-choice olfactometer	*An. gambiae*	Healy *et al.* 2002
Combination of (E/Z) 3-methyl-2-hexanoic acid isomer mixture and 7-octenoic acid	No landing response	Sweat	Dual-choice olfactometer	*An. gambiae*	Constantini *et al.* 2001
7-octenoic acid	Increased attraction of NH3 + LA blend	Axilla	Dual-choice olfactometer	*An. gambiae*	Qiu *et al.* 2011
Propanoic, Butanoic, Heptanoic, Pentanoic, Octanoic, Tetracanoic acid	Attractive when added to NH3 + LA	Sweat	Dual-choice olfactometer	*An. gambiae*	Smallegange *et al.* 2009
3-methylbutanoic acid	No landing response when added to NH3 + LA	Sweat	Dual-choice olfactometer	*An. gambiae*	Smallegange *et al.* 2009

COMPOUND	RESPONSE	SOURCE	ASSAY	SPECIES	REFERENCE
Hexanoic acid	Repellent effect when added to NH3 + LA at 0.5ml/min	Sweat	Dual-choice olfactometer	*An. gambiae*	Smallegange *et al.* 2009
Methanoic, ethanoic, propanoic, butanoic, pentanoic and hexanoic acids	Strong response	Sweat	EAG	*An. gambiae*	Cork & Park, 1996.
3-methyl-1-butanol, 3-methyl butanoic acid	Increased attraction to NH3, lactic acid and tetradecanoic acid blend with 4.5% CO_2	Sweat	Dual-choice Olfactometer	*An. coluzzi*	Van Loon *et al.* 2015
Butan-1-amine	Increased attraction to NH3, lactic acid and tetradecanoic acid blend with 4.5% CO_2	Sweat	Dual-choice Olfactometer	*An. coluzzi*	Van Loon *et al.* 2015
Ketones					
Acetone	No landing response when tested alone	Breath	Dual-choice olfacometer	*An. gambiae*	Qiu *et al.* 2011
	Attractive when combined with LA		Dual-choice olfacometer	*An. gambiae*	Qiu *et al.* 2011
	No landing response when combined with ammonia		Dual-choice olfacometer	*An. gambiae*	Qiu *et al.* 2011
6-methyl-5-heptan-2-one, geranyl acetone	Inhibited blend of NH3 + LA	Fresh sweat	Dual-choice olfacometer	*An. gambiae*	Qiu *et al.* 2011

COMPOUND	RESPONSE	SOURCE	ASSAY	SPECIES	REFERENCE
1-butanol, 2,3-butanedione, 3-methylbutanal, 2-methyl-1-butanol, 3-methyl-1-butanol, 3-methylbutanoic acid 2-methylbutanal, 2-methylbutanoic acid , 3-hydroxy-2-butanone, Benzeneethanol	Attractive	Incubated foot bacteria	Dual-choice olfactometer and MMX trap indoor experiment	*An. gambiae*	Verhulst *et al.* 2009
Indole	Inhibited blend of NH3 + LA	Sweat	Dual-choice olfactometer	*An. gambiae*	Qiu *et al*.2011
Dimethyldisulphide	No effect when added to NH3 + LA	Sweat, breath and skin	Dual-choice olfactometer	*An. gambiae*	Qiu *et al.* 2011
1-methyl-3-propylbenzene or 1-methyl-4-proylbenzene, 2-ethyl-1-hexanal, 2-nonanone, benzothiazole or 1,2-benzisothizole, [1,1'-bicyclopentyl]-2-one and tridecane	Strong response	Foot odour	CG-EAG	*An. gambiae*	Qiu *et al.* 2004
4-methyl-phenol	Dose-dependent response	Sweat	EAG	*An. gambiae*	Cork & Park, 1996

1.12 Conclusion

In recent years, there has been much interest in understanding the origin of compounds used by mosquitoes during host location, but we still do not fully understand it. For example, it is not clear if the differential attractiveness seen in humans is due to hormonal changes, genetics or skin microbiome. If we can understand how the skin microbiome or specific bacteria influences an individuals attractiveness then this could be exploited for vector control, either by developing more specific lures or by manipulating the microbiota composition.

Much research has been done investigating which compounds act as kairomones, and some of these studies have progressed into semi-field and field experiments to explore the effects of long-range host-seeking behaviour as a novel control method. Additionally, much of the work that has been done had been on *An. gambiae* mosquitoes, and far less is known about compounds that are used by *An. stephensi* mosquitoes during host location. It is important to conduct research on *An. stephensi* as it is an important malaria vector in Asia.

1.12.1 Aims and objectives:

The aim of this study is to investigate the role of skin microbiota on mosquito attractiveness to human beings. The null hypothesis to be tested is that skin microbiota does not affect mosquito attractiveness to human beings. The objectives of this study were to:

1. Investigate the behavioural effect of not washing after 4 days on mosquito attractiveness on different body sites (feet, axilla, arm and back) with *An. stephensi*
2. Investigate which compounds from the feet, axilla, arm and back that mosquitoes respond to using GC-EAG on volatiles collected from volunteers asked to not wash for four days.
3. To correlate the compounds that are important to mosquito attraction to specific bacteria.

CHAPTER 2

2 Behavioural responses of *Anopheles stephensi* mosquitoes to odour from different body sites

2.1 Introduction

Human beings have varying degrees of attractiveness to mosquitoes (Logan *et al*. 2008). Certain factors have been attributed to differential attractiveness such as pregnancy, gender, parasite infection, age and body size (Lindsay *et al.* 2000; Bryan & Smalley 1978). Studies have collected volatiles released from the skin and tested them in a laboratory setting to remove any factors that might also affect the attractiveness, such as the amount of CO_2 exhaled, heat and humidity released from volunteers as well as volunteer body size, and found variation in attractiveness (Qiu *et al.* 2006). Given this evidence, and the fact that the most important sense used by mosquitoes during host location is olfaction, it is likely that differential attractiveness of mosquitoes to humans is due to differences in human body odour.

Body odour has been investigated in previous studies and semiochemicals that have either attractive or repellent effects on mosquitoes have been identified (Bernier *et al.* 2000; Logan *et al.* 2008; Qiu *et al.* 2011; Omolo *et al.* 2013). Furthermore, the difference in attractiveness to mosquitoes between different body sites has also been investigated with various mosquito species. For example, De Jong and Knols (1995), found that *An. gambiae* had a preference for feet whereas *An. atroparvus* had a preference for the head. When feet were cleaned with an antibacterial soap and breath was removed from the experiment, the biting preferences were more uniform across the body. In contrast, Dekker *et al*. (1998) investigated the biting preference of *An. gambiae*, *An. arabiensis* and *An. quadriannulatus*, and showed all three species had a preference for feet despite their differences in host preference. Where *An. gambiae* are highly anthropophilic, *An. arabiensis* display both zoophilic and anthropophilic behaviours and *An. quadriannulatus* are zoophilic (Takken & Verhulst 2013; Pates *et al.* 2014).

In further experiments, where the volunteer was asked to lie on the floor with the feet in the air, the body area closest to the ground had a greater number of bites compared to the feet. The authors concluded that biting preference sites are influenced by convection currents, rather than specific body sites (Dekker *et al.* 1998). Similarly, Braack *et al.*, (2015) carried out whole-night seated human landing catches with *An. gambiae*, *An. fenestus* and *An. arabiensis.* The volunteers had their feet, ankles, legs and arms exposed. All three species preferred biting on the lower leg, ankles and feet over other body areas when the volunteers were sitting, but when the experiment was repeated with volunteers lying down on the ground, mosquitoes bit anywhere on the body. They concluded that mosquitoes select their biting site based on height above the ground. Verhulst *et al.* (2016) collected skin emanations from the underarms, hands and feet of volunteers. The volatile profiles were analysed and tested for their attractiveness to *An. coluzzi*. The underarm volatiles were less attractive than hands or feet volatiles and this lack of attraction was believed to be due to deodorant residue on the skin. In subsequent experiments, volunteers were asked to not wash for 5 days and no differences were found between body sites. The authors concluded that without deodorant residues, there is no difference in attractiveness between different body sites.

Studies have shown different body sites have different odour profiles, these are influenced by the skin microbiota (Shelley *et al.* 1953). The diversity and number of bacteria are affected by the types of glands present and how occluded the body site is (Dormont *et al.* 2013; Verhulst *et al.* 2011). De Jong and Knols, (1995) showed that the removal of odorants with antimicrobial soap washing affected mosquito biting preferences. The removal of bacteria during washing may also affect biting preferences as odorants produced by some bacteria have been shown to influence mosquito attraction (Verhulst et al. 2009). Bacterial communities change when the skin is not washed, so it is likely that washing regime could affect attractiveness to mosquitoes (Fierer et al. 2008). As explained above, currently there is conflicting evidence on the difference in attractiveness for different body sites. However, if there is a difference in body site attractiveness it is likely that bacteria play a role. It is therefore important to

investigate whether bacteria play a role in mosquito attractiveness and if washing, or not, could influence mosquito attractiveness to different body sites.

2.1.1 Body odour collection methods

Studying the behaviour of mosquitoes to different body sites is difficult as it is logistically challenging to isolate different parts of the body. Using live human volunteers in laboratory settings may not be convenient as it may be difficult to carry out experiments when needed, furthermore, factors such as the size of the volunteer and the heat the volunteer emits may influence the results of the study. Therefore, to test mosquito attraction to odour alone in a laboratory setting, body odour has to be collected from volunteers and tested. Some previous studies have used glass beads rubbed on hands, to collect skin emanations from volunteers, and demonstrated the same level of attractiveness as a hand up to 4 hours post collection. When frozen they remained attractive to mosquitoes for 8 weeks (Qiu *et al.* 2006; Qiu *et al.* 2004; Takken & Verhulst 2013). Cotton pads have also been used to collect body odour and can stimulate mosquito behaviour (Verhulst *et al.* 2016). Another method is to use nylon stockings worn on the feet of volunteers, which can then be used in the laboratory in olfactometer studies, or in traps in semi-field scenarios (Russell 2004; Jawara *et al.* 2009; Omolo *et al.* 2013). Cotton pads are more delicate than nylon stockings and can tear whilst being in contact with the skin, especially for areas such as the feet, where the entire weight of the volunteer is on it.

2.1.2 Behaviour experiments

Mosquito responses to human body odour are normally measured using laboratory bioassays. Dual choice olfactometers have been used extensively for *Anopheles* mosquito behavioural experiments. In these olfactometers, mosquitoes are released and they have an option between two different odour sources that enter the olfactometer with an airflow; CO_2 is added to the odour sources to activate a flight response, and it can also significantly enhance the response to some compounds (van Loon *et al.* 2015). For experiments with *An. gambiae* mosquitoes, experiments take place during the scotophase (the last 4 hours of the dark cycle) as this is when mosquitoes are highly responsive to host odours (Maxwell *et al.* 1998). Mosquitoes can host seek for a set time before researchers stop the experiment and count how

many mosquitoes there are in each port (Takken *et al.* 2001; Qiu *et al*. 2004; Qiu *et al*. 2006; Qiu, *et al.* 2011; Nyasembe *et al.* 2012).

2.2 Aims and objectives

The aim of this work was to test the behavioural response of *An. stephensi* mosquitoes to the odour collected from different body sites of volunteers who had washed recently, and then again after they had not washed for 4 days. The hypothesis is that body sites will become more attractive after not washing for 4 days, when more bacteria and odorants are likely to be present.

Objectives:

1. Collect volatiles from different parts of the body of volunteers who had washed with un-fragranced soap and then again on day 4 having not washed
2. Perform preliminary experiments, with a dual choice olfactometer and a cage bioassay, to determine the most appropriate behavioural assay to test human odour.
3. Investigate whether different body sites are more or less attractive to *An. stephensi* mosquitoes, using the odours collected in objective 1, in a cage bioassay.
4. Investigate whether odour collected from volunteers who had washed recently, and those that had not washed for 4 days collected in objective 1, were more or less attractive to *An. stephensi* mosquitoes in a cage bioassay.
5. Rank the attractiveness of volunteers for each body site between visits.

2.3 Materials and methods

2.3.1 Mosquito colony

Anopheles stephensi mosquitoes were reared under laboratory conditions. A 12:12 light/dark cycle was used. Adult mosquitoes were housed in a 30x30x30cm cage (Bugdorm). They were fed fresh human blood (no older than one week) twice a week. The larvae were fed crushed guinea pig food pellets (Tesco). Adult mosquitoes had 10% glucose solution on cotton wool available to them at all times, and they were given damp filter paper (Whatman) to lay eggs. The adults were kept in an incubator (Panasonic MLR-352H-PA) at 70% humidity and 26°C at all times. Hatched eggs were transferred into a bowl with tap water and the first larval instars were fed food for egg-laying fish (Liquifry No 1). Female mosquitoes that were 5-9 days old, unfed and mated were used for behavioural experiments.

2.3.2 Recruitment of 30 volunteers

Full ethical approval was obtained from the London School of Hygiene and Tropical Medicine (LSHTM) Research Ethics Committee (approval number: 7754) on the 17th September 2014. Thirty male volunteers were recruited via the LSHTM e-mail and posters distributed around University of London colleges, student hall accommodations, museums, galleries and libraries. Only male human subjects aged 18-65 were selected, as previous studies have shown that the body odour of females changes at different point of the menstrual cycle (Nekhotiaeva *et al.* 2004). Subjects were screened with inclusion and exclusion criteria (Appendix 1). During the screening visit, all volunteers completed an eligibility questionnaire, provided written informed consent, and retained a copy of the Participant Information sheet. If subjects were eligible, they were asked to refrain from:

- Eating strong smelling and spicy foods (such as curry) and alcohol 24 hours before and during testing.
- Wearing deodorant or cosmetic lotions 24 hours before and during testing.
- Doing vigorous exercise 24 hours before sampling.

Individual volunteer testing took place over 4 days (Figure 2-1). Subjects were asked to wash their body with an unfragranced soap (Simple) on day 0 which was provided for

them. They were asked not to wash with soap or use any cosmetic products for the remaining days. Volunteers were allowed to wash with water throughout the study. Volunteers were paid £50 pounds for taking part in the study.

Day 0		Day 1		Day 2		Day 3		Day 4	
Day	**Night**	**Day**	**Night**	**Day**	**Night**	**Day**	**Night**	**Day**	**Night**
Volunteers washed with unfragranced soap	Volunteers wear stockings						Volunteers wear stockings	Stockings collected from volunteers	

Figure 2-1 Timeline for collection of volatiles from volunteers.

2.3.3 Volatile collection

Volatiles were collected using nude colour ankle high stockings (M&S Essential Ankle Highs). The stockings were not washed prior to use. The volunteers were asked to place the stockings on the back, the forearm, an underarm and a foot before going to sleep on day 1 and 4, and removing it after 8 hours of wear (Figure 2-2). For the stocking on the back and underarm, gauze and microporous tape (Boots) was provided to allow the volunteers to place the stocking over the skin, then place the gauze over the stocking and hold it in place with microporous tape. For the forearm, volunteers were asked to cut the tip of the stockings off and insert the forearm inside the stocking. For the foot, volunteers were asked to wear the stocking. In the morning, they were asked to store the stocking in a clean glass vial upon removal and at -20°C. Stockings were collected from the volunteers on day 4 and stored at -20°C until use in the behavioural bioassay.

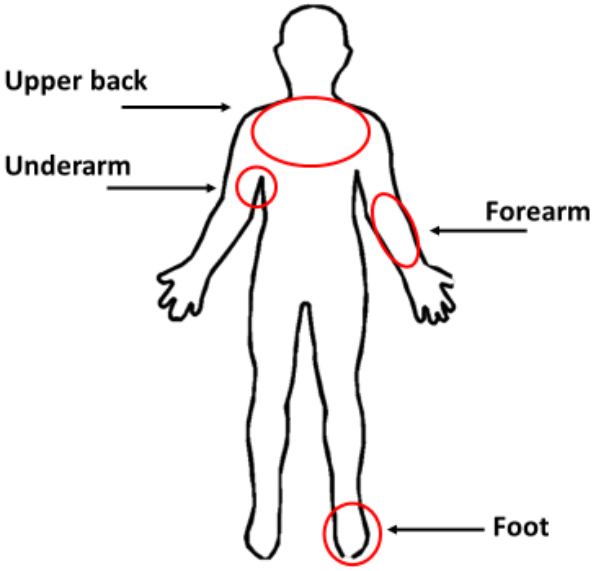


Figure 2-2. Body sites where odour was collected from volunteers.

2.3.4 Behavioural experiments

2.3.4.1 Preliminary tests: development of a behavioural test

1. Dual-choice olfactometer

First, the dual choice olfactometer was used to determine whether the response of *An. stephensi* mosquitoes could be recorded successfully. The dual choice olfactometer consists of a release chamber, windtunnel section and two trapping chambers (Figure 2-3). Charcoal-filtered warm humidified air with an airflow of 0.2m/s was blown into the windtunnel section from the trapping chambers. In addition, CO_2 (0.1m/s at 5%) was also released in a small glass tube placed infront of the trapping chambers to activate flight behaviour. One trapping chamber had a stocking that had been worn and the other trapping chamber had a blank stocking. The stockings were handled with cotton gloves to avoid contamination. Mosquitoes were placed in a release chamber and they were allowed to acclimatise in the release chamber in the room where experiments took place for one hour before experiments. Twenty-five adult mosquitoes were released into the windtunnel and given 25 minutes to respond. At the end of the 25 minutes, the number of mosquitoes in the release chamber, trapping chambers and in the windtunnel section were counted. Experiments took place during the dark phase of the light cycle. The room was kept at 60% humidity and 26±2°C. The

room had a small light to mimick moonlight during experiments and the dual-choice olfactometer was cleaned with 70% ethanol in between runs.

Two experiments were done with the dual-choice olfactometer. For the 1st experiment, the stocking was worn on the foot for 8 hours by the researcher. Ten replicates were done over 6 days. A new stocking was used for each day of testing.

To ensure that mosquitoes responded to odours not just belonging to the researcher, the experiment was repeated using stockings worn by ten volunteers on their feet on day 1. The volunteers were selected randomly.

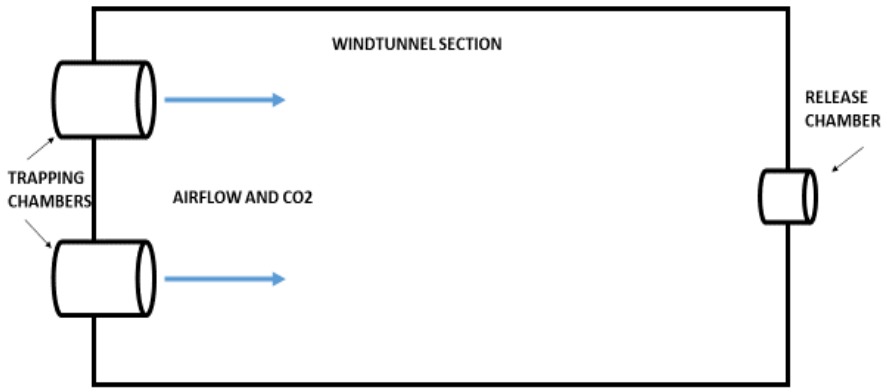


Figure 2-3. The dual-choice windtunnel olfactometer used to test measure behavioural responses of *An. stephensi* to odour stimuli.

2. Cage bioassays

Cage bioassays were also investigated as an alternative test to the dual choice olfactometer. Experiments took place during the dark phase of the light cycle in a room that was held at 26°C and 60% humidity. Five 30x30x30 cages (bugdorm) were used, and a PTFE tube with 5% CO_2 at 20cc/min was placed on top of each cage (Figure 2-4). Unfed and mated female *An. stephensi* between 5-9 days old were used for experiments. Twenty-five mosquitoes were placed in each cage one hour before experiments began to allow mosquitoes to acclimatise to their environment. A circular metallic frame (10cm diameter) was used as a sampling area, the stocking was placed over the metallic frame. The stockings were handled with cotton gloves to prevent contamination from the researcher's hands. Prior to the experiment, a stocking worn by the researcher for 8 hours on the foot was initially used, 10 replicates were performed to test the bioassay.

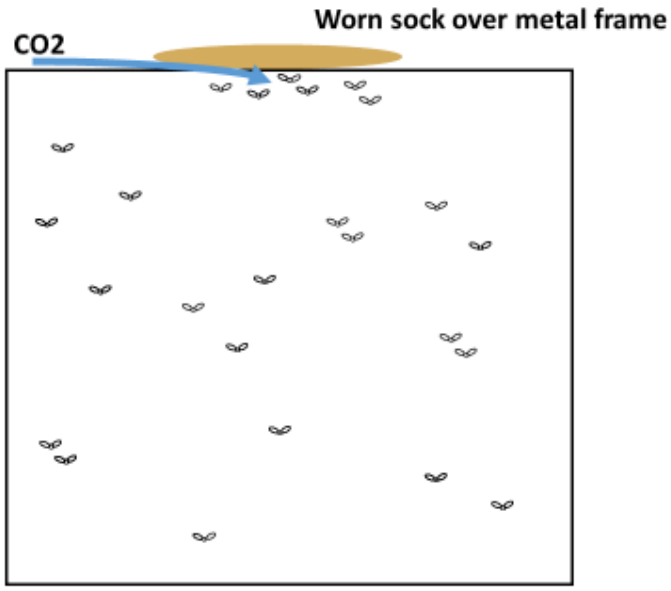


Figure 2-4. Diagram of the cage bioassay to observe the attractiveness of odour stimuli to *An. stephensi*.

2.3.4.2 Behavioural responses to odour from different body sites and days

Due to a lack of response in the dual choice olfactometer the cage bioassay (figure 2-4) was used to test the response of *An. stephensi* to odours from 30 volunteers. The samples worn by volunteers were taken out of -20°C, 8 hours prior to experiments to

allow them to reach room temperature. One volunteer had to be excluded from behavioural experiments as they did not follow the instructions given to them.

A randomised block design was used to test the stockings. Each block contained 8 volunteer samples (foot day 1 and day 4, axilla day 1 and day 4, forearm day 1 and day 4, back day 1 and day 4), a negative control (unworn stocking with CO_2) and a positive control (stocking worn by the researcher, worn on the foot the night before the experiments). The samples were allocated in a random order to a cage and the number of mosquitoes probing was counted after 6 minutes. Five cages were set up for the experiments, where one cage was used per sample. After a set of 5 samples were tested, the cages were refreshed with new mosquitoes, the metal frames were cleaned with 70% ethanol and the experiment was repeated with the remaining 5 samples. Each block was done on separate days. Each sample was tested once.

2.3.5 Statistical analysis

Preliminary experiments: an analysis of variance (ANOVA) was done for the dual-choice olfactometer tests with the researcher's and the volunteer's odour. A t-test was done to test the difference between the worn stocking and the control for the preliminary cage bioassay. Volunteer odour tested in cage bioassay: The data had a right-tale distribution, therefore, the data were log transformed. A mixed effects model on JMP was used to compare the difference between body sites and visits. The visit, the body site and the interaction between body site and visit was specified as a fixed effect, and the volunteer was specified as a random effect. A multiple comparison using Tukey test was done to investigate the difference of body sites between visits, and to test the difference of visits between body sites. An analysis of variance (ANOVA) was done to analyse the difference of the positive and negative controls against each other and against the body sites.

To test the differential attractiveness between volunteers, across all body sites and visits, a one-way ANOVA was done, and for each pair, student's t test was done for multiple comparisons using JMP. To test if the differential attractiveness was correlated between sites and visits, a Spearman's rank correlation was done for the difference in body sites between day 1 and day 4, and between body sites within the same day.

2.4 Results

2.4.1 Preliminary tests

2.4.1.1 Dual-choice olfactometer

The response to the stocking worn on the foot by the researcher varied between replicates. The mean proportion of mosquitoes responding to the stocking was 23.6% and had high variability as the lowest proportion of mosquitoes entering the trap with the stocking was 4% and the highest was 60%. The trapping chamber with the negative control had a low proportion of mosquitoes entering with an average of 0.4%. The number of mosquitoes in the trap with the researcher's foot odour was significantly higher than the negative control (P= <0.001; Figure 2-5). The highest proportion of mosquitoes (46.4%) remained in the wind tunnel section after 25 minutes and did not enter the trapping chambers.

When comparing mosquito response to the researchers odour and the volunteers odour, mosquitoes were significantly more attracted to the researchers odour than the volunteers (P =<0.001; Figure 2-5). When the mosquito response was tested using stockings worn by volunteers on their feet for 8 hours, the mean proportion of mosquitoes responding to the stocking was 6.8%, and this had high variability as the highest proportion of mosquitoes entering the trapping chamber with the worn stocking was 20% and the lowest 0%. There was no significant difference between the volunteer odour and the control (P =0.27; Figure 2-5). The highest proportion of mosquitoes (84%) remained in the wind tunnel section after 25 minutes and did not enter the capture chambers.

As a behavioural response was not recorded in the preliminary tests with the volunteer foot odour, high variability was recorded with the researcher's foot odour and a large proportion of mosquitoes remained in the wind-tunnel section, the dual-choice olfactometer was not used for further experiments.

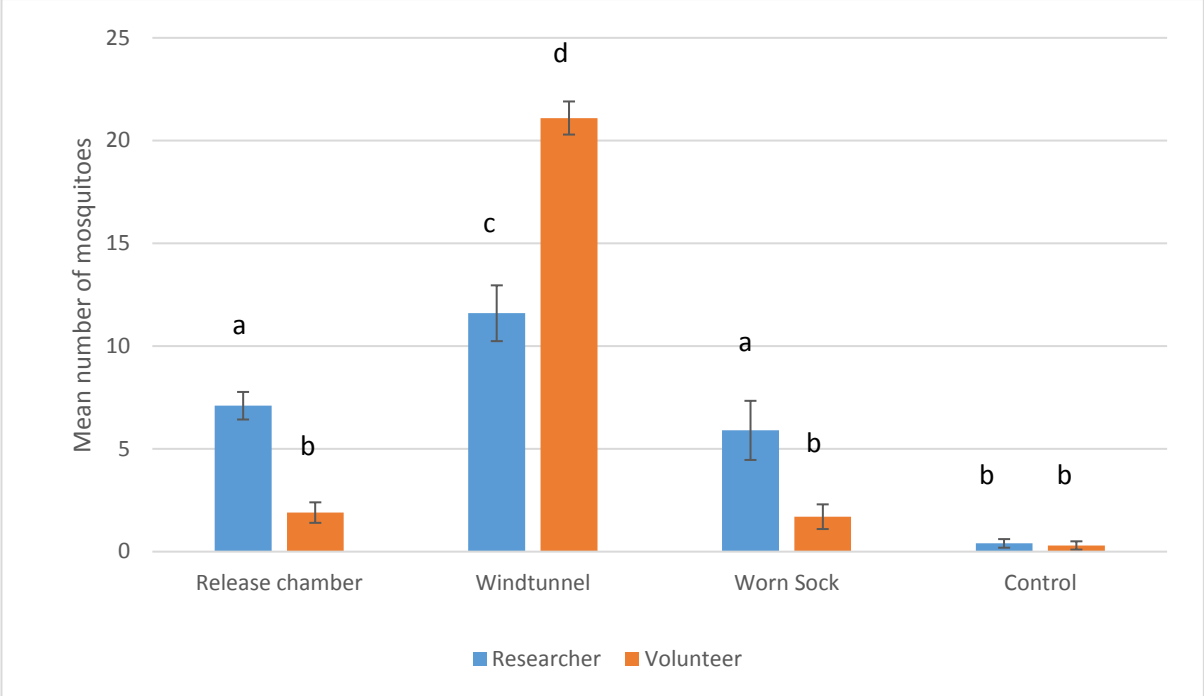


Figure 2-5. Mean number of mosquitoes in the dual-choice olfactometer, separated by area: the release chamber, wind tunnel, in the trapping chambers with the worn stocking and the control. Different letters indicate significant differences at 0.05.

2.4.1.2 Cage bioassay

In the preliminary cage bioassay, to test the response from a stocking worn by the researcher to *An. stephensi* mosquitoes, the mean proportion of mosquitoes probing was 40%. The lowest proportion of mosquitoes landing and probing was 24%, and the highest was 60%. The number of mosquitoes probing on the worn stocking was significantly higher than the control (P=<0.001; Figure 2-6). Due to a greater, and more consistent, behavioural response compared to the dual choice olfactometer, the cage bioassay was used for behavioural experiments.

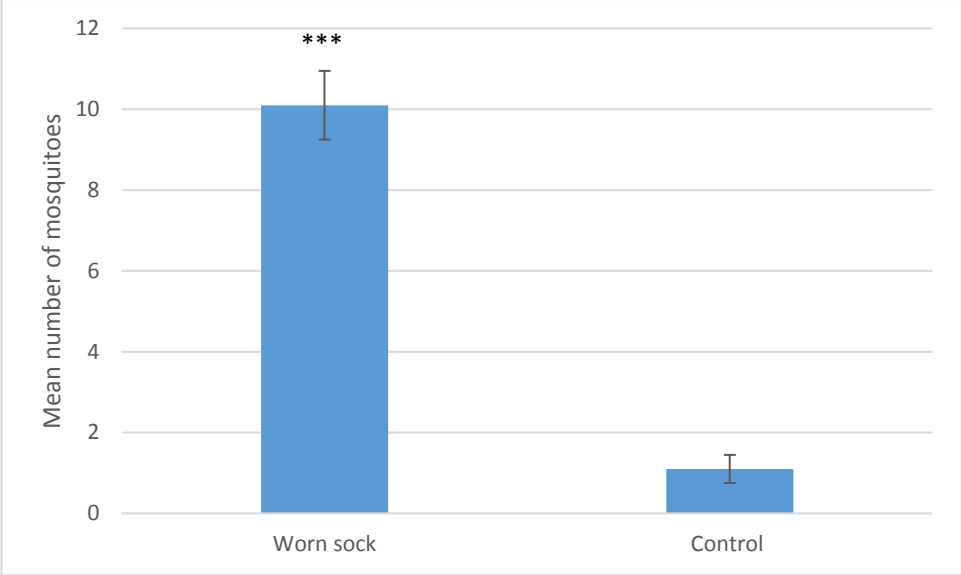


Figure 2-6. Mean number of mosquitoes probing on the worn stocking and the negative control. *P*<0.001

2.4.2 Response to stockings worn by 29 volunteers

A summary of the differences between the different body areas and day is given in Figure 2-7. The mean number of mosquitoes landing on and probing the stocking is displayed for each body area and separated by each visit.

Stockings worn by volunteers at all areas and all days were significantly different to the negative control (*P*<0.001). There was a significant difference between visits for feet, where day 4 visit was significantly greater than day 1 (*P*=0.015). There was also a significant difference between foot day 4 and all the other body sites (Table 2-1). There was no significant difference between the two visits for arm, axilla and back. The positive control was significantly greater than the negative control (*P*<0.001).

Table 2-1. Tukey analysis for the comparison of body sites and visits.

	Body Site	**Arm**		**Axilla**		**Back**		**Foot**	
Body Site	**Day**	**1**	**4**	**1**	**4**	**1**	**4**	**1**	**4**
Arm	**1**		0.884	0.732	1	0.989	1	1	0.012*
	4			1	0.731	1	0.884	0.797	<0.001 ***
Axilla	**1**				0.535	1	0.731	0.614	<0.001 ***
	4					0.941	1	1	0.03
Back	**1**						0.989	0.965	<0.001 ***
	4							1	0.012*
Foot	**1**								0.024*
	4								

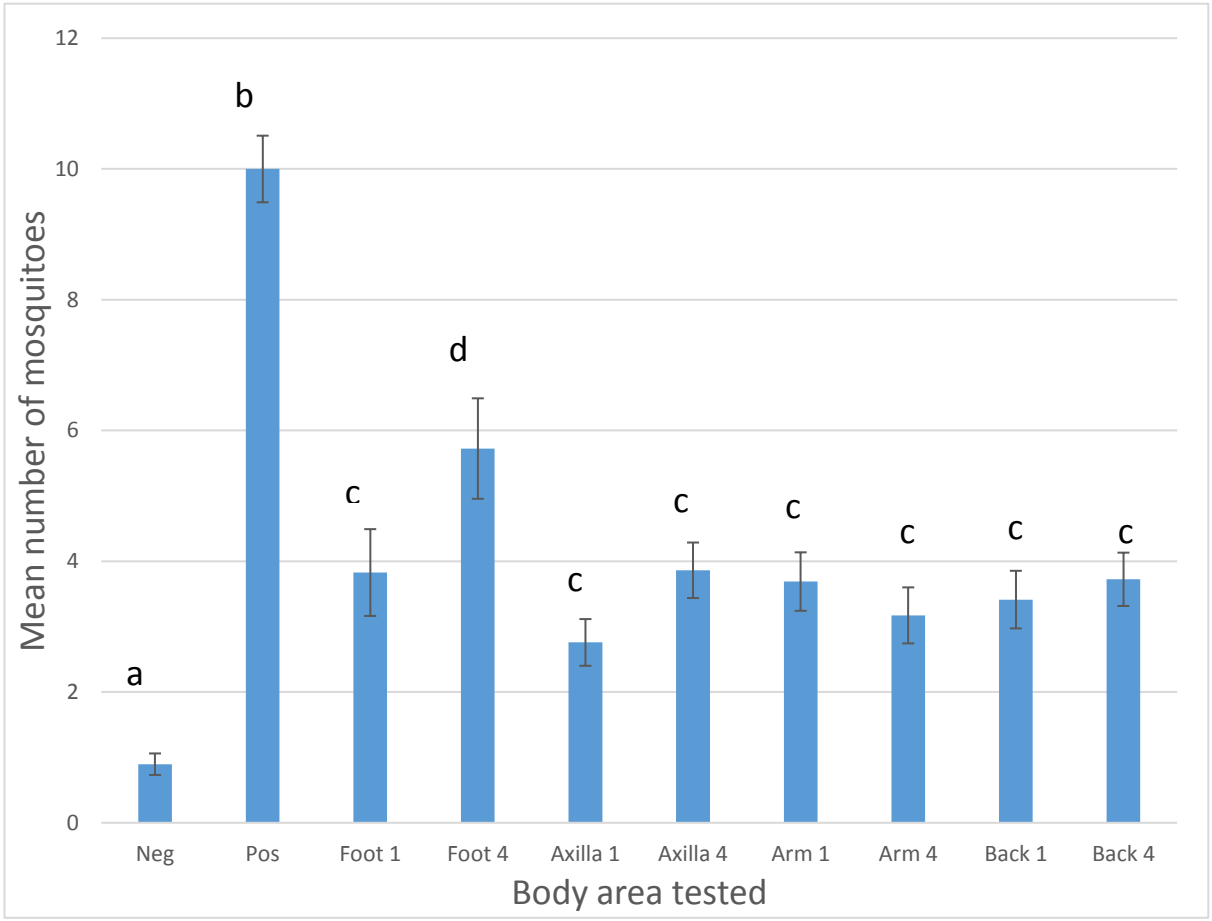


Figure 2-7. Relative attractiveness of the different body sites of volunteers by visit to *An. stephensi.* Different letter indicate differences at 0.05 significance.

2.4.3 Differential attractiveness of the volunteers to *An. stephensi*

Volunteers were ranked according to their attractiveness by using the mean mosquito response across all body sites and days (Figure 2-8, Table 2-2). There was considerable overlap in attractiveness of the volunteers, two groups of volunteers were significantly different from each other, Volunteers 14, 13, 29, 19 and 17 were significantly more attractive than volunteers 7, 9, 11, 26, 6, 1, 10, 28, 3, 2, 22, 21, 25 and 24. However, volunteer 14 was significantly more attractive than all other volunteers. Volunteer 13 was significantly less attractive than volunteer 14, but was significantly more attractive than all the volunteers apart from volunteer 29, 19 and 17.

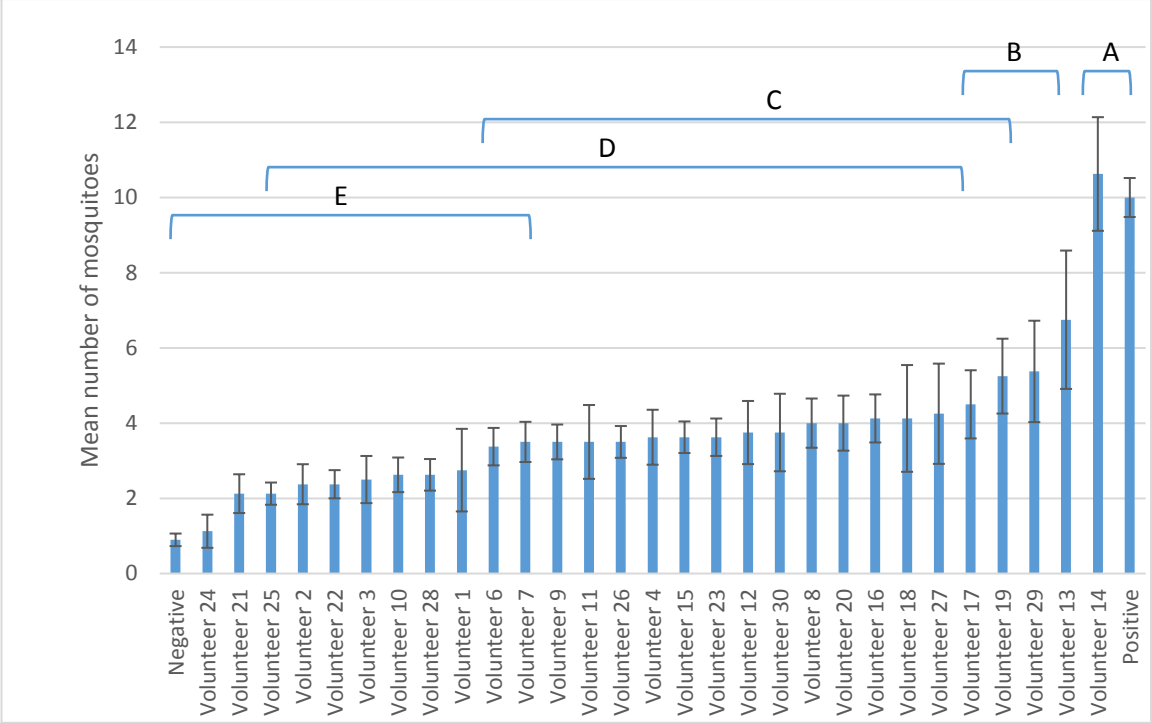


Figure 2-8. Relative attractiveness of volunteers to *An. stephensi* compared to the negative and positive control (sock worn by researcher) using the total mean of all body sites and visits combined. Significant differences of relative attractiveness between volunteers is marked with a letter. Levels not connected by the same letter are significantly different.

Table 2-2. Significant differences of relative attractiveness between volunteers at 0.05 significance. Levels not connected by the same letter are significantly different.

Volunteer	Relative attractiveness			
Positive	A			
Volunteer 14	A			
Volunteer 13		B		
Volunteer 29		B	C	
Volunteer 19		B	C	
Volunteer 17		B	C	D
Volunteer 27			C	D
Volunteer 16			C	D
Volunteer 18			C	D
Volunteer 8			C	D
Volunteer 20			C	D
Volunteer 12			C	D
Volunteer 30			C	D
Volunteer 4			C	D
Volunteer 15			C	D
Volunteer 23			C	D
Volunteer 7			C	D E
Volunteer 9			C	D E
Volunteer 11			C	D E
Volunteer 26			C	D E
Volunteer 6			C	D E
Volunteer 1				D E
Volunteer 10				D E
Volunteer 28				D E
Volunteer 3				D E
Volunteer 2				D E
Volunteer 22				D E
Volunteer 21				D E
Volunteer 25				D E
Volunteer 24				E
Negative				E

The attractiveness of the volunteers was then ranked according to body site (Figures 2-9:12). The volunteer ranking differed depending on the body site and day (1 or 4). The most consistent volunteers across body sites were volunteer 14 who was always ranked as the most attractive, regardless of body site, and volunteer 24 who was always ranked as the least attractive, except for the back.

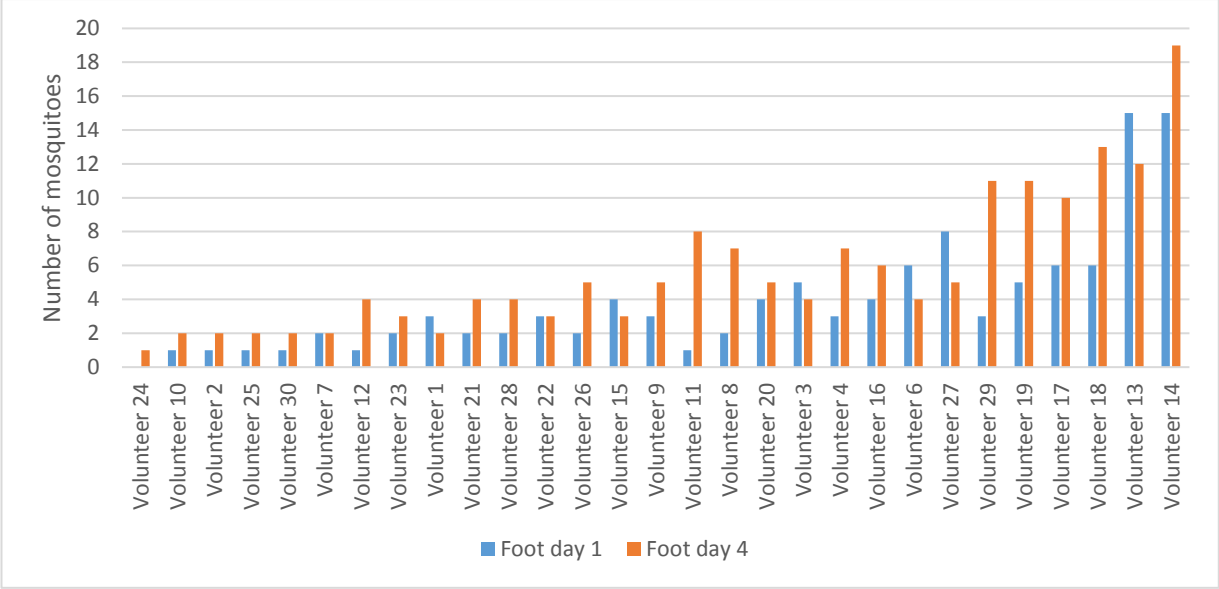


Figure 2-9. Relative attractiveness of volunteers to *An. stephensi* for the foot. Data is ranked according to overall mean and separated by day 1 and day 4.

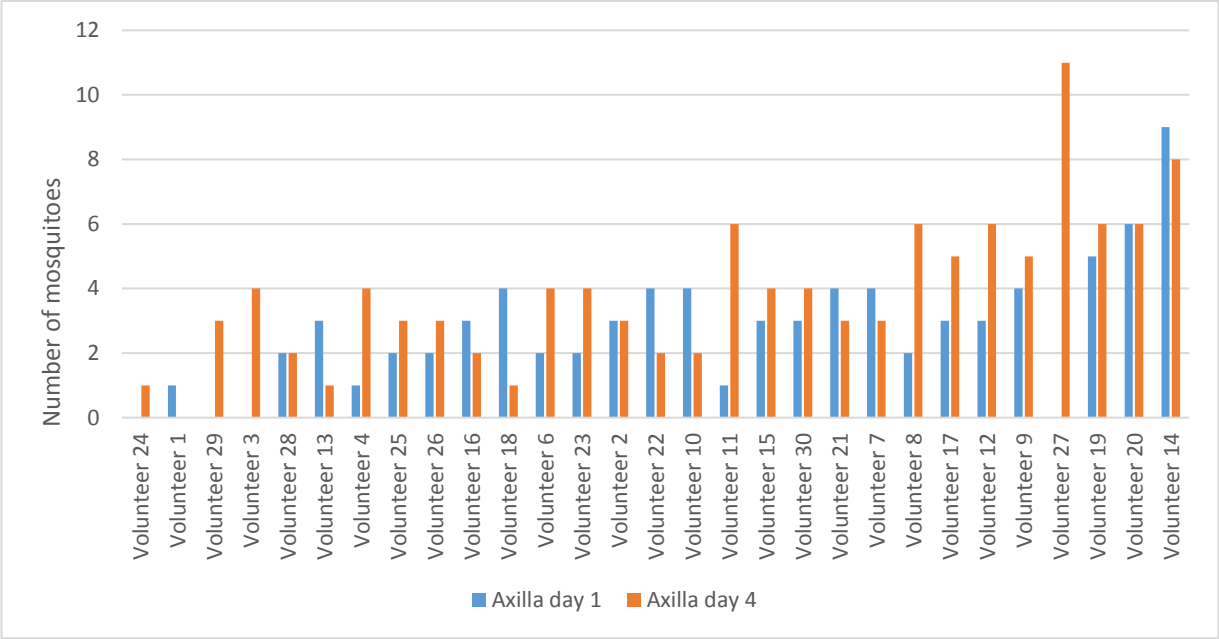


Figure 2-10. Relative attractiveness of volunteers to *An. stephensi* for the axilla. Data is ranked according to overall mean and separated by day 1 and day 4.

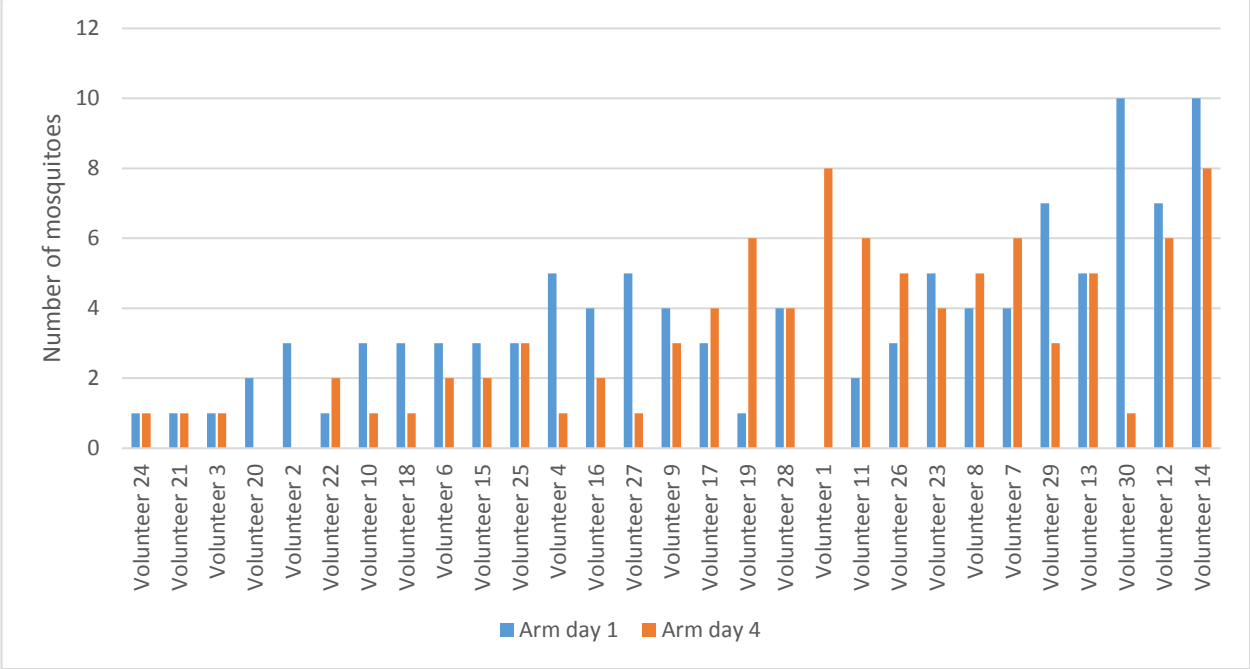


Figure 2-11. Relative attractiveness of volunteers to *An. stephensi* for the arm. Data is ranked according to overall mean and separated by day 1 and day 4.

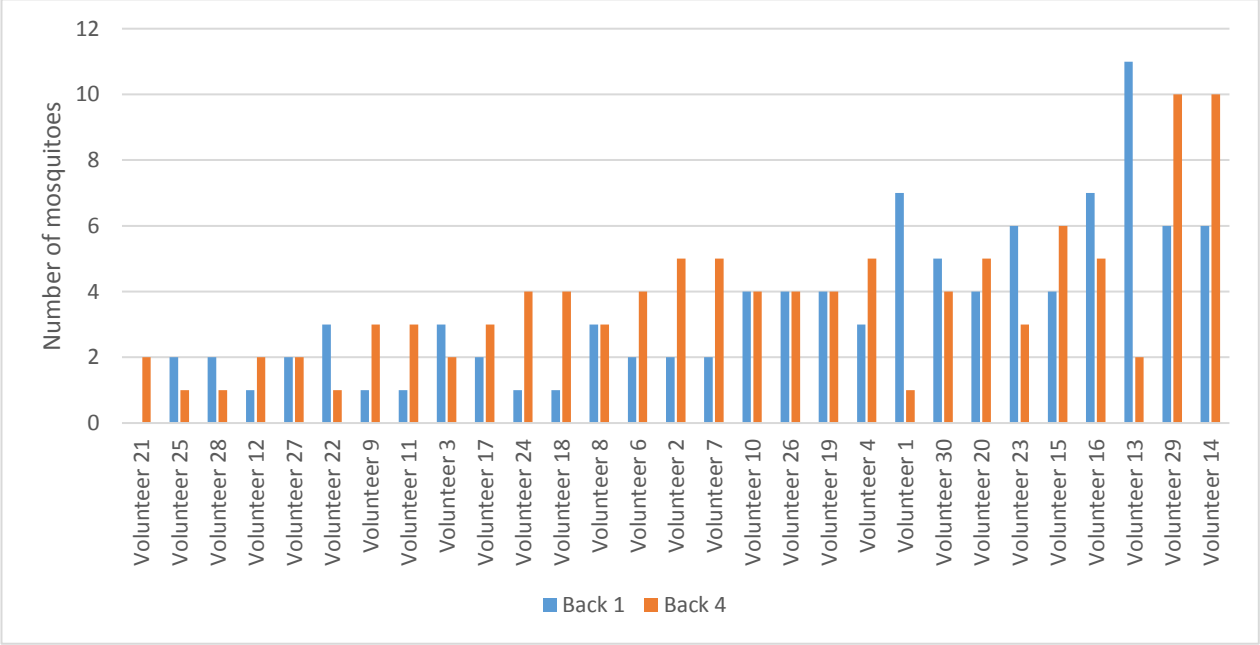


Figure 2-12. Relative attractiveness of volunteers to *An. stephensi* for the back. Data is ranked according to overall mean and separated by day 1 and day 4.

The Spearman's rank correlation of body sites between day 1 and day 4 showed that foot day 1 and foot day 4 had a positive correlation of 0.64 (*P*=0.002; Table 2-3). The other body sites did not have a correlation between day 1 and day 4. The results from the Spearman's rank correlation between the body sites for day 1 and for day 4 did not show any correlation (Table 2-4 and Table 2-5).

Table 2-3 Spearman's rank correlation of different body sites between day 1 and day 4

Body site	**Correlation**	***P* value**
Foot	0.64	0.002*
Axilla	0.09	0.638
Arm	0.16	0.408
Back	0.27	0.164

Table 2-4 Spearman's rank correlation between different body sites for day 1.

Body site	**Body site**	**Correlation**	***P* value**
Foot	Axilla	0.18	0.339
Foot	Arm	0.07	0.704
Foot	Back	0.31	0.102
Axilla	Arm	-0.02	0.915
Axilla	Back	0.02	0.899
Arm	Back	0.25	0.198

Table 2-5 Spearman's rank correlation between different body sites for day 4

Body site	**Body site**	**Correlation**	***P* value**
Foot	Axilla	0.35	0.061
Foot	Arm	0.27	0.154
Foot	Back	0.2	0.297
Axilla	Arm	0.15	0.442
Axilla	Back	0.15	0.423
Arm	Back	-0.18	0.35

2.5 Discussion

The results from this study indicate that *An. stephensi* did not respond well to a dual choice olfactometer. Mosquitoes responded to the stocking worn by the researcher, however, the response was low and varied as it ranged from 4% to 60% in the dual-choice olfactometer, and a large proportion of the mosquitoes made no-choice. Studies that have used the same dual choice olfactometer bioassay, had similar conditions in terms of temperature and humidity in the laboratory and experiments were carried out in the same part of the light cycle. However, previous studies used *An. gambiae* mosquitoes, which suggests that although the bioassay works for *An. gambiae*, it is not suitable for *An. stephensi*. Previous studies that have tested human odour in dual choice olfactometers using *An. gambiae* have had a response ranging between over 50% to over 90% (Qiu *et al.* 2006).

The reason the dual choice olfactometer did not work may be due to the windtunnel section being too large, the odour plume not being appropriate for this species, or some of the other environmental parameters were not being optimal (Spitzen *et al*. (2013). The overall aim of this study was to compare attractiveness of odours from different body sites from volunteers who had washed and not washed for 4 days, therefore, it was essential to have a bioassay that could produce consistent significant responses. The cage assay appeared to produce better results and this was chosen as a method for comparing the different odour stimuli collected.

For the large experiment involving 29 participants, each volunteer was tested in a randomised block design, with a positive and negative control. This allowed direct comparison between the different body sites for the same volunteer. The positive control was a stocking worn by the researcher for 8 hours the night before the experiment. This was chosen as the cage bioassay tests showed that mosquitoes responded strongly and consistently to the researcher's odour collected on a stocking. Moreover, the positive control was constantly available, and a new worn stocking could be obtained for each volunteer. A significant difference was found between feet day 4 and feet day 1, as well as feet day 4 and all other body parts (day 1 and 4). There was no significant difference between the forearm, the axilla and the upper back between day 1 and day 4. This suggests that the process of not washing only affects

the production of volatiles that are involved in differential attractiveness in volunteers, on the feet. Not washing a body site with soap will affect the skin microbiota, which is likely to explain the increase in attractiveness between day 1 and day 4 with feet. Skin microbiota play an important role in the production of odour. The number and type of bacteria is correlated with the concentration of odour being produced (Verhulst *et al*. 2011). In relation to mosquitoes, *An. gambiae* mosquitoes have been shown to be attracted to skin microbiota cultured on agar plates (Verhulst *et al.* 2009). Furthermore, additional experiments showed that highly attractive individuals to *An. gambiae* have a higher abundance, but lower diversity of skin microbiota, compared to poorly attractive individuals (Verhulst *et al.* 2011).

Different body areas have different glands, which affect the composition and number of microorganisms. Human skin has two types of glands, sweat and sebaceous glands. The sweat glands can be further divided into eccrine glands and apocrine glands. Eccrine glands produce sweat, which aids the body to cool down, while apocrine glands are associated with pheromone production (Smallegange e*t al.* 2011). Feet have a large number of eccrine glands on the soles, underarms have a mixture of eccrine and apocrine glands, the upper back has a large number of sebaceous glands and the forearms have low density of eccrine and sebaceous glands, and no apocrine glands. The significant increase in attractiveness in feet between day 1 and day 4 indicates that when people do not wash, the eccrine glands, found on feet, may produce an environment that is more conducive to the proliferation of bacteria that produce compounds that are attractive to mosquitoes. Alternatively, this process may produce an environment that is unfavourable to bacteria that produce compounds that are repellent to mosquitoes. The microbiota of the normal foot is composed of mainly gram positive bacteria including *Corynebacteirum, Brevibacterium, Staphylococcus* and *Micrococcus* (James *et al.* 2013), so it is likely that these bacteria are affected.

Verhulst *et al.* (2016) tested the attractiveness of the hand, foot and underarm of eight volunteers after not washing for 24 hours to *An. coluzzii*. The different body sites were tested in a dual choice olfactometer. The axilla was significantly less attractive than hands and feet. This study also asked a different set of eight volunteers to not wash for five consecutive days and the volatiles from the different body sites were tested in a

dual choice olfactometer, the researchers found no difference between body sites. A comparison between day 1 and day 5 was not done due to the use of different sets of volunteers. Volatile analysis indicated that residues of fragranced cosmetic products were found in the underarm, which may have caused the difference in attractiveness between the two experiments. The authors concluded that there was no difference in attractiveness between body sites, however, fragranced skincare products may reduce attractiveness.

In the current study, although the means of mosquito attractiveness increased for underarm between day 1 and day 4, this was not statistically significant. The difference in attractiveness was tested after four days of not washing, however, in the Verhulst *et al. (2016)* study, volunteers were asked to not wash for five days. Therefore, it is possible that an extra day could make a difference in attractiveness. Furthermore, because a different set of volunteers were used in the Verhulst *et al.* (2016) study for odour collected on day 1 versus day 5, the odours from the different visits could not be compared directly with each other.

Although both underarms and feet produce malodour in human beings, typically, people only use deodorants or antiperspirants for underarm malodour and to prevent perspiration. Foot malodour is not commonly tackled with the use of cosmetic products as they are either not readily available or there is no cultural need or habit to tackle foot malodour (James *et al.* 2013). Additionally, in this study, volunteers were specifically asked to refrain from the use of cosmetics including deodorants. Therefore, the significant increase in attractiveness between day 1 and day 4 with feet is unlikely to be attributed to the use of cosmetic products. Furthermore, an accumulation of odorants in the feet could explain the increase in attraction. This suggests that foot bacteria play a role in differential attractiveness to mosquitoes.

In the current study, the attractiveness of feet to mosquitoes on the day 4 was significantly higher than arm on day 4 axilla on day 4 and back on day 4 after washing. This suggests feet that have not been washed may produce more odour or more bacteria which then produce compounds that are attractive to mosquitoes, or fewer bacteria that produce repellent compounds. However, bacteria incubation may be greater with feet compared to other body sites as they are often in an enclosed space

(shoes) most of the day, which could lead to increased temperature, sweat and bacteria incubation. Furthermore, shoes were not standardised with volunteers as they were allowed to wear their own. Older shoes or shoes with less ventilation could be more odorous which could influence the volatiles collected on the stocking. But this should be accounted for by using the same volunteers on day 1 and day 4. In comparison, the other body sites such as the forearm or the back are not typically in an enclosed environment.

Volunteers were ranked according to their varying degrees of attractiveness. The overall mean number of mosquitoes each volunteer attracted was ranked for each individual. Differential attractiveness has been investigated previously, and these results are similar to findings by Logan *et al.* (2008), where they also found varying degrees of attractiveness to *Aedes* mosquitoes was also observed. Because different body sites were tested, individuals were ranked separately by body site and visit and Spearman's rank correlation was done on body sites between day 1 and day 4, and between different body sites within day 1 and within day 4.

The correlation showed that the same volunteers had the same level of attractiveness on day 1 and day 4 for foot odour. The ranking of volunteers between the other body sites within each day showed no correlation with each which suggests that, although body sites are likely to produce attractants (as they were all significantly more attractive than the negative control), there are sufficient differences in the compounds produced by the different body sites to alter relative attractiveness. The fact that there was also no correlation in volunteer ranking between day 1 and day 4 for any of the body sites, except feet, suggests that arm, back and axilla odours that affect relative attractiveness of the volunteers were not consistent. Since the only significant correlation was found between day 1 and day 4 for foot odour, suggests foot odour is distinct to the rest of the body, and that washing does affect the relative attractiveness of feet.

2.6 Conclusion

This study demonstrated that a dual choice olfactometer is not an effective bioassay for *An. stephensi* and that cage bioassays can be used to establish a difference in attractiveness between odour stimuli.

It is clear that feet have an important role in the attractiveness of *An. stephensi*, particularly if they have not been washed for a number of days. It also shows that after not washing, feet will become significantly more attractive than washed feet and from other body sites, and individuals may increase their relative attractiveness. This highlights that not washing regularly could significantly increase mosquito attractiveness, thus increasing the risk of mosquito borne diseases in endemic regions. If this increase in attraction between days is associated with bacteria, it could be a result of an increase in bacteria that produce attractive volatiles or a reduction in bacteria that produce repellents. Finding a link between the bacteria that produce compounds that mosquitoes are attracted to or repelled by will be investigated further in the following chapters.

CHAPTER 3

3 The identification of electrophysiologically-active compounds from human odours

3.1 Introduction

Mosquitoes use a range of external chemical and physical cues to locate their hosts including odour, temperature, humidity and visual cues. Carbon dioxide has been shown to play a primary role in activating host seeking behaviour in mosquitoes, but other body odours can be detected between 2 to 20 meters away from the host, and body heat, humidity and visual cues play a role in host seeking behaviour at a shorter distance of 1 to 2 meters (Knols & Meijerink 1997; Takken & Knols 1999). Convection currents have also been proposed to play a role in host seeking in *Anopheles* sp (Dekker *et al.* 1998). Of these cues, odour plays an important role as mosquitoes use olfaction predominantly to find hosts (Zwiebel & Takken 2004).

Human body odour is a complex matrix of hundreds of different compounds. These odorants are derived from skin, along with skin microflora secretions and include oxidation products and microbial secondary metabolites (James *et al.* 2013). Previous studies have utilised a range of approaches for the collection of odorants on human skin, Bernier *et al.* (2000) collected volatiles from volunteers' hands using glass beads (20 minutes after washing their hands) and identified 346 compounds using gas chromatography (GC). Of these compounds, the biggest peaks were carboxylic acids, many of which are fatty acids derived via the microbial breakdown of sebaceous triglycerides on skin (Bernier *et al.* 2000). Other type of compounds present included alcohols, aldehydes, aliphatics, amides, esters, halides, heterocyclics, ketones, sulphides and thioesters. Other studies have shown that fatty acids, ketones, aldehydes esters and alcohols are also present in axillary samples (Curran *et al.* 2005). Other methods of sampling body odourants on skin include headspace entrainment, solvent washing or SPME (Logan & Birkett 2007). Skin odorant samples are typically

analysed by GC which allows quantification and tentative identification of compounds. However, volatile analysis in itself does not reveal which compounds mosquitoes respond to. To determine this, a technique called coupled Gas Chromatography-electroantennography (GC-EAG) can be used. Here, half of the odour sample is detected by the GC flame ionization detector (FID), and the other half is detected simultaneously by an antennal preparation of the mosquito. This allows identification of the compounds in the sample which mosquitoes respond to (Figure 3-1). The compounds can be then identified using coupled gas chromatography-mass spectrometry (GC-MS).

The electrophysiology works by essentially using the mosquito as a biosensor (Leal & Uchida 1998; Qiu *et al.* 2004; Guerenstein & Lazzari 2009). Mosquitoes detect odours using olfactory organs, which include the antennae, maxillary palps and the proboscis. These organs are covered in sensilla which are hair-like organs and are highly sensitive to odour molecules. (Guidobaldi *et al.* 2014). The sensilla on the maxillary palps contain receptors that detect CO_2 which activates flight. Sensilla that detect semiochemicals contain olfactory sensory neurons (ORNs) and they are found in abundance on the antennae, and fewer numbers are found on the proboscis and maxillary palps. One sensillum contains two or more bipolar ORNs, the semiochemical information received by the ORN is processed in the olfactory lobe which can then activate a behaviour in the mosquito. GC-EAG involves attaching microelectrodes to the base of the mosquito head and to the antennae and tests online EAG responses simultaneously with the FID signals (Qiu & van Loon 2010).

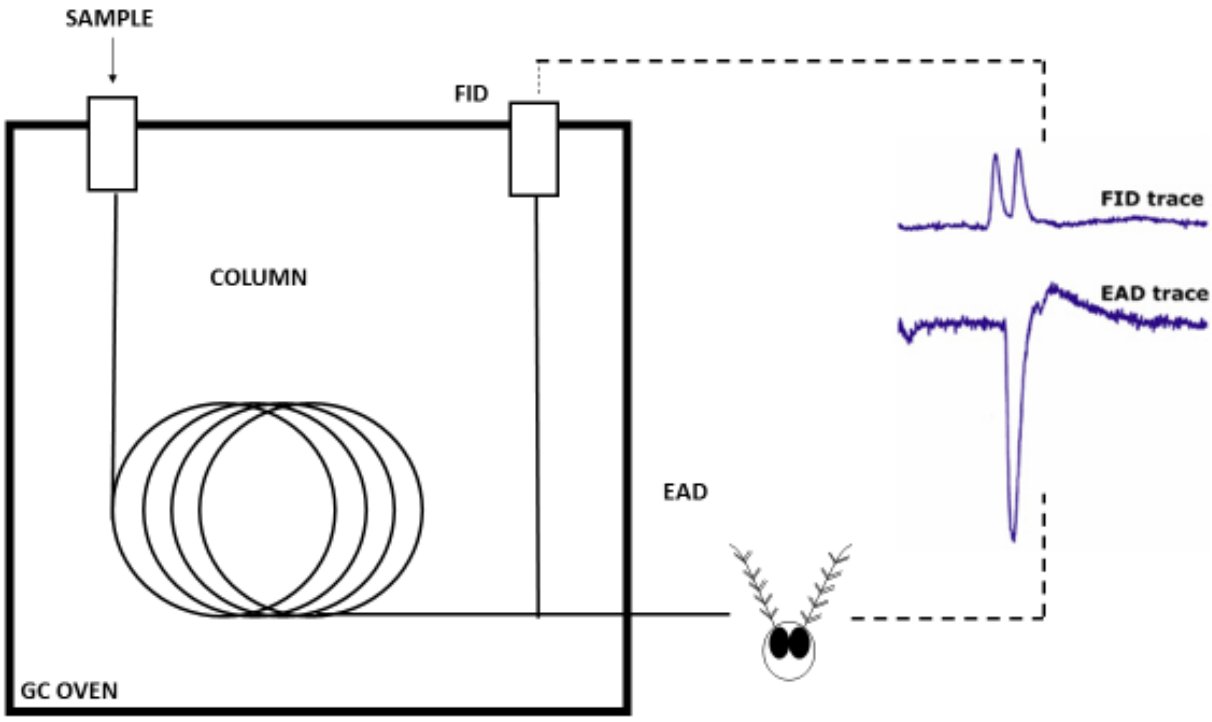


Figure 3-1. Gas chromatography coupled with electroanntenography (GC-EAG).

Many compounds that play a role in host seeking behaviour have been identified using GC-EAG. Compounds collected from human sweat samples were identified using GC-EAG with *An. gambiae* previously; this revealed that short chain carboxylic acids had a larger response compared to long chain carboxylic acids. The short chain carboxylic acids included methanoic, ethanoic, propanoic, butanoic, pentanoic and hexanoic acids. Furthermore, two nonacids (1-octen-3-ol and 4-methylphenol) also elicited an EAG response (Cork & Park, 1996). Costantini *et al.* (2001), also collected volatiles from human hosts and identified which compounds were physiologically relevant to *An. gambiae;* two carboxylic acids (E)-and (Z)-3-methyl-2-hexenoic acid and 7-octenoic acid were found to elicit EAG responses. Other responsive compounds identified using GC-EAG with *An. gambiae* include octanal, 1 methyl-3-propylbenzene, 2-ethyl-1-hexanal, 2-nonanone, decanal, benzothiazole, tridecane, indole, 6-methyl-5-hepten-2-one, geranyl acetone, 4-methylcyclohexanol, 2-ethyltoluene and 2-acetylpyridine, benzaldehyde, octanal, lonalool oxide (Meijerink *et al*. 2000; Qiu *et al.* 2004; Suer, 2011). By identifying which compounds mosquitoes respond to, further behavioural experiments in laboratory and semi field settings can further determine which combinations of compounds act as attractants or repellents to mosquitoes (Meijerink

et al. 2001; Smallegange *et al*. 2009; Qiu *et al.* 2011; Mburu *et al.* 2017. In this study *An. stephensi* was used for the GC-EAG experiments.

3.2 Aims and Objectives

The aim of this work was to identify compounds in body odour collected from volunteers which are electrophysiologicaly active to *An. stephensi*

The objectives of this chapter were:

1. To use coupled GC-EAG to locate electrophysiologically active compounds for each pooled sample (foot day 1 and day 4, axilla day 1 and day 4, arm day 1 and day 4, back day 1 and day 4).
2. To Identify the electrophysiologically active compounds using GC-MS

3.3 Materials and methods

3.3.1 Mosquitoes

Details of the mosquitoes used in this experiment can be found in Chapter 2 (page 46; 2.3.1). Female mosquitoes that were 5-9 days old, unfed and mated females were used for GC-EAG experiments.

3.3.2 Volatile collection

3.3.2.1 Participant Recruitment

The recruitment of participants is described in Chapter 2 (page 46; section 2.3.2).

3.3.2.2 Air entrainment

Volatiles were collected using headspace entrainment. Areas of the back, the underarm, the forearm and the foot were sampled on days 1 and 4, as described in Chapter 2 (Volatile collectionpage 48; section 2.3.3). The collection methods for feet and forearms differed from the axilla and back due to the nature of the different body sites. As feet and forearms are extremities they could be placed in a bag and entrained without difficulty. However, the entrainment of the axilla and the upper back was more challenging as it is impossible to place them in a bag. Consequently, two different methods were used for headspace entrainment which are explained below.

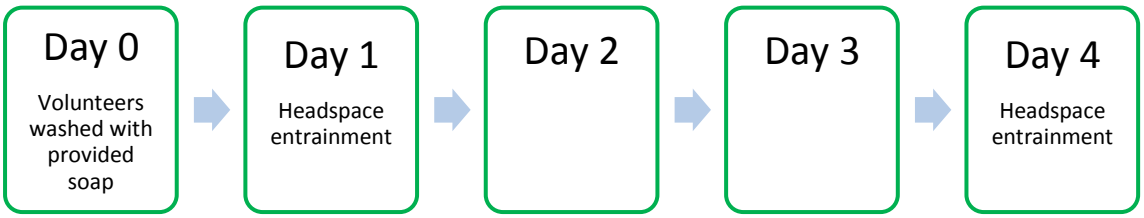


Figure 3-2. Timeline of headspace entrainment

3.3.2.3 Feet and forearm volatile collection

Feet and forearms were sampled using oven bags (Figure 3-4). Cooking oven bags (Sainsbury's) were opened and cleaned by baking them in an oven at 150°C for a minimum for 2 hours. Inlet and outlet ports were made on opposite sides of the bag using Swagelock bulkhead union fittings sealed with a PTFE rubber ring to ensure the fittings were airtight. The swagelock fittings were tightened using a spanner. The foot

or forearm was placed in the bag and tightened around the ankle or the arm using bulldog clips. The oven bag was then connected to a headspace entrainment kit.

3.3.2.4 Axilla and back volatile collection

The axilla and the back were sampled using a glass funnel (Figure 3-4). The glass funnel used for the underarm and the upper back were previously cleaned with 70% ethanol and acetone and baked at 150 °C for two hours. The funnel had inlet and outlet ports, one to push charcoal-filtered air in and the other for the Porapak Q filter. The funnels were placed on the volunteers and held in place with microporous tape and elastic bands, and it was connected to a headspace entrainment kit.

3.3.2.5 Headspace entrainment kit

The entrainment kit had two inlet and outlet ports connected the oven bag; one pushed charcoal-filtered air and the other extracted air. Porapak Q filters were used to capture volatiles from samples; 50mg was placed in a glass tube and glass wool was used as a stopper at each end. The filters were cleaned with 2ml of dichloromethane, and placed in a heating block at 150°C with nitrogen at 600m/sec for 2 hours. This cleaning protocol was then repeated twice using diethyl ether to remove porapak contaminants before first use. Filters were re-used following cleaning with 2ml of diethyl ether and heating in a block at 150°C with nitrogen at 600m/sec for 2 hours. A porapak Q filter (50mg) was connected to the outlet port that extracted air to capture volatiles. The oven bag or funnel was purged of any contaminated air before using on a volunteer. The volunteers were entrained for two hours and a positive pressure was maintained throughout the sampling. The volatiles captured on the porapak Q filters were eluted with 700µl of re-distilled ether and stored in glass vials at -20°C.

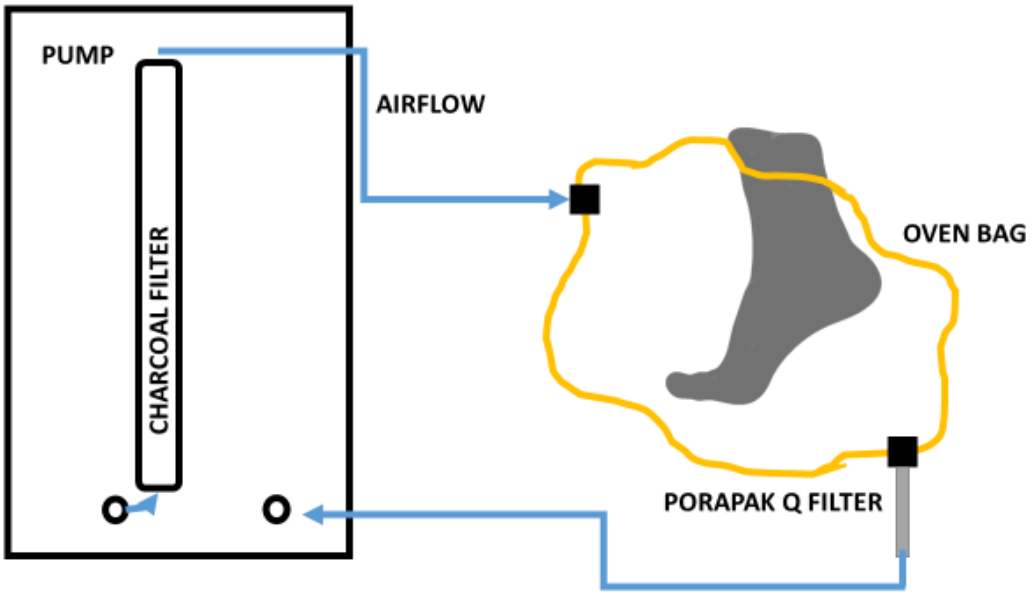


Figure 3-3. Headspace entrainment set up of feet and forearms. The airflow leaving the pump was purified with a charcoal filter, the airflow leaving the bag passed through a Porapak q filter where the volatiles were captured.

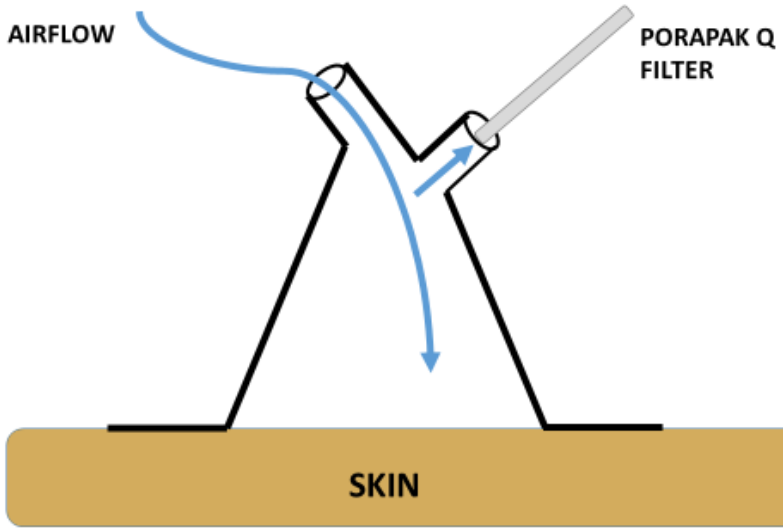


Figure 3-4. Headspace entrainment set up for the upper back and the axilla. The airflow leaving the pump was purified with a charcoal filter, the airflow leaving the bag passed through a Porapak Q filter where the volatiles were captured.

3.4 Sample analysis

The air entrainment samples collected from volunteers were concentrated to 50µl using charcoal filtered nitrogen. After concentrating, 1µl of sample was analysed using GC (see Chapter 5). After all the samples were run on the GC, the samples were diluted to 1ml using re-distilled ether and stored in glass ampoules under nitrogen for storage until further use. For GC-EAG, 500µl of the diluted samples from the same body site and visit were pooled together. The pooled sample was then concentrated with charcoal-filtered nitrogen to 1ml.

3.4.1 GC-EAG

An Agilent 7890A gas chromatograph was used for the GC-EAG. It was equipped with a cool-on-HP1 column (50m x 0.320 mm x 0.52µm). Nitrogen was used as a carrier gas and hydrogen was used as a make-up gas for the flame ionization detector (FID). The injected sample was split 50:50 between the FID detector and EAG. The GC method was as follows: the sample was injected at 40°c which was held for 0.5 minutes, the temperature was increased by 10°C per minute to 230°C. This was held for 20 minutes. The total run time for each sample was 40 minutes.

Two borosilicate glass capillaries (2mm x 1.16mm) were used as electrodes for EAG. Both capillaries had one end sharpened using an electrode puller. An indifferent and a recording electrode were used for EAG. The indifferent electrode had the end finely trimmed off and was very sharp. The recording electrode was trimmed further, the tip of the electrode had a 'shelf' shape to allow enough space for both antennae to rest on the inside of the electrode. Both electrodes were filled with ringers solution (7.55 gl^{-1} sodium chloride, 0.64 gl^{-1} potassium chloride, 0.22 gl^{-1} calcium chloride, 1.73 gl^{-1} magnesium chloride, 0.86 gl^{-1} sodium bicarbonate, 0.61 gl^{-1} sodium orthophosphate) (Maddrell, 1969).

Individual mosquitoes were placed in Eppendorf tubes and placed on ice for 1 minute to knock them down. Upon removal, the head, proboscis and palps were removed. The last segment of the antennae was also cut off. The indifferent electrode was inserted at the base of the head and was mounted on the EAG. The recording electrode was

mounted and with the aid of a silver wire, the antennae were placed inside the recording electrode. After setting up the mosquito on the EAG, a glass tube with a 600ml/min humidified air was placed close to the mosquito head. The glass tube had an opening on the side where the GC transfer line was placed. The EAG set up was carried out in a Faraday cage which had sand under the legs of the table to minimise electrical interference. Once the mosquito head was set up, 1µl of sample was injected into the GC, the GC and EAG started recording by pressing start on the GC and pressing the foot pedal simultaneously. The room was vacated once the GC-EAG had started to minimise environmental interference.

3.4.1.1 Replicates

Each sample was replicated 11 times to ensure that a response from a mosquito could be replicated, and therefore was a true response. Each replicate used a new mosquito. Only a response that was present in over 5 traces was considered a true response. Traces were overlaid using a light box (MiniSun light pad) to visualise the responses.

3.4.2 GC-MS

Coupled gas chromatography-mass spectrometry (GC-MS) was performed on a Micromass Autospec Ultima, magnetic sector mass spectrometer, and Agilent 6890N GC (fitted with a non-polar HP1 column 50m length x 0.32mm inner dia. x 0.52µm film thickness, J & W Scientific). Sample injection was via cool-on-column and MS ionization was by electron impact at 70 eV and 220^0C. The GC oven temperature was maintained at 40^0C for 0.5 min and then programmed at 10^0C min^{-1} to 230^0C, run time 40 minutes. The GC-MS was done by Dr John Caulfield at Rothamsted Research, who guided Christina Due with the interpretation.

3.5 Results

Examples of GC-EAG traces are shown in Figures 3-5, 3-6, 3-7 and 3-8. Foot day 1 and foot day 4 (Figure 3-5), axilla day 1 and axilla day 4 (Figure 3-6), arm day 1 and arm day 4 (Figure 3-7), and back day 1 and back day 4 (Figure 3-8). Fifty-two compounds were identified as EAG-active all samples (Table 1). Of these, peak numbers 1, 3, 6, 11, 12, 17, 18, 23, 24, 27 and 29 had more than 1 possible identification, and 25 peaks were unidentified, thus 16 identified (Table 3-1). The mass spectra of these compounds can be seen in Appendix 2.

Three compounds were active for the foot sample for both day 1 and day 4 including peak number 12, 16 and 25, which were identified as 1-ethyl-2-methyl benzene or p-ethyltoluene, phenol and nonanal respectively. Compounds that were active in foot samples from day 1only were peak numbers 1 (2-methyl-2-pentenal, 4-methyl-3-pentenal or 2-ethyl-2-butenal), peak number 2 (siloxane), peak number 5 (Ethyl-cyclohexane), peak number 14 (benzaldehyde), peak number 20 (unidentified), peak number 22 (dihydromyrecenol), peak number 27 (2-nonenal or camphor), peak number 33 (unidentified), and peak number 38 (geranylacetone). Compounds that were only present in day 4 for foot were peak number 3 (2,4-dimethyl heptane, 2,3,4 trimethyl hexane or 2,3,5 trimethyl hexane), peak number 6 (ethylbenzene), peak number 28 (menthol), and peaks 8, 9, 19, 35, 36, 46, 49, 51 and 52 (all of which remain unidentified).

The compounds that were active for axilla in both day 1 and day 4 were peak number 8, (2-heptanone), peak number 13 (unidentified), peak number 16 (phenol), peak number 28 (menthol). Compounds that were only present in day 1 for axilla were peak number 10 (unidentified), peak number 18 (indene, 3-methylphenylacetylene or p-ethynytoluene), peak number 21 (gamma-terpinene), peak number 23 (2-phenylisopropanol or 1-acetyl-2-methylcyclopentene), peak number 24 (methyl-(E)-3,5-heptadien-2-one, 1-acetylcuclohexane), peak number 25 (nonanal) and peaks 39, 47 and 48 (all of which remain unidentified). Compounds that were only present in day 4 for axilla were peak number 4 (unidentified 1), peak number 9 (1,2-dimethyl benzene), peak number 30 (dodecane), peak number 38 (geranylacetone) and peaks 40 and 43 (unidentified).

The compounds that were active for forearm in both day 1 and day 4 were peak number 16 (phenol), peak number 28 (menthol), peak number 29 (naphthalene or 1-methylene-1H-indene) and peak number 47 (unidentified). Compounds that were active in day 1 for arm were peak number 2 (siloxane), peak number 15 (propylbenzene), peak number 17 (o-ethyl toluene or m-Ethyl toluene), peak number 26 (terpinenol), peak number 34 (triacetin) and peaks 20, 32 and 39 (all of which are unidentified). Compounds that had an EAG response in day 4 only in arm were peak number 18 (Indene, 3-methylphenylacetylene or p-ethyntoluene), peak number 22 (dihydromyrcenol), peak number 38 (geranyl acetone), peaks, 10, 41, 42 and 47 (all of which are unidentified).

The compounds that were active for back in both day 1 and day 4 include peak number 3 (2,4-dimethyl heptane, 2,3,4-trimethyl hexane or 2,3,5 trimethyl hexane), peak number 28 (menthol), peak number 29 (napthalene or 1-methylene-1H-indene) and peak number 44 (unidentified). Compounds that had an EAG response in day 1 only for samples collected from the back were peak number 1 (2-methyl-2-pentenal, 4-methyl-3-pentenal, 2-ethyl-2-butenal), peak number 22 (dihydromyrcenol), peak number 25 (nonanal), peak number 31 (benzothiazole), peak number 34 (triacetin), peaks 10, 42, 45 and 50 (all of which are unidentified). Compounds that had an EAG active response in day 4 include peak number 7 (cyclohexanone or 2-methyl cyclopentanone), peak number 9 (1,2-dimethyl benzene), peak number 11 (dihydro-5-methyl-2-(£H)-furanone or cumene), peak number 16 (phenol), peak number 37 (dodecanal), peaks , 19, 33, 41, 43 and 46 (all of which are unidentified).

Table 3-1. GC-EAG active compounds for *An. stephensi* from the foot, axilla, arm and back from 30 volunteers and the number of responses for each sample.

Peak no.	RI	Tentative ID (Chemical name)	Foot		Axilla		Arm		Back	
			Day 1	Day 4	Day 1	Day 4	Day 1	Day 4	Day 1	Day 4
1	810	2-methyl-2-pentenal 4-methyl-3-pentenal 2-ethyl-2-butenal	6	0	0	0	0	0	8	0
2	820	Siloxane	5	0	0	0	5	0	0	0
3	829	2,4-dimethyl heptane 2,3,4-trimethyl hexane 2,3,5 trimethyl hexane	0	5	0	0	0	0	7	6
4	835	Unidentified 1	0	0	0	6	0	0	0	0
5	841	Ethyl-cyclohexane	6	0	0	0	0	0	0	0
6	851	Ethylbenzene	0	5	0	0	0	0	0	0
7	865	Cyclohexanone 2-methyl cyclopentanone	0	0	0	0	0	0	0	7
8	870	2-heptanone	0	0	6	5	0	0	0	0
9	880	1,2-dimethyl benzene	0	0	0	6	0	0	0	6
10	889	Unidentified 2	0	0	3	0	0	5	6	0
11	910	Dihydro-5-methyl-2(3H)-furanone Cumene	0	0	0	0	0	0	0	6
12	918	1-ethyl-2-methyl benzene P-ethyltoulene	3	5	0	0	0	0	0	0
13	924	Unidentified 3	0	0	8	5	0	0	0	0
14	933	Benzaldehyde	5	0	0	0	0	0	0	0
15	953	Propylbenzene	0	0	0	0	5	0	0	0
16	958	Phenol	5	9	10	11	5	9	0	10
17	965	O-ethyl toluene M-ethyl toluene	0	0	0	0	5	0	0	0
18	1022	Indene 3-methylphenylacetylene P-ethynytoluene	0	0	5	0	0	5	0	0
19	1033	Unidentified 4	0	0	0	0	0	0	0	10
20	1044	Unidentified 5	5	0	0	0	8	0	0	0
21	1052	Gamma-terpinene	0	0	6	0	0	0	0	0
22	1060	Dihydromyrcenol	5	0	0	0	0	5	5	0
23	1063	2-phenylisopropanol 1-acetyl-2-methylcyclopentene	0	0	5	0	0	0	0	0
24	1073	Methyl-(E)-3,5-heptadien-2-one 1-acetylcuclohexane	0	0	5	0	0	0	0	0

Peak no.	RI	Tentative ID (Chemical name)	Foot		Axilla		Arm		Back	
			Day 1	Day 4	Day 1	Day 4	Day 1	Day 4	Day 1	Day 4
25	1085	Nonanal	5	5	8	0	0	0	5	0
26	1120	Terpinenol	0	0	0	0	6	0	0	0
27	1130	2-nonenal Camphor	5	0	0	0	0	0	0	0
28	1172	Menthol	0	10	11	9	5	8	11	11
29	1187	Naphthalene 1-methylene-1H-indene	0	0	0	0	5	7	6	5
30	1200	Dodecane	0	0	0	5	0	0	0	0
31	1213	Benzothiazole	0	0	0	0	0	0	5	0
32	1225	Unidentified 6	0	0	0	0	5	0	0	0
33	1232	Unidentified 7	5	0	0	0	0	0	0	6
34	1301	Triacetin	0	0	0	0	5	0	3	0
35	1354	Unidentified 8	0	7	0	0	0	0	0	0
36	1374	Unidentified 9	0	8	0	0	0	0	0	0
37	1404	Dodecanal	0	0	0	0	0	0	0	7
38	1432	Geranyl acetone	6	0	0	3	0	6	0	0
39	1499	Unidentified 11	0	0	8	0	6	0	0	0
40	1519	Unidentified 12	0	0	0	4	0	0	0	0
41	1539	Unidentified 13	0	0	0	0	0	8	0	5
42	1586	Unidentified 14	0	0	0	0	0	5	4	0
43	1612	Unidentified 15	0	0	0	7	0	0	0	5
44	1628	Unidentified 16	0	0	0	0	0	0	5	5
45	1640	Unidentified 17	0	0	0	0	0	0	5	0
46	1711	Unidentified 19	0	5	0	0	0	0	0	6
47	1725	Unidentified 20	0	0	4	0	4	5	0	0
48	1817	Unidentified 21	0	0	5	0	0	0	0	0
49	1843	Unidentified 22	0	5	0	0	0	0	0	0
50	1856	Unidentified 23	0	0	0	0	0	0	5	0
51	1880	Unidentified 24	0	7	0	0	0	0	0	0
52	1935	Unidentified 25	0	8	0	0	0	0	0	0

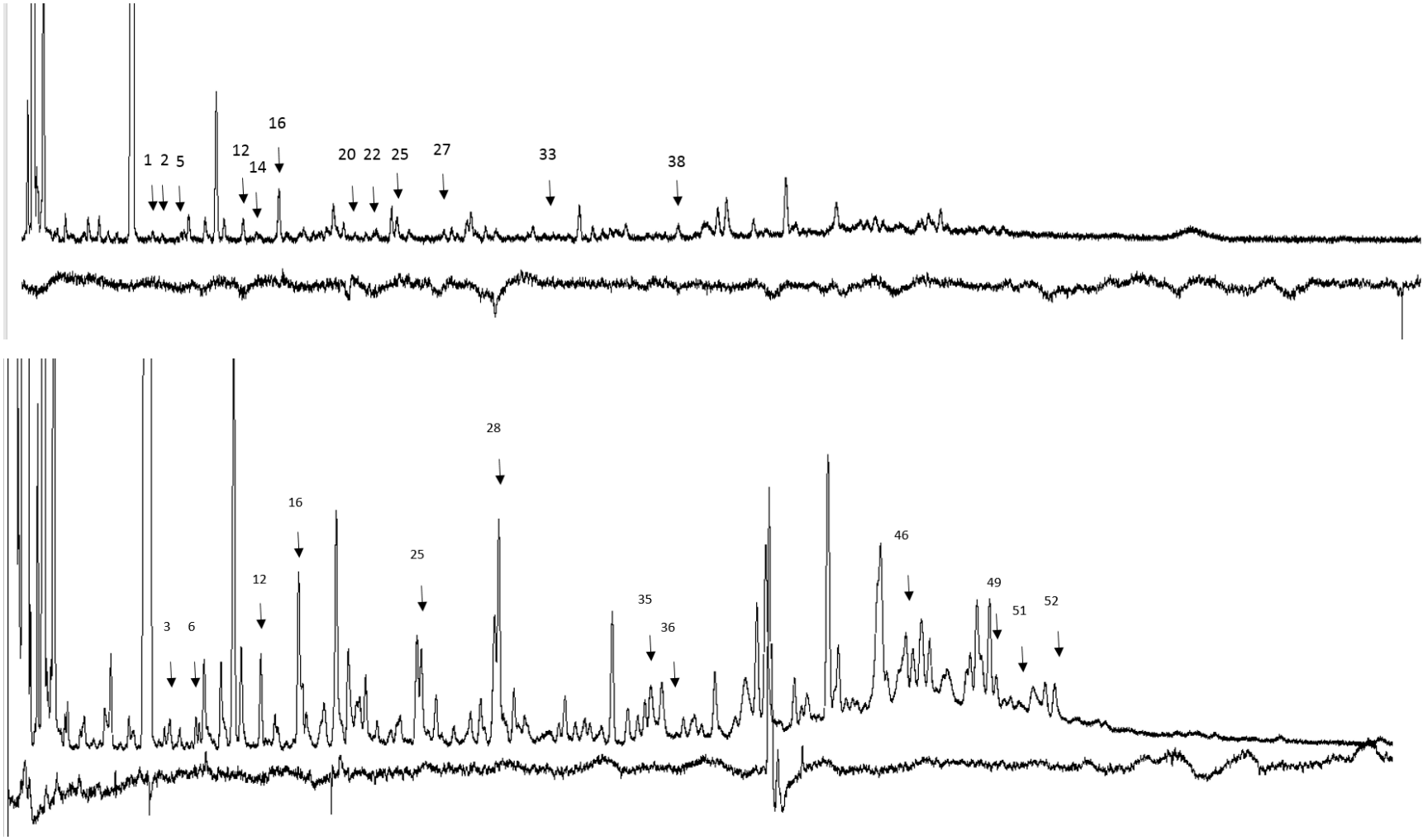


Figure 3-5. Examples of coupled GC-EAG traces of *An. stephensi* responses to human air entrainment extracts from foot day 1 (top figure) and foot day 4 after washing (bottom figure).

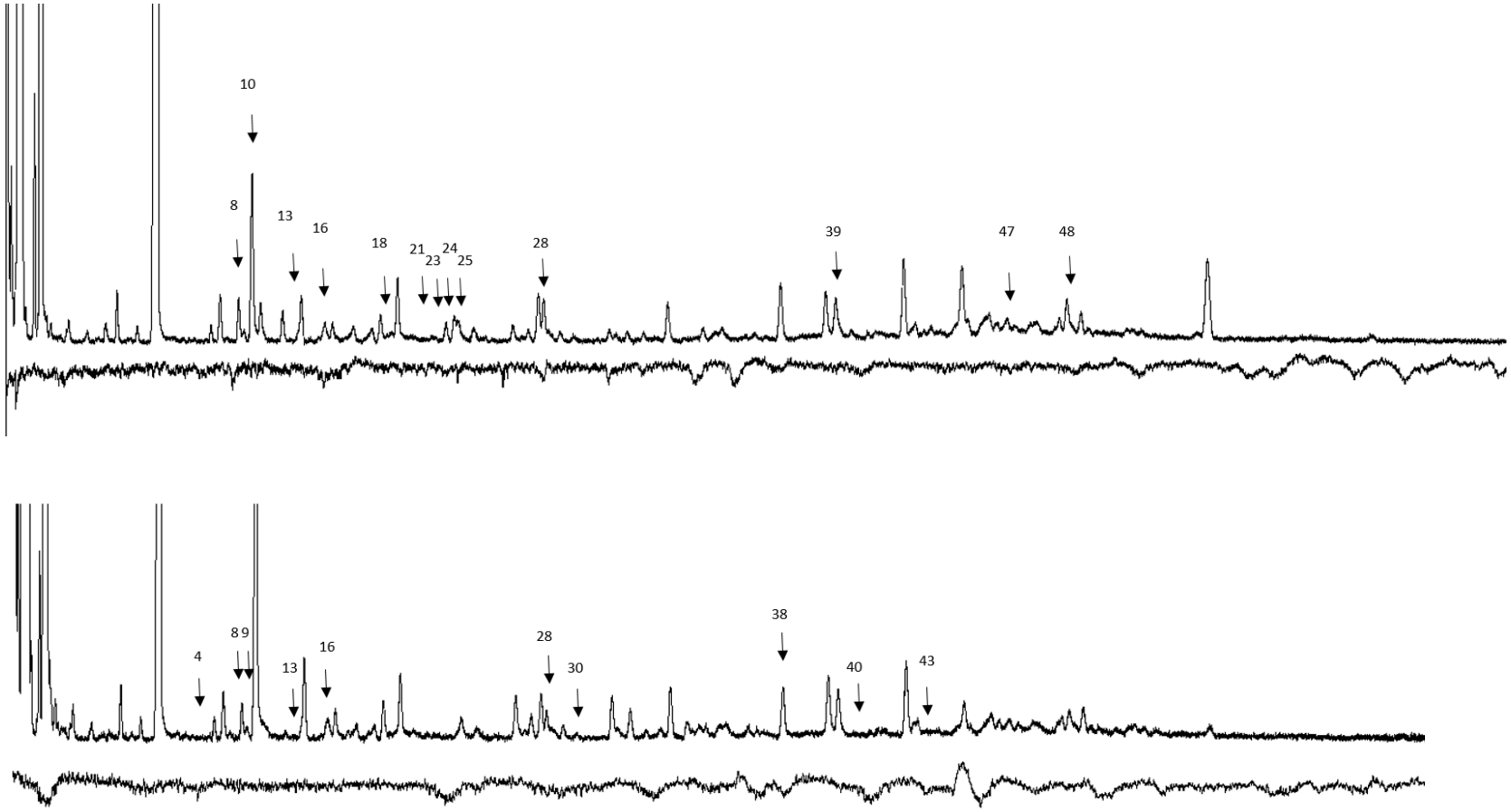


Figure 3-6. Examples of coupled GC-EAG traces of *An. stephensi* responses to human air entrainment extracts from axilla day 1 (top figure) and axilla day 4 after washing (bottom figure).

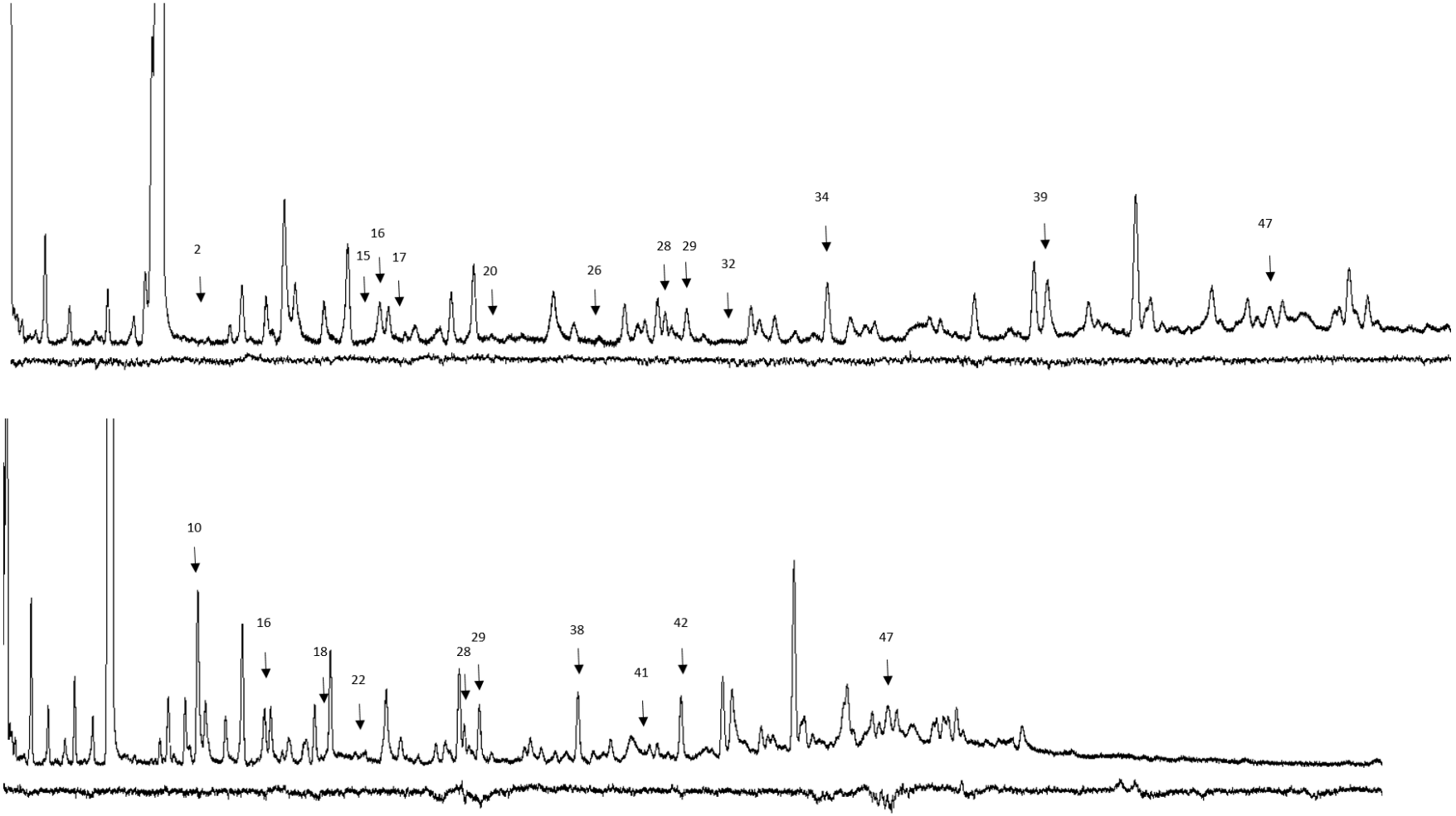


Figure 3-7. Examples of coupled GC-EAG traces of *An. stephensi* responses to human air entrainment extracts from forearm day 1 (top figure) and axilla day 4 after washing (bottom figure).

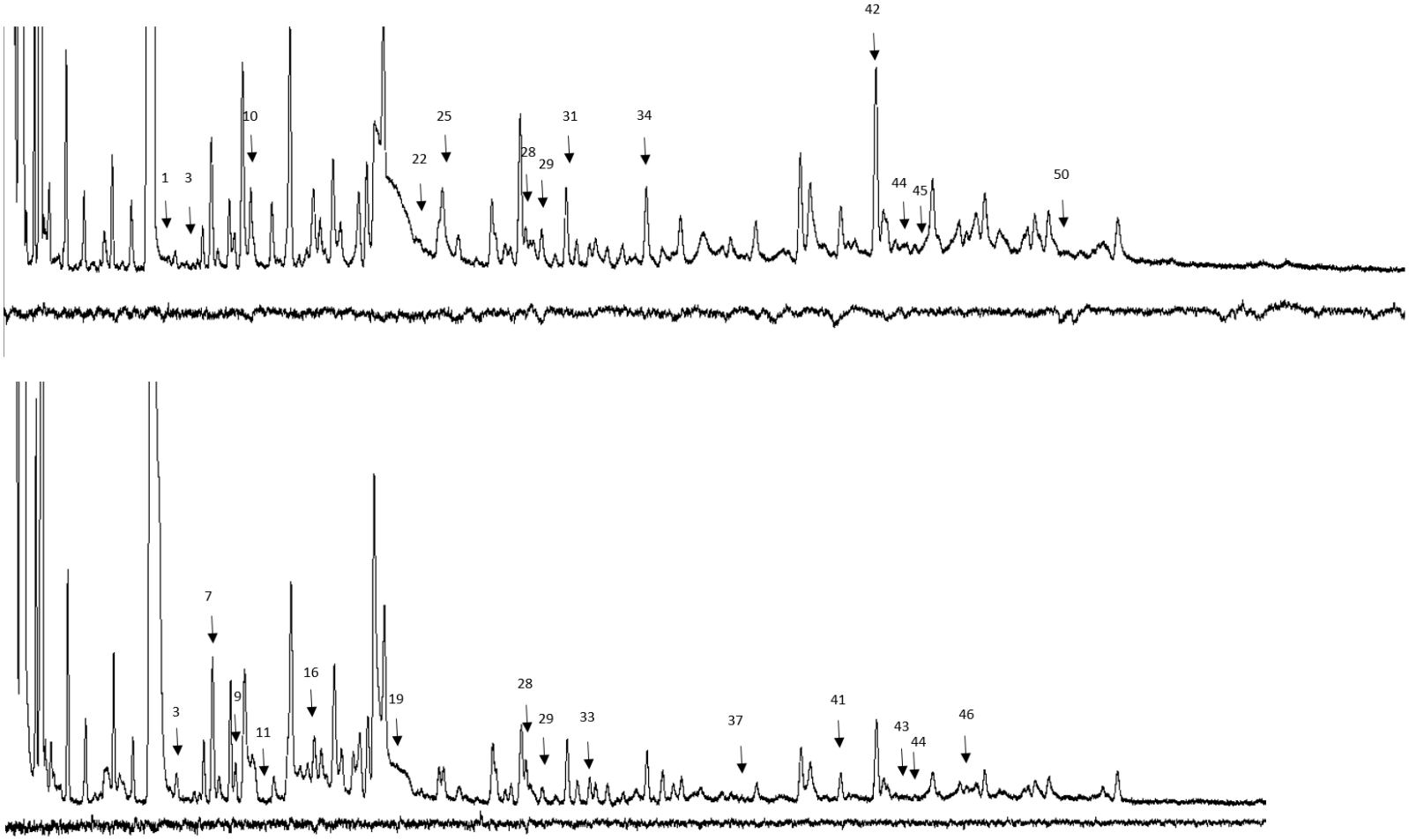


Figure 3-8. Examples of coupled GC-EAG traces of *An. stephensi* responses to human air entrainment extracts from upper back day 1 (top figure) and axilla day 4 after washing (bottom figure).

3.6 Discussion

3.6.1 Human derived compounds

A total of 52 compounds were found to be EAG active in the 8 samples tested, and some of those (27) were tentatively identified by GC-MS. The remaining 25 compounds could not be identified due to the amount of compound in the sample being too small. Of the compounds that were tentatively identified, some have been previously associated with human skin including benzaldehyde, propylbenzene, phenol, nonanal, menthol, 2-nonenal, benzothiazole, napthalene, dodecane, dodecanal and geranylacetone. Furthermore, benzaldehyde, menthol, geranylacetone, dodecanal, naphthalene and benzothiazole have been previously identified as EAG active compounds with mosquitoes (Bernier *et al.* 2000; Gallagher *et al.* 2008; Logan *et al.* 2008; Smallegange *et al.* 2005; Haze *et al.* 2001). EAG responses were not consistent across the samples for most of the compounds, except for menthol, nonanal and phenol where responses were in all samples, or the majority of samples. This variation in responses across samples has been seen commonly in other studies (Logan *et al*. 2009) and may reflect qualitative or quantitative differences between the samples, rather than differences in mosquito responses. The quantification of compounds in the samples is described in Chapter 5. Here, the focus is on the identification of the compounds.

Benzaldehyde has been found in human odour and has been shown to have an effect on mosquito behaviour (Logan *et al.* 2008). When the compound was tested behaviourally with *Culex quinquefasciatus* mosquitoes, it was found to have no response in a Y-tube olfactometer, however a blend of six compounds including benzaldehyde was attractive to *C. pipiens* in a dual choice olfactometer (Otienoburu *et al.* 2012). Logan *et al.*, (2008) found it to be EAG-active with *A. aegypti* and it was found in greater amounts in less attractive individuals, suggesting it may have a repellent effect on this species. However, Moraes *et al.* (2014) found it to be associated with mice that were chronically infected with malaria, suggesting it may be an attractant. Smallegange *et al.* (2012) found that benzaldehyde significantly reduced the attractiveness of a basic blend of ammonia, lactic acid and tetradecanoic acid against *An. gambiae*.

In this study, there was an EAG response to benzaldehyde with the sample from foot day 1 only. In addition, in behavioural experiments (Chapter 2), the behavioural response for worn socks on feet was significantly lower on day 1 than day 4. This supports the association observed by Logan *et al.* (2008) of higher levels of benzaldehyde with a reduction in attractiveness of people and could explain the significant behavioural increase between day 1 and day 4 observed by others (Bernier *et al*. 2000; Gallagher *et al.* 2008; Curran *et al.* 2005). The quantitative and qualitative differences in this compound are discussed further in Chapter 5.

Peak number 16 (phenol) had the greatest number of responses observed in all samples except for samples from back day 1 after washing. Phenol has been previously detected in hands, axilla, upper back and forearms (Bernier *et al.* 2000; Gallagher *et al.* 2008; Curran *et al*. 2005). Although it has been tested with mosquitoes, no studies have shown behavioural activity associated with this compound. For example, Cork & Park, (1996) tested phenol with EAG using *An. gambiae* mosquitoes and found no response, similarly, Puri *et al.*, (2006) tested phenol in a Y-tube olfactometer with *C. quinquefasciatus* and found no response. Phenol has, however, been shown to attract other insects, such as *Culicoides furens* and tsetse flies (*Glossina*) when tested in traps in the field (Kline *et al.* 1990; Jordan, 1995). Furthermore, phenol has been shown to be attractive to *Culicoides nubeculosus* in a dual-choice behavioural experiment (Isberg *et al.* 2016; Isberg *et al.* 2017). The literature suggests that phenol is an important semiochemical for various insects, however, the role it plays for *Anopheles* mosquitoes remains unclear and further behavioural experiments are needed to test the role in play in attractiveness.

Peak number 25 (nonanal) has been reported to be a dominant volatile in the human odour complex. Several previous studies have detected it in hands, axilla, upper back and forearm extracts (Bernier *et al.* 2000; Curran *et al.* 2005; Gallagher *et al.* 2008; Logan *et al.* 2008; Dormont *et al.* 2013). Furthermore, it has been found to be significantly more abundant in older individuals (Gallagher *et al*. 2008). Logan *et al.*, (2008) found it to be EAG active with *A. aegypti* and it was found in greater amounts in less attractive individuals. When tested behaviourally, nonanal had a significant repellent effect for all three genera of mosquitoes, *Aedes, Culex* and *Anopheles*. Two

studies tested nonanal behaviourally with *C. quinquefasciatus* in both a Y-tube olfactometer and a dual choice olfactometer (Syed & Leal 2009; Puri *et al.* 2006). One study demonstrated that it was attractive, however, the other showed no response. This could be due to the difference in methods used or it could also be due to variability or concentration. A study that tested the addition of nonanal to a basic blend of ammonia, lactic acid and tetradecanoic acid showed no reduced catches at any concentrations used (Smallegange *et al*. 2012). Nonanal has also been found to be an important ovipositioning compound for *C. quinquefasciatus* (Syed & Leal 2009). The behavioural responses of mosquitoes to nonanal are variable in the literature. It appears that in certain circumstances it can attract mosquitoes and at other times it can repel. In this study, nonanal was tentatively present in foot day 1 and day 4, axilla day 1 and back day 1 samples.

Two further compounds, peak number 27 (2-nonenal) and peak number 31 (benzothiazole), have been associated with human body odour previously, and specifically with ageing bodies. For example, 2-nonenal was collected from a sample on the back of t-shirts worn by volunteers for 3 days (Haze *et al.* 2001)To our knowledge, this is the first time 2-nonenal has been shown to elicit an EAG response in mosquitoes. Benzothiazole, has been found in upper back and forearm extracts (Gallagher *et al.* 2008). Furthermore, Qiu *et al.* (2004) found it to be EAG active with *An. gambiae*. Further behavioural experiments revealed that benzothiazole attracted fewer mosquitoes compared to the solvent control. Benzothiazole was found in lower amounts in malaria infected mice which had an enhanced attraction to *An. stephensi* mosquitoes. So overall previous studies suggest it to be associated with a reduction in attractiveness. In this study benzothiazole was EAG active in samples on back day 1 after washing.

Peak number 29 (naphthalene) has been previously extracted from axillary samples and from whole body air entrainments. Furthermore, it has elicited EAG responses with *Ae. aegypti* and *An. gambiae* mosquitoes (Qiu *et al.* 2004; Curran *et al.* 2005; Logan *et al.* 2008). In this study, it was found to elicit EAG responses in arm day 1, arm day 4, back day 1 and back day 4. Studies that have found naphthalene in their samples conclude that it is likely to be a contaminant from sorbents used, however, it

has been found in studies that haven't extracted the volatiles using sorbents, which could suggest that it does have human origin (Qiu *et al.* 2004; Logan *et al.* 2008; Prada *et al.* 2010). Interestingly, it is a compound that is used as an insect repellent, therefore it likely decreases the attractiveness to mosquitoes (Daisy *et al.* 2002).

Peak number 30 (dodecane) has been found in axillary samples in a previous study Curran *et al.* (2005); in this study only axilla day 4 samples produced an EAG response), which could suggest that dodecane is a compound that is important for mosquitoes but is specific to the axilla only. There have been no studies that have investigated the behavioural response to dodecane.

Peak number 37 (dodecanal) has been found previously from hands, upper back, forearms and whole body entrainments (Bernier *et al.* 2000; Dormont *et al.* 2013; de Lacy Costello *et al.* 2014). Furthermore, (Logan *et al.* 2008) found it had an EAG response with *A. aegypti.* Dodecanal has been associated with malaria infected individuals (De Boer *et al.* 2017). In this study only samples from back day 4 after washing had an EAG response.

Peak number 38 (geranylacetone) has been previously detected from both fresh and incubated sweat, feet, forearms axilla and whole body entrainments. Furthermore, it was also found to be EAG active with *A. aegypti* and *An. gambiae.* When tested behaviourally for both species, it was shown to reduce attractiveness (Logan *et al.* 2008; Gallagher *et al.* 2008; Meijerink *et al.* 2000; Qiu *et al.* 2004). In this study, EAG responses were detected in foot day 1, axilla day 4 and forearm day 4. The EAG-activity of geranylacetone in foot day 1 could explain the significant increase of attraction between day 1 and day 4 (chapter 2). Further information relating to the quantification of this compound is described in Chapter 5.

Peak number 28 (menthol) is commonly associated with flavourings and essential oils, however it has also been associated with human odour. Meijerink *et al.* (2000) found it was a component of fresh sweat but not incubated sweat, and Logan e*t al.* (2008) extracted it from whole body entrainments and found it was an EAG active compound with *A. aegypti*. In this study, an EAG response to menthol was observed in all samples except foot day 1. It was surprising to observe this in all samples, as we would have not

expected any residuals from cosmetics in day 4 samples, therefore this may give more evidence that it is a human derived compound and not a contaminant.

3.6.2 Non-human derived compounds

The following compounds elicited EAG responses in the samples, however, they have never been associated with human body odour in previous studies: Peak 1 (2-methyl-2-pentenal, 4-methyl-3-pentenal or 2-ethyl-2-butenal), siloxane, Peak 3 (2,3,4-trimethyl hexane or 2,3,5 trimethyl hexane), ethyl-cyclohexane, peak 7 (cyclohexanone or 2-methyl cyclopentanone), 2-heptanone, 1,2-dimethyl benzene, dihydro-5-methyl-2(3H)-furanone, peak 12 (1-ethyl-2-methyl benzene or P-ethyltoulene), peak 17 (O-ethyl toluene or M-ethyl toluene), peak 18 (indene, 3-methylphenylacetylene or p-ethynytoluene), gamma-terpienene, dihydromyrcenol, peak 23 (2-phenylisopropanol or 1-acetyl-2-methylcyclopentene), peak 24 (methyl-(E)-3,5-heptadien-2-one or 1-acetylcuclohexane), terpinol and triacetin. The fact that they have never been described before in association with human odours may mean that they are contaminants or that they simply have not been found before. Strict protocols were used to try and minimise contaminants in this study, however, inevitably the samples may have contained contaminants because the volunteers were allowed to wear their own clothing and socks. Furthermore, the study took place over 4 days and the volunteers were allowed to go home when they were not sampled, this study relied on volunteers in adhering to the protocol.

Peak number 26 (terpinenol) and peak number 21 (gamma-terpinene) are terpenoid compounds with plant origins, and they are particularly associated with conifers. Terpinenol has been previously associated with lapsang souchong tea (Yao *et al.* 2005). It had an EAG response with forearm day 1. Other active EAG compounds in this study are classified as petrochemicals including, 1,2-dimethyl benzene, cumene or peak 12 (1-ethyl-2-methyl benzene). Benzene derivatives were also found to be EAG active with *An. gambiae* from entrained worn nylon stockings (Qiu et al. 2004). The authors concluded these compounds were non-host origin contaminants from the nylon stockings, and that they may have obstructed the detection of relevant kairomones. In their study, they include naphthalene as a contaminant, however, it has been found to be an EAG active compounds in various studies which could suggest that it is a

compound of human origin. Similarly, peak 7 (cyclohexanone) was found to be an EAG active in this study on back day 4, it is commonly used as a precursor in the synthesis of nylon which suggests it could be a contaminant from the nylon stocking.

Other compounds that elicited an EAG response in this study are commonly used for cosmetic or flavouring purposes, including peak number 34 (triacetin) and peak number 8 (2-heptanone). Triacetin is used as carrier for flavours and fragrances (Fiume, 2003). In this study, EAG responses were observed in forearm day 1 and back 1. Compound 2-Heptanone has been found in cheddar cheese (Januszkiewicz *et al.* 2008). Furthermore, the compound 2-hepanone is a ketone that is normally present in the urine of mice and humans, and it is thought to be a pheromone in mice (Gutiérrez-García *et al.* 2007). This is the first instance it has been detected from human skin. In this study, it was only found in axilla day 1 and day 4, where apocrine glands are present.

Peak number 2 (siloxane) was found to be an EAG active compound in this study. Previous studies have also found similar findings. Bernier *et al.* (2000) found it in samples taken from human hands, and Qiu *et al.* (2004) also found it to be an EAG active compound. Siloxanes have often been observed as a contaminant from the column (Qiu *et al.* 2004). Other contaminants could be compounds present in the air where the air entrainments took place, (Gallagher *et al.* 2008) found 14 compounds present in room air, therefore it is likely that siloxane is a contaminant.

In the samples tested, certain compounds that have been found to be important kairomones for *Anopheles* mosquitoes such as carboxylic acids, oxocarboxylic acids, lactic acid and ammonia were not detected by EAG (Smallegange *et al.* 2005). Carboxylic acids were detected by Bernier *et al.* (2000) however they used thermal desorption to collect volatiles and not headspace entrainments, which could explain the difference in compounds detected. Furthermore, oxocarboxylic acids are not suited for GC analysis due to the absorption in the column, detecting oxocarboxylic acids requires a two stage derivitazation (Healy *et al.* 2002).

This study had many unidentified compounds, especially towards the end of the traces; this could be due their less volatile nature. Many of the unidentified

compounds were also present in very small amounts and therefore difficult to identify them with GC-MS due to poor spectra. Furthermore, the method used for GC-EAG had a total run time of 40 minutes compared to a 70 minute run when analysing the samples by GC only. A shorter method was necessary to ensure that the mosquitoes head was viable throughout the entire run. This could have impacted the separation of compounds and could have allowed for certain compounds to co-elute, making identification difficult.

3.7 Conclusion

There are many compounds in this study that have been described as EAG active for the first time in mosquitoes, specifically, most of the compounds discussed have never been found to be EAG active for *An. stephensi,* as most studies have been done with *A. aegypti* and *An. gambiae*. Some compounds identified in this study have been previously identified as human derived compounds, such as benzaldehyde, propylbenzene, phenol, nonanal, menthol, 2-nonenal, benzothiazole, napthalene, dodecane, dodecanal and geranylacetone, the role they play in the association of bacteria will be discussed in chapter 5. Some compounds that have been previously identified as unattractive were observed in foot day 1 which could explain the significant behavioural difference between foot day 1 and foot day 4 discussed in chapter 2. Future research is needed to confirm the identification by doing co-injections and to test behavioural responses with behavioural experiments.

CHAPTER 4

4 Method development for bacteria and compound analysis

4.1 Introduction

A complex set of data was collected in Chapter 3, involving several variables that included body sites, days of collection, bacteria and volatile compounds. This type of data is highly complex as there are hundreds of bacterial species and hundreds of volatiles compounds associated with the human body. An analysis that takes into account bacteria, volatiles and body sites has never been done before. Such analysis would be useful in determining correlations between bacteria and volatiles.

Previous studies that have collected microbiota have used different methods to analyse the data. Adams *et al*. (2017) tested the effect of toothpaste against a control with oral microbiota. Beta diversity was used to investigate the differences between sample groups and the data were visualised using ordination plots with random forest analysis. This is a useful tool for exploratory visualisation in 2D. The beta diversity measures how similar the bacterial communities are based on relative abundance. It uses multidimensional scaling which visualises the level of similarity of individual samples in the dataset on a two-dimensional representation and uses a dissimilarity matrix to make a plot. When used with metric scaling the distance between two communities indicates how similar they are to each other.

Furthermore, parallel coordinates can be used as another visualisation tool, which was also used by Adams *et al.* (2017). It provides an overview of the microbial profile of the samples and allows the visualisation of changes in the relative abundance. Individual samples are displayed as polylines that meet the axes that correspond to their microbial count (Fernstad *et al.* 2011).

In this chapter, an exploratory pilot analysis was done with only one compound (RI 966) and the bacteria present on day 1 and day 4 on different body sites. This compound was selected as it was tentatively identified using a Kovats index database as 6-methyl-5-hepten-2-one, an important mosquito semiochemical.

4.2 Aims and objectives

The aim of this chapter was to explore different ways to analyse the dataset, by using visualisation tools that explore patterns in the dataset.

Objectives:

1. To visually explore the dataset using MicrobiViz and create parallel coordinates
2. To create 2D ordination plots using Beta diversity
3. To determine if the presence or absence of bacteria is responsible for the presence of compound RI 966
4. To fit a model (using a Dirichlet Multinomial distribution) that predicts if the increase of a bacteria increases the compound present.

4.3 Methods

The volatile and bacteria sampling and analysis can be found in chapter 3, page 70-72, section 3.3.2, and 5, page 109, section 5.21.1, respectively.

4.3.1 Visualisation parallel coordinates

Parallel coordinates were done using MicrobViz. This tool was used to visualise the compound and bacteria data together by providing an outline of abundance patterns and distributions. Parallel coordinates display a summary of the bacteria present based on the median and their confidence intervals. The compound data was plotted for each bacterium or bacteria genus to determine any associations between the amount of compound present and the bacteria. This tool allowed data to be filtered by different variables. The bacteria distribution was visualised for RI 966 at operational taxonomic unit (OTU) and genus level. MicrobViz does not support the use of continuous data for the visualisation, therefore in order to visualise the data, the compound data was changed from continuous data to discreet data by categorising the amount of compound into 5 separate categories where 1 represents the lowest amount of compound, and 5 the highest based on the median.

4.3.2 Beta diversity plots

In order to explore the similarities between two communities by analysing the amount of variation in species composition, 2D ordination plots were done using Random forest metric scaling on JMP to explore the beta diversity of the samples. Beta diversity plots were done for all samples and divided by visit (day 1 and day 4 set as separate communities). Furthermore, RI 966 was overlaid as a contour across all samples. This was repeated for each site (foot, axilla, forearm and upper back).

4.3.3 Presence or absence of bacteria

To investigate if a bacteria genus was wholly responsible for the production of compound RI 966, bacteria data were visualised by showing presence and absence for each bacteria genus. Both the compound and bacteria data were transformed to binary data where a label of present/absent was given. If a bacteria genus had 10 CFUs or more it was considered to be present, if it had less than 10 it was considered to be

absent. Ten CFUs were chosen as a cut off. Bacteria and compound RI 966 were plotted against each other to observe if the presence of a bacteria was associated with the compound.

4.3.4 Comparative testing

The data for compound RI 966 were split into two categories based on the median compound level. Samples with values below the median were labelled 'low' and samples above the median were labelled 'high'. For each bacteria genus, a statistical test (using a Dirichlet Multinomial distribution) fitted a model that predicted the mean relative abundance (%) for bacteria and compound. An adjusted p-value (to account for error inflation due to performing multiple statistical tests) was given when comparing the mean relative abundance in the low and high category. Therefore, if the mean relative abundance in the low category was lower than in the high category, and the p-value was significant, there was statistical confidence that the mean relative abundance increased with a higher concentration of compound. This test was done across all samples for compound RI 966.

4.4 Results

4.4.1 Parallel coordinates

The output from this analysis gave a graphical summary of the bacteria present by displaying the median compound level and their confidence intervals. The parallel coordinates showed that the most abundant OTU's in all the samples were OTU 1 (*Staphyloccocus*), 868 (*Corynebacterium*), 1386 (*Corynebacterium mucifaciens*) 1459 (*Micrococcaceae*) and 1420 (*Micrococcaceae*), these OTU's were associated with compound RI 966 (Figure 4-1). This was seen for the 5 levels of compound amount. When this was done at the genus level, it revealed that *Staphylococcus* and *Corynebacterium* were the most abundant bacteria associated with RI 966 (Figure 4-2).

4.4.2 Beta diversity

When the beta diversity plots for bacteria were separated for day 1 and day 4, the day 1 samples were similar to one another, and the day 1 samples differed from visit 4 (Figure 4-3). When a contour plot was overlaid for RI 966, it showed some data points were associated with higher amounts of compound RI 966, however, no clear pattern was observed (Figure 4-4). This plot was filtered further by sites (Figure 4-5). For the foot and axilla, some samples had higher amounts of compound RI 966 that were associated with day 4, however, this was only observed for a few samples. For the upper back and forearm, there was no clear pattern observed.

4.4.3 Presence or absence of bacteria

Two examples of the plots showing the presence or absence of bacteria associated with compound RI 966 are shown in Figure 4-6. If the entire presence of a compound would have been affected by the presence of a bacteria, only one cloud would be seen in one box, however, the samples were distributed across all boxes, therefore the The presence of *Flavobacterium* or *Dermabacter* did not influence the presence of compound RI 966. This was done for all bacteria, however, very similar plots were produced for all genera, therefore, only two examples are presented.

4.4.4 Comparative testing

The mean relative abundance predicted for the 'High' and 'Low' categories for compound RI 966 were calculated (Table 4-1). In total, 52 bacteria had significant differences between the high and low categories, however, only 10 are displayed as the others had extremely low abundance. *Pseudofulvimonas, Nosocomiicoccus, Sulfrimonas, Parvulacula, Fluviicola, Actinobaculum* and *Paludibacter* all had higher mean relative abundance with the higher category, which suggests that when there is a higher abundance of these bacteria, the compound amount increases. However, *Salinicoccus, Fusobacterium* and *Truepera* have a lower mean relative abundance with the high category which suggests that the presence of these bacteria may lower the concentration of compound 966.

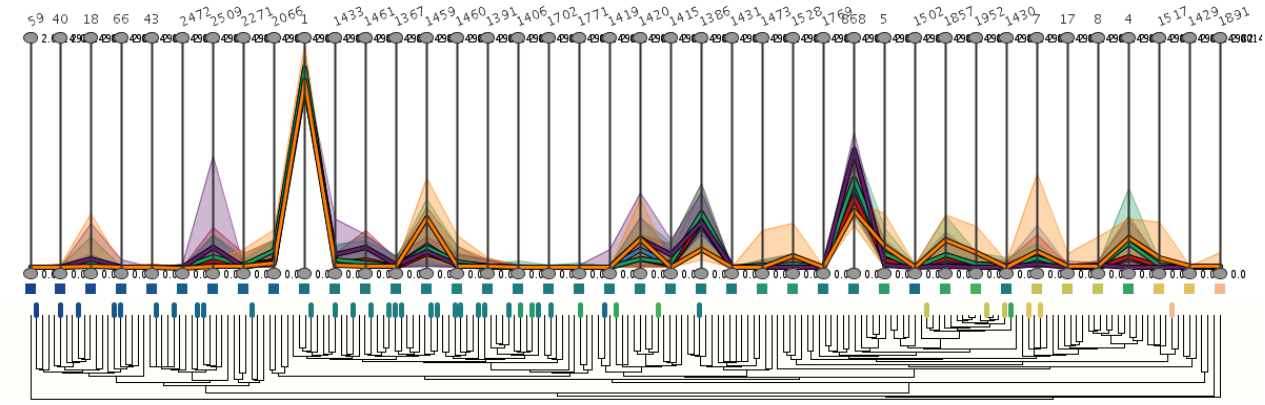


Figure 4-1. Parallel coordinate for compound KI 966 at OTU level. The compound was split into 5 categories (1 representing the lowest amount and 5 the highest); orange= 5, purple= 4, green= 3; red=2, blue=1

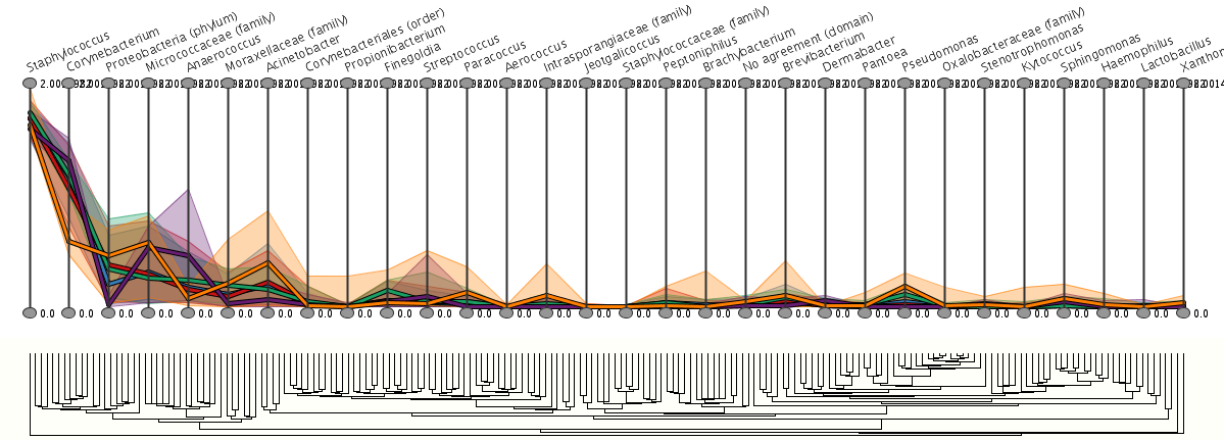


Figure 4-2. Parallel coordinate for compound RI 966 at genus level. The compound was split into 5 categories (1 representing the lowest amount and 5 the highest); orange= 5, purple= 4, green= 3; red=2, blue=1

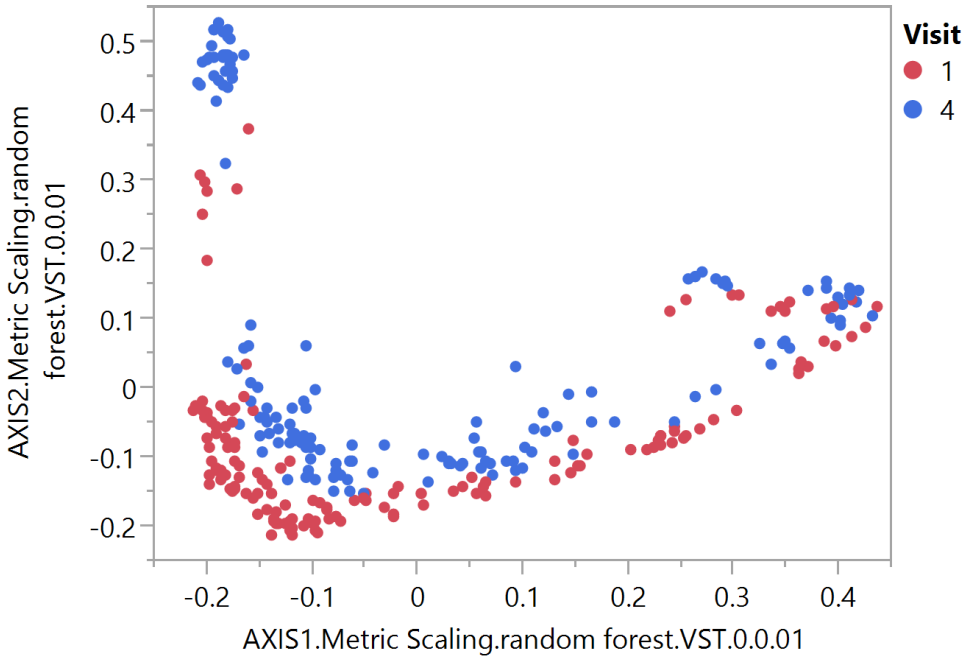


Figure 4-3. Beta diversity plot for all samples for visit 1 and 4. Red dots represent day 1, blue dots represent day 4.

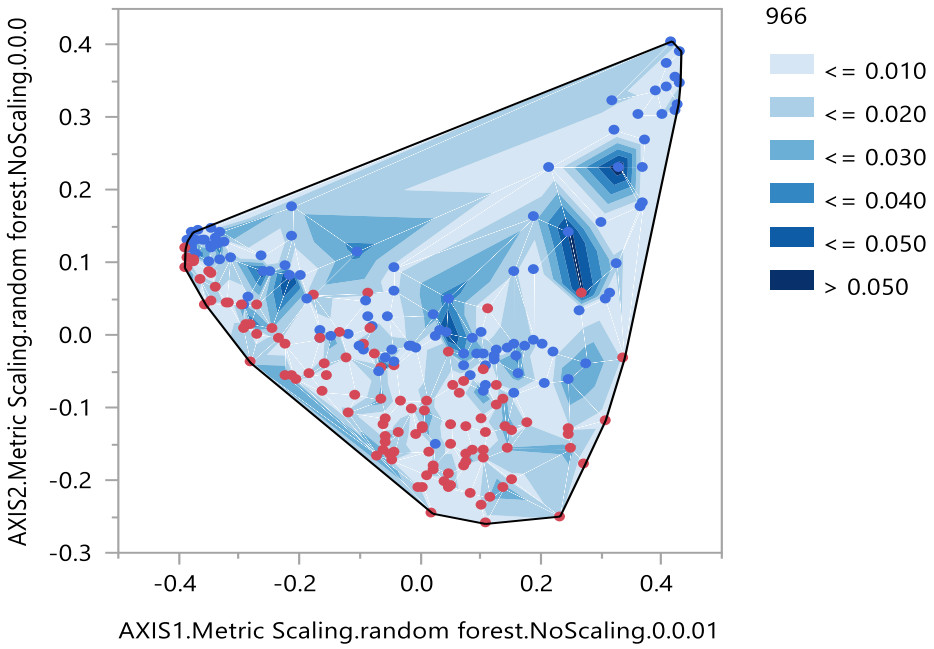


Figure 4-4. Beta diversity plot for all samples for visit 1 and visit 4 with compound RI 966 overlaid. Red dots represent day 1, blue dots represent day 4.

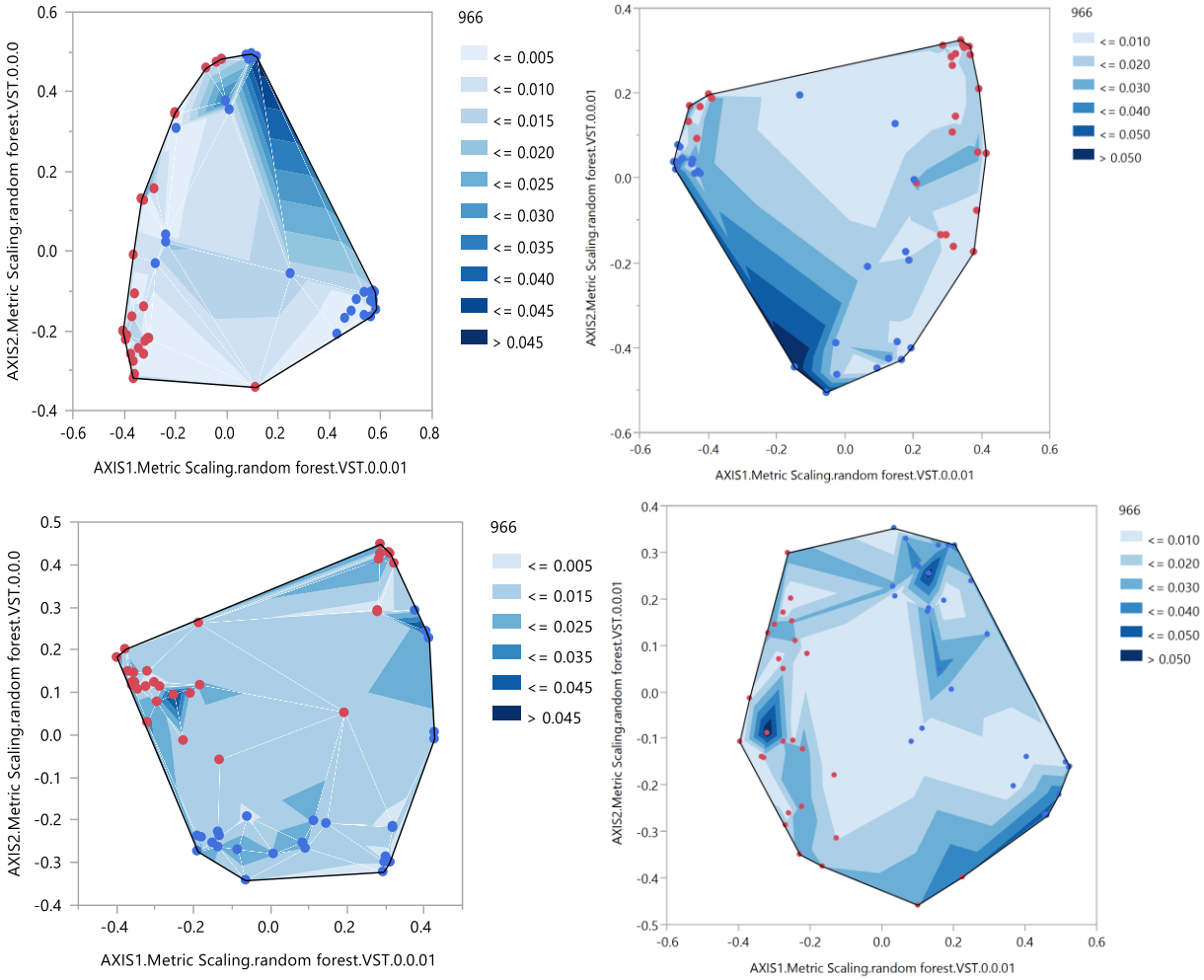


Figure 4-5. Beta diversity plot for per site for day 1 and day 4 with compound RI 966 overlaid as a contour. The darker the contour the higher amount of compound present. Upper left: foot; upper right: axilla; lower left: upper back; lower right: forearm. Red dots represent day 1, blue dots represent day 4.

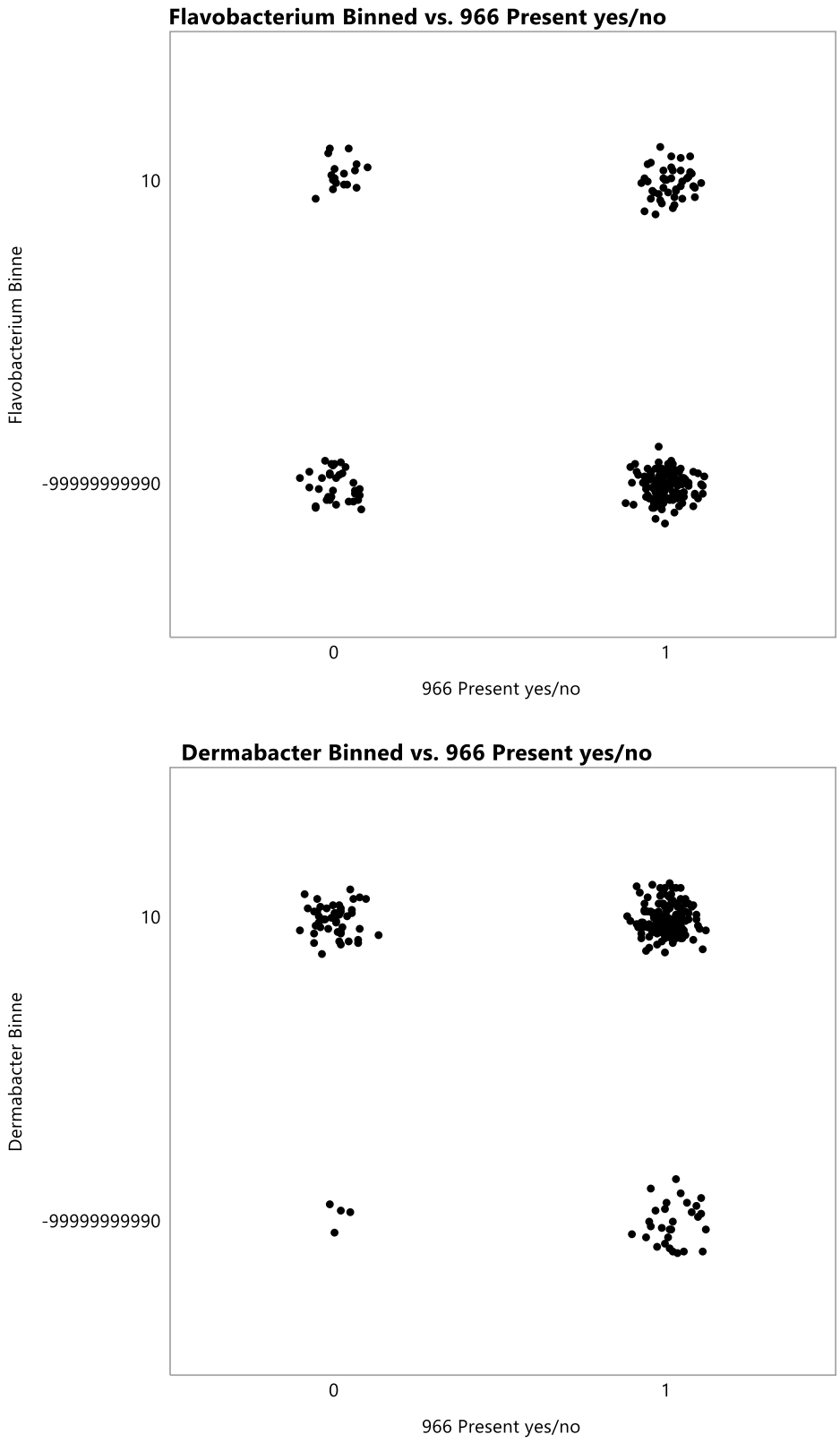


Figure 4-6. Presence and absence of *Flavobacterium* and *Dermabacter* against the presence or absence of compound RI 966. Compound RI 966 0=absent; 1=present. Bacteria 10=present; -9999990= absent. Dots represent individual samples.

Table 4-1. Mean relative abundance predicted for the 'High' and 'Low' categories for compound RI 966.

Genus	**High**	**Low**	**Q value**	**Higher or lower?**
Pseudofulvimonas	0.0692%	0.0029%	<0.001	higher
Salinicoccus	0.0424%	0.3621%	0.005	lower
Nosocomiicoccus	0.0213%	0.0006%	<0.001	higher
Sulfurimonas	0.0212%	0.0031%	0.02	higher
Fusobacterium	0.0207%	0.0565%	0.003	lower
Truepera	0.0050%	0.0268%	0.003	lower
Parvularcula	0.0036%	0.0001%	<0.001	higher
Fluviicola	0.0033%	0.0002%	<0.001	higher
Actinobaculum	0.0031%	0.0005%	0.003	higher
Paludibacter	0.0024%	0.0001%	<0.000	higher

4.5 Discussion

The aim of this exploratory analysis was to determine an appropriate method for visualising and describing the data, to investigate the association between bacteria and volatile production, from different body sites on multiple volunteers, on day 1 and day 4 after not washing. Due to the number of factors, the dataset was highly complex and several methods were trialled with one compound, RI 966 (6-methyl-5-hepten-2-one).

The use of MicrobiViz, to create parallel coordinates, is a useful tool to determine trends in a dataset. In this study, many OTUs were missing so the visualisation could only be done at genus level. It was difficult to determine if the amount of compound was associated with a particular bacteria, as the *Staphylococcus* and *Corynebacterium* were the most abundant bacteria in all the samples, and was, therefore, associated across all samples. Adams *et al.* (2017) successfully used this tool to explore a dataset, however, they only had two groups (control and treatment group) to compare, therefore, a change in bacterial communities between the two groups was easier to detect as a treatment that affected microbiota was applied. This study aimed to do an exploratory analysis of associations (where no treatments were applied), which could be the reason why no clear patterns were observed. This method was not taken forward as no clear patters were observed and no clear statistical observations could be done.

The aim of the ordinal plots was to visualise if the bacteria in one group were similar to one another, and to determine if they differed from another group. In this study, the bacteria from day 1 were similar to one another and they differed from bacteria in day 4. The purpose of this study was to explore associations between compounds and bacteria, therefore, the compound was overlaid to create a contour plot. This was repeated for each body site, however, no clear patterns were observed for all samples, and for each body site. This visualisation would have allowed observation of a pattern only if a compound was particularly associated with either day 1 or day 4 across all bacteria samples. However, this was not observed as it is likely that the associations between compounds are bacteria are highly complex and not associated with day 1 or day 4 only. Therefore, this analysis seemed inappropriate and was not taken any further.

The analysis investigating presence or absence of bacteria showed that one bacteria was not responsible for the production of this compound. Although only two figures are displayed here, the results for all bacteria were similar. It is likely that a single genus of bacteria is not wholly responsible for the production of one compound, and it is likely to be an interaction between different bacterial communities, and it was not possible to observe these complex interactions using this method.

The results from the comparative testing did show that when some bacteria increased, compound RI 966 either increased or decreased, suggesting that the increase of a bacteria led to an increase in the compound as it is produced by the bacteria. However, because this method used the median to determine low and high categories, it may not be accurate in predicting the increase of compound associated with the increase of bacteria. Furthermore, this method only allowed the associations between one compound and bacteria, and did not allow an exploration of the interactions between various bacteria and various compounds. It is important to investigate this effect as it has been reported that one single bacteria can produce blends of volatiles (Schmidt et al. 2015). Furthermore, the skin bacteria exist in communities where different species likely interact with one another and may increase or decrease the compound production depending on the competition of bacteria species.

4.6 Conclusion

Although the tools used in this chapter are useful to explore microbiota data, they were not appropriate for an investigation into the associations between multiple bacteria and multiple volatiles, as no clear statistical comparisons allowed the exploration of the association between compounds and bacteria. Due to the high amount of *Staphylococcus* and *Corynebacterium* in the samples, they were always associated with the compound as they were present in abundance in all samples, therefore, the analysis is likely to have missed any other associations present. The compound amount was not associated with one particular visit. Furthermore, a bacteria was not wholly responsible for the production of one compound. A more inclusive analysis, which accounts for bacteria, volatile amount and visit was therefore needed and is described in chapter 5.

CHAPTER 5

5 Associations of bacteria and compounds for foot

5.1 Introduction

The human skin produces many volatile organic compounds (VOCs) which contribute to human body odour. Different body sites produce their own characteristic odour, which is caused by the presence or absence (or concentration) of different compounds. Certain glands can be found at different sites on the body and this is likely to influence the production of VOCs through alteration of the composition and abundance of skin microbiota. For example, it is known that areas that have glands present have a high abundance of bacteria, which metabolise gland secretions (Wilson, 2008). Odourless compounds secreted from glands undergo biotransformations into VOCs. The involvement of bacteria in the production of VOCs is well established, and studies have shown that mosquitoes respond to odours that are associated with bacteria. However, no study has fully characterised bacteria on different parts of the body and linked this directly to differences in VOC production, and mosquito behaviour.

To investigate this complex system, a series of experiments are needed to collect VOCs and bacteria, and analysis is required to determine whether there is a correlation between the two. Methods have been used in the past to collect volatiles from volunteers, however, they have differed depending on the body site targeted. For example, SPME and headspace entrainment (using polymeric filters) have been used to collect VOCs from feet, axilla, upper back and forearm in the past (Curran *et al.* 2005; Gallagher *et al.* 2008; James *et al.* 2013). This has traditionally been followed by gas chromatography coupled with mass-spectrometry (GC-MS) to identify the VOCs. One study collected over 300 compounds from volunteers' hand emanations, the majority of which were carboxylic acids. Other compound groups present included alcohols, aldehydes, aliphatics, amides, esters, halides, heterocyclics, ketones and sulphides (Bernier *et al.* 2000). Similarly, Curran *et al.* (2005) collected volatiles from axillary sweat samples, and found organic fatty acids, ketones, aldehydes, esters and alcohols. The authors also found that (*E*)-3-methyl-2-hexenoic acid was not detected in axillary

samples, however, other studies found it was a major odour causing compound (Zeng et al. 1996). Zeng *et al.*, (1991) found that characteristic axillary malodour in males consists of C6-C11 saturated and unsaturated branched acids, with (E)-3-methyl-2-hexenoic acid being a major component. Zhang *et al.*, (2005) collected volatiles from the underarm of volunteers and identified 35 compounds using SPME extraction and GC-MS. The groups of compounds identified included alkenes, alkanes, alcohols, aldehydes and esters. Furthermore, they found 10 compounds that contributed to seasonal differences in odour profiles. In another study, the volatile profile of the forearm and the upper back was investigated with 25 volunteers using SPME extraction and GC-MS. Fewer than 100 compounds were identified, and both body sites had similar types of compounds. However, there were some differences. Nine compounds were observed only in upper back samples, and there were significant differences of dimethylsulphone, hexyl salicylate and α-hexyl cinnamaldehyde between the upper back and the forearm. Furthermore, three compounds were found significantly more in older volunteers (dimethylsulphone, benziothiazole and nonanal). Although the authors remarked that these differences could be due to cosmetic use (Gallagher *et al.* 2008), it could be due to genuine differences in compounds produced at different body sites.

5.1.1 Bacteria on different body sites

Bacteria that exist on the skin mainly consist of gram-positive bacteria including *Staphylococcus, Micrococcus, Corynebacterium* and *Propionibacterium* (Leyden *et al.* 1981). However, microbial communities on the skin are extremely varied due to the diverse habitats which can be influenced by skin thickness, folds, hair follicle density and glands. Eccrine glands that are found in large concentrations on the feet, mostly excrete water, salt and electrolytes. In comparison, apocrine glands excrete a milky viscous odourless secretion, which develops into the stereotypical sweat odour after it is incubated with bacteria. Sebaceous glands connected to hair follicles excrete sebum which is rich in lipids. The variation of microbial composition is dependent on the body site. Generally, sites that are partially enclosed are very stable over time in terms of microbial profile, such as the inside of the ear. Sites that have a high diversity of

microbiota tend to be less stable over time, such as the forearm and the plantar heel, this could because they are exposed areas (Grice & Segre 2011).

The foot plantar is dominated by *Staphylococcu*s and *Proteobacteria*, and the space in between the toe web is dominated by *Corynebacteria, Actinobacteria and Staphylococcus*. The axilla, is dominated with *Proteobacteria, Bacteroidetes* and *Corynebacteria*. The forearm is dominated by *Proteobacteria* and *Bacteroidetes* and the upper back is mostly dominated by *Actinobacteria* (Grice & Segre 2011).

Studies that have investigated the skin microbiota that are associated with volatiles have mostly focussed on compounds that are perceived as malodour, especially in axilla and feet. Medium chained volatile fatty acids (VFAs), in particular (E) 3-methyl-2-hezenoic acid bind to L-glutamine residues found in apocrine glands secretions and are released by a *Corynebacteria* enzyme (Natsch *et al.* 2005). Further research has shown that 16-androstene steroids and thioalcohols are associated with malodour, in which members of the *Corynebacterium* genus are largely responsible (James *et al.* 2013). In feet, volatile fatty acids are responsible for foot malodour, in particular isovaleric acid. *Staphylococcus* bacteria have been found to be responsible for the production of isovaleric acid by degrading foot callus into soluble amino acids including L-leucine (James *et al.* 2013). Studies that have investigated the effects of washing on skin bacteria have mostly focused on the removal of pathogenic bacteria using soap (Burton *et al.* 2011). Few studies have investigated the effect of washing on skin microbiota communities, one study found that the skin microbiota compostion was significantly affected by the time since last handwashing (Fierer *et al.* 2008). Another study found that there is minimal temporal variability with individuals (Costello *et al.* 2009). However, there is a lack of research on the effect of not washing on skin microbiota communities.

5.1.2 Association of bacteria and volatiles in relation to mosquitoes

Bacteria that are responsible for malodour in human beings may not be responsible for the production of kairomones used by mosquitoes. Verhulst *et al.*, (2009) collected skin microbiota from human feet, incubated the bacteria on agar plates and tested it behaviourally with *An. gambiae.* The agar was found to be attractive, a headspace entrainment was done on the agar plates and analysed with GC-MS. A synthetic blend

of the compounds was then tested behaviourally and was also found attractive. The synthetic blend consisted of 1-butanol, 2,3-butanedoine, 2-methyl-1-butanol, 2-methylbutanal, 3-hydroxy-2-butanone, 3-methyl-1-butanol, 3-methylbutanal, 3-methylbutanoic acid and benzeneethanol (Verhulst *et al.* 2011). Qiu, *et al.*, (2011) ranked the attractiveness of 48 volunteers to *An. gambiae* mosquitoes and analysed the foot bacteria using the hypervariable V2 region of bacterial 16S rRNA genes that determine the sequence diversity of different bacteria. Results showed that highly attractive volunteers had a higher abundance but lower diversity of bacteria on their skin compared to poorly attractive volunteers. A limitation to this study is that the agar plates allow the growth of some selected bacteria, but other bacteria may not have been able to grow, therefore some compounds may have been missed. However, this study did not investigate which bacteria are responsible for the attractant compounds that attractive individuals produce. Doing this would allow the detections of compounds associated with bacteria.

In this project, four different body sites were investigated (the foot, the forearm, the upper back and the axilla). However, results from Chapter 2 showed that the most attractive body part was the foot, and with the only difference in mosquito behaviour found between samples collected on day 1 and day 4 was also the foot, this Chapter will focus entirely on foot bacteria and foot volatiles. Analysis for forearm, upper back and axilla has also been done, and this is presented in Chapter 6.

5.2 Aims and objectives

The first aim of this study was to investigate the bacteria and odours found on the feet of volunteers and determine whether there is an association between them which may explain the differential attractiveness to mosquitoes observed in Chapter 2. The second aim was to determine whether there were any differences between day 1 (washed participants) and day 4 (unwashed participants).

Objectives:

1. To collect bacteria samples using swabs and buffer from the feet of volunteers and, using 16S rRNA analysis, identify, and quantify, the bacteria species present
2. To collect odour samples using air entrainment, from the feet of volunteers and identify and quantify, using gas chromatography (GC) and coupled GC-mass spectrometry (GM-MS), the volatile compounds present.
3. To determine whether there are qualitative or quantitative differences in bacteria and/or volatiles between day 1 and day 4.
4. To perform statistical analysis to determine whether there is an association between the bacteria presence or absence or quantity and volatiles between day 1 and day 4.

5.3 Methods

5.3.1 Bacteria analysis

5.3.1.1 Bacteria collection

Skin bacteria samples were collected from volunteers on the same days that air entrainments were done on the volunteers (see Chapter 3, section 3.3.2.2, page 71), before the air entrainment took place. Bacteria were collected from the plantar of the feet. Sterile buffer (2ml; 50mM Tris-HCl + 0.1% Triton-X100, pH 7.9, Sigma) was placed in a sterile tube (4ml; Eppendorf). A sterile PTFE ring (2cm diameter) was used as a sampling area and was placed on the foot plantar of the volunteer. A swab (Catch-all sample collection Epicentre) that had been dipped in the buffer was gently scrubbed inside the sampling ring for 1 minute. The tip of the swab was then cut off into the tube with buffer using sterile scissors. The same site was repeated using a different swab and a different tube. Samples were stored at -20°C. This method was modified from the Williamson and Kligman (1956) scrub-cup technique.

5.3.1.2 Bacteria DNA extraction

Each sample consisted of two Eppendorf tubes with 2ml each of buffer which were pooled together and spun in a centrifuge for 10 minutes at 11000 rpm. The supernatant was removed, and the pellet was re-suspended with 500µl of TE buffer (10 mM Tris-HCl; 1 mM EDTA, pH 7.4). The samples were then transferred to a 96 well Lysing Matrix B plate (MP Biomedicals).

The samples were lysed using Ready-Lyse lysozyme (250U/µl) and incubated for 18 hours at 37°C with a 300rpm agitation using Eppendorf Thermomixer. Following overnight incubation, the temperature of the shaker was increased to 68°C. The samples were transferred to the TissueLyser (Qiagen) and beat at 20Hz for 3 minutes. An additional lysis step was done, where 640µl of a lysis buffer (Table 5-1) was added to a Qiagen S block, and 400µl of the sample was added into the wells.

Table 5-1. Ingredients for the lysis

Ingredient (Qiagen)	96 sample (+4) (ml)
Proteinase K	4.4
ATL buffer	18.15
Carrier RNA	13.2
ACL buffer	34.65

The well plate was incubated for 15 minutes at 68°C (Eppendorf Thermomixer) with gentle agitation at 300rpm for 10 seconds. The plate was then centrifuged at 1000rpm for 1 minute to remove droplets from the lid. The samples were then loaded into the QIAsymphony using the QIAsymphony Bacteria Midi kit (931055) and the complex fix 400 protocol. On completion, the Qiagen EMTR plate was removed from the robot deck, the tubes capped with rubber strip caps and stored in the -25°C freezer.

5.3.1.3 DNA quantification

The DNA was quantified using a qPCR assay. This step was required to determine the number of PCR cycles needed for illumina sequencing. A qPCR programme was set up using the QIAagility robot using the volumes described in Table 5-2, qPCR was carried out using the Rotorgene Q using the following parameters: 95°C for 5 minutes then 40 cycles of 95°C for 5 seconds, 65°C for 10 seconds.

Once completed, the rotordisc was sealed using the Qiagen Heat sealer.

Table 5-2. Components used in bacteria qPCR assay

Reagent	Volume (µl)
Rotorgene SYBR Green Mastermix	10
Forward primer 515f_10uM	2
Reverse primer 1061r_10uM	2
RNase free water	1
Sample DNA	5

5.3.1.4 DNA normalisation

The PCR cycle number for bacterial samples was determined from the qPCR. A minimum of 1ng/µl is usually required to normalise the DNA, however, if there is no signal detected above baseline after 10 cycles then the number of cycles needs to be increased. The samples had low amounts of DNA, so they were therefore normalised

to 0.5ng/µl. Because of the low amount of DNA, the samples had 30 cycles done at Unilever and an extra 15 were done at CGR (Centre for Genomic Research, Liverpool University), giving a total of 45 cycles. Any samples with lower DNA than 0.5ng/µl, were left at the original concentration.

5.3.1.5 PCR amplification for Illumina sequencing

Two rounds of PCR were done on the samples, the first was done in Unilever (Port Sunlight) and the second was done at CGR. For the first PCR round, the bacterial primers used targeted the V3 and V4 region of the 16S rRNA gene. The V3 region has been shown to be the most appropriate hypervariable region for distinguishing all bacterial species to genus level (Chakravorty *et al.* 2007). The designed primer sequences (red) along with the Illumina recognition sequence (blue) for the forward and reverse primers are shown below.

341F
F:5'ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNCCTACGGGAGGCAGCAG 3'

806R
R:5'GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGACTACHVGGGTWTCTAAT3'

Samples were amplified in triplicate, therefore, for 96 samples there were 3 separate runs. Controls were included including the buffer control from the DNA extraction step, a positive axilla mock community control and a non-template control. The reagents used in the PCR are found in Table 5-3.

Table 5-3. Components in end-point PCR assay for illumina sequencing

Reagent	**Volume (µl)**
HotStar Taq Mastermix	10
Forward primer_10uM	0.25
Reverse primer_10uM	0.25
RNase free water	4.5
Sample DNA	5

End point PCR was carried out using the Biorad-T100 using the following parameters: 95°C for 5 minutes then 30 cycles of 94°C for 45 seconds, 65°C for 30 seconds, 72°C for 60 seconds, 72°C for 10 minutes. Samples from the 3 runs were pooled together, the plate was stored at -25°C. The second round of PCR was done at CGR where the primers incorporated a recognition sequence to allow a secondary nested PCR process. The second PCR was done to incorporate Illumina adapter sequences for samples on the Illumina Sequencing platforms.

N501 f
5'AATGATACGGCGACCACCGAGATCTACAC*TAGATCGC*ACACTCTTTCCCTACACGACGCTC3'

N701 r
5'CAAGCAGAAGACGGCATACGAGAT*TCGCCTTA*GTGACTGGAGTTCAGACGTGTGCTC3'

A mapping file was created with the sequencing data from CGR. Raw reads from CGR were processed through a non-oral bacterial processing pipeline. PCR primers were trimmed before analysis due to the presence of degenerate bases using Cutadapt. Short reads were trimmed using Sickle, reads with less than 100bp were discarded. Pandaseq was used to align forward and reverse Illumina reads with PCR primers embedded in the sequence which was then merged into a single fasta file. Chimeras and singleton reads were removed using Vsearch. Finally, Megablast tool was used to match sequences and blasts to different databases found online including NCBI, DDBJ, EMBL, RDP and Greengenes. The sequences were classified according to the lowest common ancestor. DNA extraction, sample preparation and sequence processing were done in accordance to Stevens *et al.* (2015).

5.4 Gas chromatography analysis

5.4.1 Gas chromatography

The volatile collection from volunteers is described in Chapter 3, Section 3.3.2. The air entrainment samples collected on Porapak Q filter were eluted with 700µl of re-distilled diethyl ether. The samples were concentrated to 50µl with charcoal-filtered nitrogen. After concentrating, 1µl of sample was injected into the GC. The GC method was as follows: The oven temperature was maintained at 40°C for 0.5 minutes, increased by 5°C until it reached 150°C then held at this temperature for 0.1 minutes.

The temperature then increased further by 10°C until it reached 230°C where it was maintained for 30 minutes. A syringe that had been previously cleaned 20 times with re-distilled hexane was used to inject 1 µl of sample.

5.4.2 Quantitative analysis of volatiles in air entrainment samples

A 1µl injection of C7-C25 alkanes in hexane (100 ng μl^{-1}) was run every week to calculate the amount of compound in the samples. Peaks in samples were aligned by the use of Retention index (RI). The RI of compounds within samples were calculated by using the difference between the retention indices or alkanes eluting before and after the compound:

$$RI = \left(\frac{100(\log rt(x) - \log rt(z-1))}{(\log rt(z+1) - \log rt(z-1))} \right) + 100(z-1)$$

Rt= Retention time

x= Compound of interest

z+1= Alkane after the compound of interest

z-1= Alkane before the compound of interest

To match up retention index of the same compounds in different samples, the traces were aligned. A trace that had the greatest number of compounds was chosen as a representative trace and all the traces were aligned to the representative trace by matching Retention Index value and checked by eye.

5.5 Statistical analysis

Bacteria count data were log transformed and the volatile data were expressed as proportions out of the total amount of all compounds present per sample. A t-test was carried out for all the bacteria counts (Table 5-4) and compounds (Table 5-5) for the difference between day 1 and day 4. A partial least square (PLS) analysis, used to find essential relation between two matrices, X and Y, was done for the foot analysis. Any compound that had only zeros was eliminated. Bacteria count data were log transformed using log(x+1). Bacteria was set as a predictor and compounds as a response. In the PLS analysis, only the first 5 dimensions were taken into account for

each of the responses as these are the ones that explain the most responses. A cut off of 66.67% was chosen, therefore anything that explains more than 2/3rds was selected to be analysed further. Finally, for each group, each individual Y was used to calculate the correlation (and its associated p value). The results were analysed according to the level of significance, 0.001% significance was chosen. The results were separated according to the correlation level: positive correlation (0.5-0.69), strong positive correlation (0.7-1), negative correlation (-0.5-0.69), strong negative correlation (-0.7-1). The results from the correlation were plotted in graphs for each mean compound and mean bacteria counts by volunteer visit (day 1 and day 4). The graphs show the correlation trend.

5.6 Results

5.6.1 Bacteria found on feet

The analysis from the 16S rRNA sequencing revealed 414 different bacteria collected from the volunteers. Table 5-4 shows the mean bacteria counts for feet on the different visits. The majority were *Staphylococcus, Corynebacterium, Acinetobacter, Anaerococcus, Paracoccus, Propionibacterium and Aerococcus* and these bacteria dominated the samples and were found in all volunteers. Many other bacteria were found but these were in much lower quantities and were not consistently found in all volunteers.

5.6.2 Volatiles found on feet

The GC analysis revealed the presence of 117 different compounds. Table 5-5 shows the mean proportion for each compound for the different body sites and the different visits. Compounds RI 1657 (unidentified), RI 1586 (unidentified), RI 1817 (unidentified) and RI 1701 (unidentified) dominated the samples and were found in all volunteers. Many other volatiles were found but these were in much lower quantities and were not consistently found in all volunteers.

5.6.3 Comparison of bacteria between day 1 and day 4

There was a significant increase between day 1 and day 4 for the following bacteria found on feet (Table 4-4): *Actinomycetospora* (p=0.024), *Actinoplanes* (p=<0.001), *Adhaeribacter* (p=0.016), *Aliihoeflea* (p=0.016), *Aminobacter* (p=0.023), *Ancylobacter* (p=0.026), *Acrobacter* (p=0.031), *Arenimonas* (p=0.048), *Brukholderia* (p=0.011), *Candidatus* (p=0.028), *Caulobacter* (p=0.002), *Cellvibrio* (p=0.005), *Conexibacter* (p=0.01), *Crauococcus* (p=0.023), *Curtobacterium* (p=0.003), *Defluviicoccus* (p=0.033), *Devosia* (0.004*), Flavihumibacter* (p=0.012), *Flavitalea* (p=0.002), *Gemmatimonas* (p=0.002), *Herminiimonas* (p=0.036), *Hyphomicrobium* (p=0.003), *Ilumatobacter* (p=0.012), *Kaistia* (p=0.007), *Kineococcus* (p=0.008), *Kineosporia* (p=0.023), *Legionella-like* (p=0.007), *Leuconostoc* (p=0.009), *Lewinella* (p=0.046), *Luteolibacter* (p=0.003), *Lysobacter* (p=0.009), *Methylobacterium* (p=0.025), *Methylocystis* (p=0.026),

Methylophilus (p=0.004), *Methylotenera* (p=0.015), *Modestobacter* (p=0.028), *Nakamurella* (p=0.034*), Nitrosomonas* (p=0.018), *Ohtaekwangia* (p=0.007), *Ornithinimicrobium* (p=0.035), *Oryzihumus* (p=0.012), *Paenibacillus* (p=0.023), *Parasegetibacter* (p=0.018), *Pedomicrobium* (p=0.006), *Phaselicystis* (p=0.009), *Piscicoccus* (p=0.003), *Polaromonas* (p=0.009), *Pseudofulvimonas* (p=0.015), *Pseudonocardia* (p=0.031), *Pseudoxanthomonas* (p=0.034), *Ramilibacter* (p=0.005), *Rathayibacter* (p=0.013), *Rhizobium* (p=0.002), *Rhodoblastus* (p=0.022), *Rhodoferax* (p=0.002), *Rhodoplanes* (p=0.005), *Rhodopseudomonas* (p=0.028), *Roseococcus* (p=0.002), *Sanguibacter* (p=0.012), *Solirubrobacter* (p=0.031*), Solobacterium* (p=0.033*), Sphaerobacter* (p=0.008), *Sphingobium* (=0.042), *Sutterella* (p=0.036), *Terrimonas* (p=0.005*), Thermononas* (p=0.006).

There was a significant decrease between day 1 and day 4 for the following bacteria found on feet: *Acidisphaera* (p=0.006), *Dolosigranulum* (p=0.008), *Halomonas* (p=<0.001), *Paracraurococcus* (p=0.010), *Tsukamurella* (p=0.037).

Out of these bacteria, the following had the highest abundance: *Methylobacterium* (0.23%), *Acidisphaera* (0.16%), *Hyphomicrobium* (0.04%), *Nitrosomonas* (0.04%), *Craurococcus* (0.03%), *Halomonas* (0.03%), *Ilumatobacte*r (0.03) and *Lysobacter* (0.02%).

5.6.4 Comparisons of volatiles between day 1 and day 4

For feet, only two compounds showed a significant difference of quantities between day 1 and day 4: RI 1164 (p=0.034) and RI 1259 (p=0.017), both are unidentified (Table 4-5)

5.6.5 Correlation between bacteria and volatiles

The correlation between foot volatiles and bacteria between day 1 and day 4 is shown in Table 5-6. The correlation was split in four different categories, strong positive correlation (0.7-1), strong correlation (0.5-0.699), strong negative correlation (-0.7-1) and negative correlation (-0.5-0.699). The compounds and bacteria correlated are significant at p=0.001. Figures 4-1:21 display the mean for each compound and bacteria that is correlated between day 1 and day 4.

5.6.6 Strong positive correlations

Compound RI 841 was correlated (0.761) with *Sutterella*. (Figure 5-1); compound RI 977 was correlated with bacteria *Ralstonia* (0.714) and *Tuberibacillus* (0.751) (Figure 5-2), compound RI 1172 was correlated (0.916) with bacteria *Tuberibacillus* (Figure 5-3); compound RI 1232 was correlated (0.719) with bacteria *Megasphera* (Figure 5-4); compound 1712 was correlated (0.74) with bacteria Peptococcus (Figure 5-5). A comparison between day 1 and day 4 could not be done for *Tuberibacillus* as there was only 1 data point for day 1. The only associations where the increase in bacteria and compounds between day 1 and 4 was significant by t test was Bacteria *Sutterella* (p=0.036) (Figure 4-6).

5.6.7 Positive correlations

Compound RI 849 was correlated with *Phascoloarctobacterium* (0.694) and *Tyzzerella* (0.662) (Figure 5-6); compound RI 1172 was correlated with *Paludibacter* (0.657) and *Ralstonia* (0.668) (Figure 5-7); compound RI 1232 was correlated with *Oryzihumus* (0.645) and Parabacteroides (0.663) (Figure 5-8); compound RI 1250 was correlated with *Nakamurella* (0.652), *Parabacteroides* (0.665) and *Tyzzerella* (0.653) (Figure 5-9); compound RI 1348 was correlated with Tuberibacillus (0.649) (Figure 5-10); compound RI 1489 was correlated with *Pascolarctobacteirum* (0.652) and *Sutterella* (0.662) (Figure 5-11); compound RI 1601 was positively correlated with Bacteria *Peptococcus* (0.678) (Figure 5-12). A comparison could not be done between day 1 and day 4 for bacteria *Tuberibacillus*, *Paludibacter, Parabacteroides, Tyzzerella, Tuberibacillus* as there were either only 1 data point or no data points on day 1. The only associations where the increase in bacteria and compounds between day 1 and 4 was significant by t test was Bacteria, *Oryzihumus* (p=0.012), *Nakamurella* (p=0.034) and *Sutterella* (p=0.036).

5.6.8 Strong negative correlations

Turicella was correlated with compounds RI 1134 (-0.708) (Figure 5-13), RI 1148 (-0.746) (Figure 5-14) and RI 1232 (-0.732) (Figure 5-15). Compound RI 1494 was correlated with *Megasphaera* (-0.706) (Figure 5-16).

5.6.9 Negative correlation

Sphingopyxis was correlated with compounds RI 1148 (-0.671) (Figure 5-17) and RI 1232 (-0.672) (Figure 5-18). Compound RI 1348 was correlated with *Pseudohodoferax* (-0.645) and *Terrabacter* (-0.695) (Figure 5-19). Compound RI 1812 was correlated with *Schlegelella* (-0.687) (Figure 5-20). Compound RI 2010 was correlated with R*hodococcus* (-0.680) (Figure 5-21).

Table 5-4. Mean amount of bacteria counts collected from the foot on day 1 and day 4. P values indicates significant difference between day 1 and day 4. Bacteria with significant changes between days are in bold.

Bacteria	Day 1	Day 4	P value	Bacteria	Day 1	Day 4	P value	Bacteria	Day 1	Day 4	P value
Abiotrophia	0.00	0.13	n/a	*Enterococcus*	240.20	103.43	0.579	*Panacagrimonas*	0.00	2.67	n/a
Acetivibrio	0.00	11.43	n/a	*Eremococcus*	3131.83	867.87	0.653	*Pandoraea*	9.00	0.00	n/a
Acetobacter	3.20	19.93	0.545	*Erysipelatoclostridium*	0.00	17.67	n/a	*Pantoea*	1794.97	7404.03	0.203
Achromobacter	2.97	59.67	0.083	*Erythrobacter*	1.87	37.23	n/a	*Parabacteroides*	0.00	15.53	n/a
Acidipila	0.00	40.13	n/a	*Erythromicrobium*	13.77	37.23	0.186	*Paracoccus*	19798.60	5769.60	0.934
Acidisphaera	200.00	3233.60	0.006	*Eubacterium*	58.03	45.57	0.221	*Paracraurococcus*	70.80	23.97	0.010
Aciditerrimonas	36.33	208.63	0.165	*Facklamia*	577.67	648.03	0.640	***Parasegetibacter***	5.60	14.73	0.018
Acidobacterium	0.03	51.67	n/a	*Faecalibacterium*	86.07	553.00	0.349	*Parvimonas*	79.67	52.37	0.436
Acidothermus	7.60	34.13	n/a	*Fastidiosipila*	0.37	0.27	n/a	*Parvularcula*	0.00	82.10	n/a
Acidovorax	34.73	56.63	0.230	*Ferrimicrobium*	0.57	18.77	n/a	*Patulibacter*	21.33	13.83	0.079
Acinetobacter	35876.50	23420.87	0.329	*Ferruginibacter*	26.33	79.77	0.013	*Pedobacter*	142.23	335.47	0.293
Actinobaculum	0.00	46.63	n/a	*Fibrella*	0.77	66.50	n/a	***Pedomicrobium***	1.17	24.40	0.006
Actinomadura	0.80	142.53	0.051	*Filifactor*	4.17	0.33	n/a	*Pelistega*	11.77	284.90	0.380
Actinomyces	847.87	1364.63	0.812	*Fimbriimonas*	0.73	23.80	n/a	*Pelomonas*	59.33	67.57	0.055
Actinomycetospora	66.53	338.30	0.024	*Finegoldia*	6195.60	6221.00	0.875	*Peptococcus*	41.50	32.83	0.328
Actinoplanes	2.30	194.43	0.000	*Flaviflexus*	0.27	12.93	n/a	*Peptoniphilus*	3659.53	2738.50	0.799
Actinotignum	1.53	18.10	0.715	***Flavihumibacter***	5.80	14.13	0.012	*Peptostreptococcus*	277.43	124.07	0.652
Adhaeribacter	1.27	33.20	0.016	*Flavisolibacter*	19.83	31.70	0.188	*Perlucidibaca*	255.90	1734.57	0.616
Advenella	0.40	2.40	n/a	***Flavitalea***	11.27	130.20	0.002	*Phascolarctobacterium*	0.10	34.40	0.226
Aerococcus	11490.87	12873.43	0.459	*Flavobacterium*	173.80	560.83	0.144	***Phaselicystis***	2.03	37.63	0.009
Aeromicrobium	212.33	194.10	0.585	*Flectobacillus*	1.67	1.90	n/a	*Phenylobacterium*	50.40	236.73	0.157
Aeromonas	564.33	2833.27	0.261	*Flexithrix*	0.00	1.70	n/a	*Photobacterium*	42.80	83.47	0.138
Aetherobacter	4.63	1.97	0.451	*Flexivirga*	3.47	22.97	n/a	***Piscicoccus***	7.83	59.77	0.003
Agaricicola	0.00	5.63	n/a	*Fluviicoccus*	33.47	0.00	n/a	*Planifilum*	119.97	0.00	n/a
Aggregatibacter	18.53	81.17	0.469	*Fluviicola*	2.17	0.23	n/a	*Planococcus*	175.17	55.00	0.263
Agromyces	1.93	0.57	0.913	*Fontimonas*	0.00	0.00	n/a	***Polaromonas***	2.80	38.93	0.009

Akkermansia	0.00	9.37	n/a	*Frankia*	1.70	7.60	n/a	*Polynucleobacter*	12.77	0.00	n/a
Alcaligenes	24.67	1.27	0.609	*Frondihabitans*	0.10	10.77	n/a	*Pontibacter*	0.00	0.10	n/a
Alcanivorax	10.30	0.17	0.353	*Fusobacterium*	214.87	326.53	0.893	*Porphyromonas*	83.63	124.90	0.668
Algiphilus	23.27	21.40	0.439	*Gaiella*	106.07	426.70	0.154	*Prevotella*	431.60	1162.10	0.462
Algoriphagus	8.53	16.50	0.259	*Gallionella*	0.00	0.00	n/a	*Promicromonospora*	0.00	3.47	n/a
Aliihoeflea	2.53	74.33	0.016	*Gardnerella*	828.67	59.60	0.372	*Propionibacterium*	11538.20	14069.37	0.928
Alistipes	1.97	0.00	n/a	*Gelidibacter*	0.00	0.00	n/a	*Pseudoalteromonas*	2.20	0.33	0.884
Alkaliphilus	0.00	4.07	n/a	*Gelria*	0.00	0.00	n/a	*Pseudoclavibacter*	320.90	499.97	0.296
Alkanindiges	31.23	89.00	0.237	***Gemmatimonas***	4.37	76.57	0.002	***Pseudofulvimonas***	36.43	66.40	0.015
Allisonella	0.00	0.00	n/a	*Gemmatirosa*	10.23	68.33	0.081	*Pseudomonas*	9661.43	10972.77	0.833
Allocatelliglobosispora	0.03	35.50	n/a	*Gemmiger*	101.50	26.27	0.670	***Pseudonocardia***	45.30	181.27	0.031
Alloiococcus	4.07	24.17	0.875	*Gemmobacter*	157.27	201.40	0.435	*Pseudorhodobacter*	17.40	40.03	0.811
Alloprevotella	2.63	2.73	0.682	*Geobacillus*	0.00	0.00	n/a	*Pseudorhodoferax*	29.57	188.07	0.118
Altererythrobacter	680.23	943.63	0.072	*Geobacter*	4.33	27.40	n/a	*Pseudoxanthobacter*	5.90	0.13	n/a
Amaricoccus	925.47	3029.00	0.217	*Geodermatophilus*	0.00	0.00	n/a	***Pseudoxanthomonas***	223.50	262.30	0.034
Aminobacter	53.93	223.13	0.023	*Georgenia*	3.27	21.90	0.148	*Psychrobacter*	554.17	564.80	0.483
Anaerococcus	22724.70	14784.00	0.324	*Globicatella*	1.73	0.47	n/a	*Ralstonia*	252.10	319.17	0.619
Anaeroglobus	0.00	0.00	n/a	*Gluconobacter*	4.00	7.73	n/a	***Ramlibacter***	0.90	63.80	0.005
Anaeromyxobacter	0.00	0.03	n/a	*Glycomyces*	0.00	3.43	n/a	***Rathayibacter***	30.37	76.63	0.013
Ancylobacter	46.97	115.67	0.026	*Gordonia*	1011.07	404.77	0.832	*Rheinheimera*	732.50	1750.23	0.093
Aquamicrobium	15.60	38.43	0.183	*Granulicatella*	19.23	413.83	0.070	***Rhizobium***	25.90	165.67	0.022
Aquaspirillum	0.00	0.00	n/a	*Granulicella*	38.90	0.13	n/a	*Rhodanobacter*	0.37	38.03	0.066
Aquicella	0.00	14.93	n/a	*Gulbenkiania*	0.03	0.00	n/a	*Rhodobacter*	1587.70	1923.33	0.097
Aquihabitans	43.40	101.27	0.120	*Haematobacter*	235.10	2744.33	0.182	***Rhodoblastus***	0.37	102.83	0.022
Arcicella	69.57	0.80	0.512	*Haemophilus*	2315.73	1912.00	0.763	*Rhodococcus*	7312.23	891.13	0.151
Arcobacter	7.83	172.50	0.031	*Hahella*	0.00	0.03	n/a	*Rhodocytophaga*	2.30	47.37	0.546
Arenimonas	29.20	89.10	0.048	*Haliscomenobacter*	0.93	7.77	n/a	***Rhodoferax***	52.47	426.57	0.002
Arsenicicoccus	80.13	30.07	0.529	*Halochromatium*	0.07	0.86	n/a	***Rhodoplanes***	1.93	95.90	0.005
Arthrobacter	484.03	513.40	0.240	*Haloechinothrix*	0.03	3.83	n/a	***Rhodopseudomonas***	29.20	248.50	0.028
Asaia	1.93	17.63	0.390	*Halomonas*	595.73	131.17	0.00	*Rhodovulum*	662.30	530.43	0.381

Asticcacaulis	0.73	39.70	0.002	*Helcococcus*	52.60	38.37	0.658	*Rickettsiella*	3.93	77.40	0.060
Atopobium	68.00	19.20	0.257	*Herbaspirillum*	9.33	82.67	0.090	*Roseburia*	0.07	176.90	n/a
Atopostipes	0.67	7.03	0.267	***Herminiimonas***	1.13	10.00	0.036	*Roseiarcus*	0.00	22.93	n/a
Azonexus	2.67	1.50	0.806	*Hydrocarboniphaga*	13.40	19.97	0.554	***Roseococcus***	25.33	124.87	0.002
Azorhizobium	316.33	76.70	0.339	*Hydrogenophaga*	6.67	25.70	0.120	*Roseomonas*	3034.50	1247.07	0.318
Azospira	14.30	2.90	0.460	*Hydrogenophilus*	31.07	206.50	0.870	*Rothia*	848.73	822.07	0.495
Azospirillum	31.50	21.60	0.353	*Hymenobacter*	640.23	534.53	0.378	*Rubellimicrobium*	1863.50	2119.27	0.713
Azovibrio	7.77	75.33	0.203	***Hyphomicrobium***	162.37	878.93	0.003	*Rubrobacter*	37.77	71.27	0.270
Bacillus	2298.53	814.03	0.729	*Hyphomonas*	8.50	0.87	n/a	*Rufibacter*	0.43	0.27	0.486
Bacteriovorax	41.97	81.53	0.975	*Iamia*	164.83	170.00	0.147	*Ruminococcus*	86.63	3668.60	0.072
Bacteroides	46.37	81.23	0.832	*Ignavigranum*	2.03	3.40	0.173	*Saccharopolyspora*	16.53	0.70	0.205
Bavariicoccus	0.30	0.00	n/a	***Ilumatobacter***	91.30	621.17	0.012	*Salinicoccus*	3179.40	508.37	0.893
Bdellovibrio	85.43	197.57	0.133	*Isoptericola*	44.77	185.47	0.085	*Salmonella*	28.43	341.43	0.290
Bergeyella	4.27	8.37	0.271	*Jeotgalicoccus*	9593.37	1612.60	0.579	*Sandaracinobacter*	9.23	47.30	0.185
Bifidobacterium	166.07	356.57	0.524	*Jiangella*	115.60	21.57	0.475	*Sandaracinus*	0.00	7.90	n/a
Blastococcus	23.37	34.70	0.052	*Jonquetella*	14.27	0.00	n/a	***Sanguibacter***	10.07	108.07	0.012
Blautia	131.17	1065.20	0.170	***Kaistia***	1.00	122.97	0.007	*Schlegelella*	56.37	31.70	0.976
Bombiscardovia	10.93	0.07	0.432	*Kaistibacter*	7.93	52.87	0.058	*Sediminibacterium*	0.00	4.30	n/a
Brachybacterium	4501.67	4553.27	0.946	*Ketogulonicigenium*	25.47	150.47	0.164	*Selenomonas*	0.30	20.37	0.255
Brachymonas	0.00	0.00	n/a	***Kineococcus***	24.50	93.33	0.008	*Serratia*	32.03	58.80	0.093
Branchiibius	53.77	43.50	0.088	***Kineosporia***	8.60	33.40	0.023	*Shewanella*	111.67	745.13	0.495
Brevibacillus	276.90	45551.03	0.774	*Kingella*	88.63	16.30	0.452	*Simonsiella*	0.00	0.00	n/a
Brevibacterium	4027.63	11339.37	0.590	*Kribbella*	0.00	35.33	n/a	*Skermanella*	683.60	1519.17	0.553
Brevundimonas	667.27	639.63	0.228	*Kurthia*	0.53	1.50	0.511	*Snodgrassella*	340.67	79.37	0.333
Buchnera	2072.30	8.37	0.863	*Kytococcus*	1369.37	2343.17	0.701	*Solimonas*	2.13	50.10	0.333
Bulleidia	0.00	0.00	n/a	*Lachnoclostridium*	0.00	24.07	n/a	***Solirubrobacter***	81.77	381.50	0.031
Burkholderia	15.57	105.23	0.011	*Lactobacillus*	8166.00	2880.20	0.777	***Solobacterium***	0.93	38.53	0.033
Butyrivibrio	0.00	0.13	n/a	*Lactococcus*	169.43	695.87	0.161	***Sphaerobacter***	9.37	21.47	0.008
Byssovorax	0.00	1.53	n/a	*Lautropia*	408.03	495.73	0.205	*Sphingobacterium*	95.57	164.43	0.343
Camelimonas	3.50	16.87	0.139	*Legionella*	25.57	105.27	0.173	***Sphingobium***	10.53	39.90	0.042

Campylobacter	29.33	71.43	0.343	***Legionella-like***	0.17	19.77	0.007	*Sphingomicrobium*	0.00	0.00	n/a
Candidatus	55.17	135.33	0.028	*Leptotrichia*	46.30	139.77	0.824	*Sphingomonas*	4370.37	6196.00	0.476
Capnocytophaga	7.10	10.10	0.265	*Leucobacter*	32.07	36.53	0.074	*Sphingopyxis*	81.33	2.83	0.367
Cardiobacterium	3.30	21.37	0.263	***Leuconostoc***	7.20	346.90	0.009	*Spirosoma*	454.77	314.07	0.258
Carnobacterium	5.00	46.63	0.312	***Lewinella***	0.23	11.73	0.046	*Sporichthya*	19.37	71.57	0.072
Catonella	0.20	0.23	n/a	*Luedemannella*	6.33	16.83	0.119	*Sporocytophaga*	4.10	134.90	0.062
Caulobacter	3.47	89.43	0.002	*Luteimonas*	370.70	246.70	0.091	*Stackebrandtia*	0.00	0.23	n/a
Cellvibrio	35.33	168.37	0.005	***Luteolibacter***	3.57	51.53	0.003	*Staphylococcus*	650430.07	575994.67	0.841
Cetobacterium	7.43	0.83	n/a	***Lysobacter***	153.33	399.60	0.009	*Stenotrophomonas*	5437.97	1567.70	0.775
Chelatococcus	2.97	93.87	n/a	*Marinococcus*	5.37	71.10	0.410	*Steroidobacter*	21.53	89.20	0.053
Chiayiivirga	48.93	74.10	0.365	*Marinomonas*	0.00	0.00	n/a	*Streptococcus*	4700.13	9173.70	0.650
Chitinimonas	0.00	8.17	n/a	*Marmoricola*	223.30	404.57	0.252	*Streptomyces*	62.20	277.30	0.083
Chondromyces	1.27	52.23	n/a	*Massilia*	477.87	121.00	0.397	*Subtercola*	1.70	14.37	0.093
Christensenella	8.97	0.00	n/a	*Megasphaera*	0.70	2.97	0.546	*Succinivibrio*	0.00	1.57	n/a
Chryseobacterium	793.73	213.93	0.862	*Meiothermus*	0.00	0.00	n/a	*Sulfuricurvum*	0.30	0.07	n/a
Clostridium	85.30	401.80	0.070	*Mesorhizobium*	123.77	194.03	0.081	*Sulfurimonas*	36.60	81.10	0.915
Cohnella	0.97	7.43	n/a	*Methylobacillus*	0.40	30.50	0.053	***Sutterella***	10.60	42.40	0.036
Collinsella	9.17	67.13	0.112	***Methylobacterium***	1186.30	3809.43	0.025	*Tahibacter*	7.53	17.07	0.839
Comamonas	305.80	694.97	0.233	***Methylocystis***	34.60	40.90	0.026	*Tannerella*	1.00	2.07	0.761
Conchiformibius	12.27	1.03	n/a	***Methylophilus***	0.90	8.23	0.004	*Tepidiphilus*	9.60	45.97	0.739
Conexibacter	17.57	124.20	0.010	***Methylotenera***	4.07	24.43	0.015	*Terrabacter*	173.90	372.00	0.092
Coprococcus	0.13	58.07	n/a	*Micromonospora*	0.03	0.23	n/a	*Terriglobus*	6.87	6.97	0.179
Corynebacterium	169872.50	246227.27	0.516	*Mobiluncus*	46.20	87.37	0.303	***Terrimonas***	4.63	119.90	0.005
Coxiella	0.00	0.20	n/a	***Modestobacter***	3.57	14.23	0.028	*Thauera*	6.13	14.33	0.583
Craurococcus	137.77	595.60	0.023	*Moraxella*	95.10	906.47	0.125	*Thermoactinomyces*	10.00	16.53	0.080
Cryptosporangium	0.00	0.43	n/a	*Morococcus*	517.00	241.90	0.248	*Thermomicrobium*	1.60	29.67	0.424
Curtobacterium	0.63	142.57	0.003	*Moryella*	0.17	0.13	n/a	***Thermomonas***	0.90	40.03	0.006
Curvibacter	8.77	27.00	0.081	*Mucilaginibacter*	0.13	40.30	0.083	*Thermomonospora*	0.00	4.10	n/a
Cystobacter	1.30	0.67	n/a	*Mycobacterium*	1006.13	1447.00	0.107	*Thermovum*	0.03	77.97	n/a
Cytophaga	2.57	13.10	n/a	***Nakamurella***	60.20	198.63	0.034	*Thermus*	0.27	11.97	n/a

Dechloromonas	0.07	3.77	n/a	*Nannocystis*	0.10	0.23	0.639	*Thiobacillus*	0.00	4.23	n/a
Defluviicoccus	17.37	67.03	0.033	*Negativicoccus*	155.70	153.67	0.251	*Thioprofundum*	4.50	0.43	0.608
Deinococcus	290.40	682.63	0.099	*Neisseria*	694.77	1037.90	0.987	*Toxopsis*	4.97	20.20	0.297
Delftia	252.93	151.10	0.337	*Nesterenkonia*	1165.73	208.67	0.824	*Treponema*	10.23	1.40	0.418
Denitratisoma	0.00	0.03	n/a	***Nitrosomonas***	23.70	1012.90	0.018	*Trichococcus*	13.37	21.20	0.056
Dermabacter	2082.30	2949.47	0.619	*Nitrosospira*	0.00	6.27	n/a	*Truepera*	498.07	652.80	0.095
Desulfosporosinus	2.43	32.03	n/a	*Nitrospira*	13.67	46.37	0.063	*Tsukamurella*	36.13	19.50	0.037
Desulfotomaculum	0.00	0.00	n/a	*Nocardia*	18.80	7.20	0.118	*Tuberibacillus*	1.10	86.13	n/a
Desulfovibrio	0.00	0.00	n/a	*Nocardioides*	303.20	1048.50	0.138	*Tumebacillus*	0.00	2.87	n/a
Devosia	33.90	238.03	0.004	*Nocardiopsis*	3.83	32.00	0.144	*Turicella*	111.73	11.43	0.062
Dialister	232.27	399.10	0.833	*Nosocomiicoccus*	8.90	15.60	0.712	*Turicibacter*	56.90	64.67	0.725
Dickeya	0.03	32.87	n/a	*Nostoc*	2.03	1.33	0.877	*Tyzzerella*	0.00	36.40	n/a
Dietzia	1007.00	1533.13	0.867	*Novispirillum*	3.03	39.53	0.136	*Uliginosibacterium*	14.50	21.90	0.088
Dokdonella	10.67	15.90	0.653	*Novosphingobium*	518.53	410.53	0.061	*Variovorax*	538.33	842.53	0.514
Dolosigranulum	348.70	125.93	0.008	***Ohtaekwangia***	0.47	26.97	0.007	*Veillonella*	272.83	460.77	0.589
Dorea	7.33	147.10	0.290	*Oligella*	37.43	0.03	n/a	*Vibrio*	99.70	329.90	0.646
Duganella	1.17	88.23	n/a	*Oribacterium*	3.43	41.33	0.378	*Virgibacillus*	0.00	6.60	n/a
Dyadobacter	87.93	97.80	0.265	***Ornithinimicrobium***	4.63	43.67	0.035	*Weissella*	37.30	38.77	0.595
Eggerthella	0.00	13.10	n/a	***Oryzihumus***	4.97	19.20	0.012	*Williamsia*	1769.40	164.47	0.061
Eikenella	1.37	4.03	n/a	***Paenibacillus***	43.60	412.60	0.023	*Wolbachia*	35.70	9.40	0.057
Emticicia	0.33	2.57	n/a	*Paludibacter*	1.10	73.93	n/a	*Xanthomonas*	21.53	15.70	0.154

Table 5-5. Mean amount of volatile compound collected from each body site on day 1 and day 4. P values indicates significant difference between day 1 and day 4.

RI	Day 1	Day 4	P value	RI	Day 1	Day 4	P value	RI	Day 1	Day 4	P value
810	0.11	0.13	0.93	1159	0.79	0.97	0.592	1553	1.04	1.15	0.499
820	0.24	0.21	0.933	1164	0.23	0.24	0.034	1562	0.07	0.09	0.952
841	0.75	1.13	0.466	1172	1.2	2.07	0.186	1568	0.26	0.35	0.815
851	1.26	1.2	0.959	1180	0.03	0.08	0.143	1574	0.16	0.18	0.758
865	0.1	0.02	0.285	1187	1.33	1.17	0.806	1586	5.66	5.28	0.949
870	0.25	0.86	0.07	1200	0.11	0.12	0.861	1591	0.47	0.33	0.693
879	0.2	0.28	0.51	1213	0.11	0.06	0.464	1601	1.63	1.69	0.885
882	0.05	0.09	0.457	1217	0.03	0.05	0.658	1612	0.04	0.31	0.061
889	0.37	0.35	0.849	1225	0.02	0.03	0.874	1618	0.78	0.59	0.433
910	0.15	0.21	0.883	1232	0.29	0.91	0.168	1628	0.44	0.76	0.451
930	0.42	0.31	0.483	1250	0.62	1.27	0.088	1640	0.19	0.39	0.246
933	0.18	0.26	0.621	1259	0.19	0.06	0.017	1657	6.15	7.17	0.883
943	0.03	0.04	0.747	1269	0.28	0.27	0.96	1667	0.57	0.46	0.824
953	0.09	0.11	0.786	1275	0.28	0.28	0.919	1676	1.06	1.07	0.479
958	0.06	0.06	0.935	1282	0.01	0.13	0.826	1684	0.61	0.72	0.888
962	1.76	1.49	0.221	1287	0.23	0.36	0.571	1688	0.32	0.21	0.334
965	0.67	0.9	0.178	1301	0.07	0.03	0.214	1693	0.7	0.58	0.848
972	0.05	0.08	0.286	1305	0.46	0.58	0.662	1701	2.8	2.3	0.587
974	0	0	n/a	1317	0.21	0.26	0.866	1711	2.22	2.19	0.874
977	0.06	0.04	0.559	1331	0.15	0.2	0.795	1720	1.16	0.78	0.472
982	1.03	0.74	0.135	1336	1.22	1.24	0.421	1725	1.98	2.35	0.375
990	0.07	0.86	0.055	1348	0.22	0.26	0.682	1740	0.48	0.63	0.65
995	0.73	0.84	0.555	1354	1.56	1.55	0.514	1747	0.31	0.83	0.111
1000	0.15	0.14	0.781	1374	0	0.03	n/a	1754	1.04	0.44	0.409
1005	0.76	0.85	0.386	1381	0.04	0.23	0.126	1761	1.93	2.26	0.451
1015	1.13	0.87	0.184	1389	0.16	0.18	0.415	1790	1.27	0.84	0.161

RI	Day 1	Day 4	P value	RI	Day 1	Day 4	P value	RI	Day 1	Day 4	P value
1033	0.47	0.42	0.558	1404	0.42	0.46	0.448	1817	4.23	3.62	0.375
1052	0.02	0.02	0.651	1422	0.08	0.26	0.199	1830	2.37	2.36	0.337
1060	1.03	0.6	0.166	1432	0.85	1.01	0.538	1843	0.51	0.49	0.885
1063	0.05	0.13	0.435	1451	0.08	0.15	0.693	1867	0.45	0.67	0.445
1073	0.05	0.04	0.799	1458	0.22	0.2	0.543	1874	1.28	0.85	0.302
1085	2.19	2.34	0.532	1462	0.54	1.5	0.619	1880	0.04	0.12	0.355
1086	0.26	0	n/a	1470	2.75	1.57	0.291	1901	0.93	0.98	0.738
1102	0.36	0.23	0.128	1489	1.51	2.37	0.26	1909	0.39	0.54	0.373
1120	0.11	0.18	0.424	1494	4.95	5.19	0.984	1920	0.57	0.82	0.443
1130	0.59	1.19	0.169	1499	0.99	0.77	0.225	1935	0.42	0.42	0.968
1148	0.18	0.7	0.119	1519	0.14	0.32	0.21	2001	0.07	0.12	0.617
1154	0.14	0.17	0.47	1539	0.44	0.45	0.878	2010	0.25	0.18	0.312

Table 5-6. Correlation between bacteria and compounds between day 1 and day 4 at 0.001% significance.

	Positive correlation		Strong positive correlation		Negative correlation		Strong negative correlation	
Compound	**Bacteria**	**Correlation**	**Bacteria**	**Correlation**	**Bacteria**	**Correlation**	**Bacteria**	**Correlation**
841	*Phascolarctobacterium*	0.694	*Sutterella*	0.761	-	-	-	-
	Tyzzerella	0.662	-	-	-	-	-	-
977	-	-	*Ralstonia*	0.714	-	-	-	-
	-	-	*Tuberibacillus*	0.751	-	-	-	-
1134	-	-	-	-	-	-	*Turicella*	-0.708
1148	-	-	-	-	*Sphingopyxis*	-0.671	*Turicella*	-0.746
1172	*Paludibacter*	0.657	*Tuberibacillus*	0.916	-	-	-	-
	Ralstonia	0.668	-	-	-	-	-	-
1232	*Oryzihumus*	0.645	*Megasphaera*	0.719	*Sphingopyxis*	-0.672	*Turicella*	-0.732
	Parabacteroides	0.663	-	-	-	-	-	-
1250	*Nakamurella*	0.652	-	-	-	-	-	-
	Parabacteroides	0.665	-	-	-	-	-	-
	Tyzzerella	0.653	-	-	-	-	-	-
1348	*Tuberibacillus*	0.649	-	-	*Pseudorhodoferax*	-0.645	-	-
	-		-	-	*Terrabacter*	-0.695	-	-
1489	*Phascolarctobacterium*	0.652	-	-	-	-	-	-
	Sutterella	0.662	-	-	-	-	-	-
1494	-	-	-	-	-	-	*Megasphaera*	-0.706
1601	*Peptococcus*	0.678	-	-	-	-	-	-
1712	-	-	*Peptococcus*	0.74	-	-	-	-
1812	-	-	-	-	*Schlegelella*	-0.687	-	-
2010	-	-	-	-	*Rhodococcus*	-0.68	-	-

Positive strong correlation

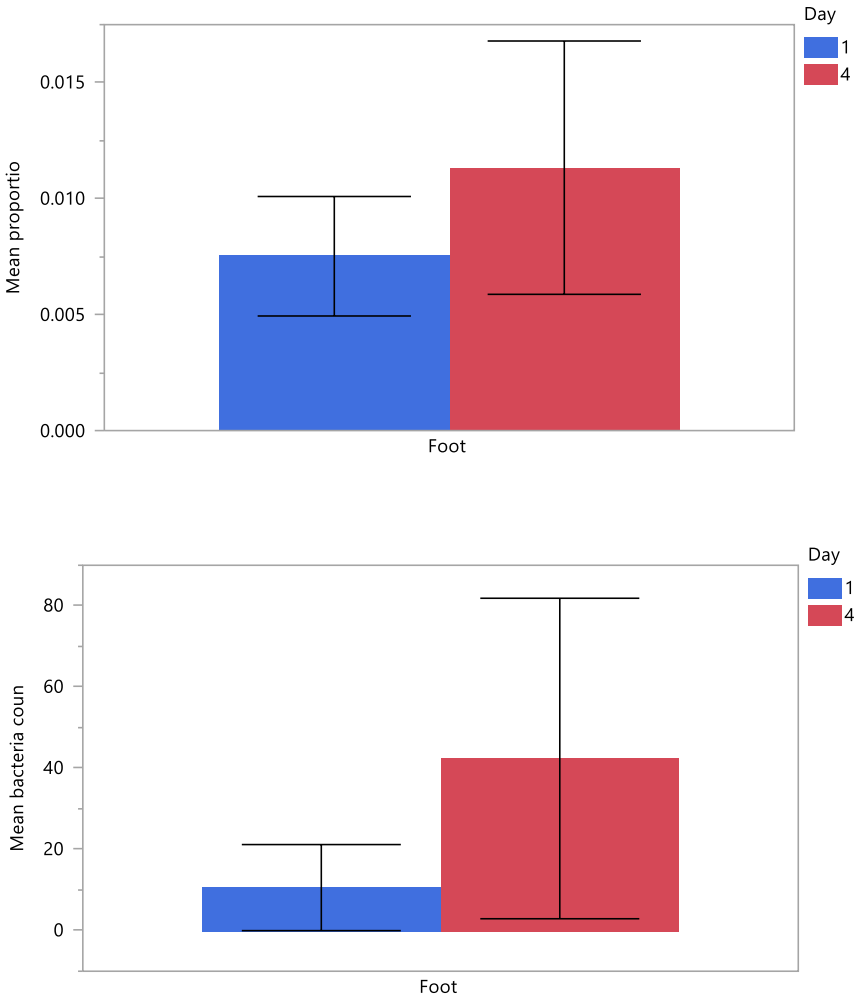


Figure 5-1. Top graph shows the mean amount of compound RI 841 collected from the feet of 30 volunteers on day 1 and day 4. Bottom graph shows mean *Sutterella* bacteria counts between foot day 1 and day 4. Standard error bars are shown.

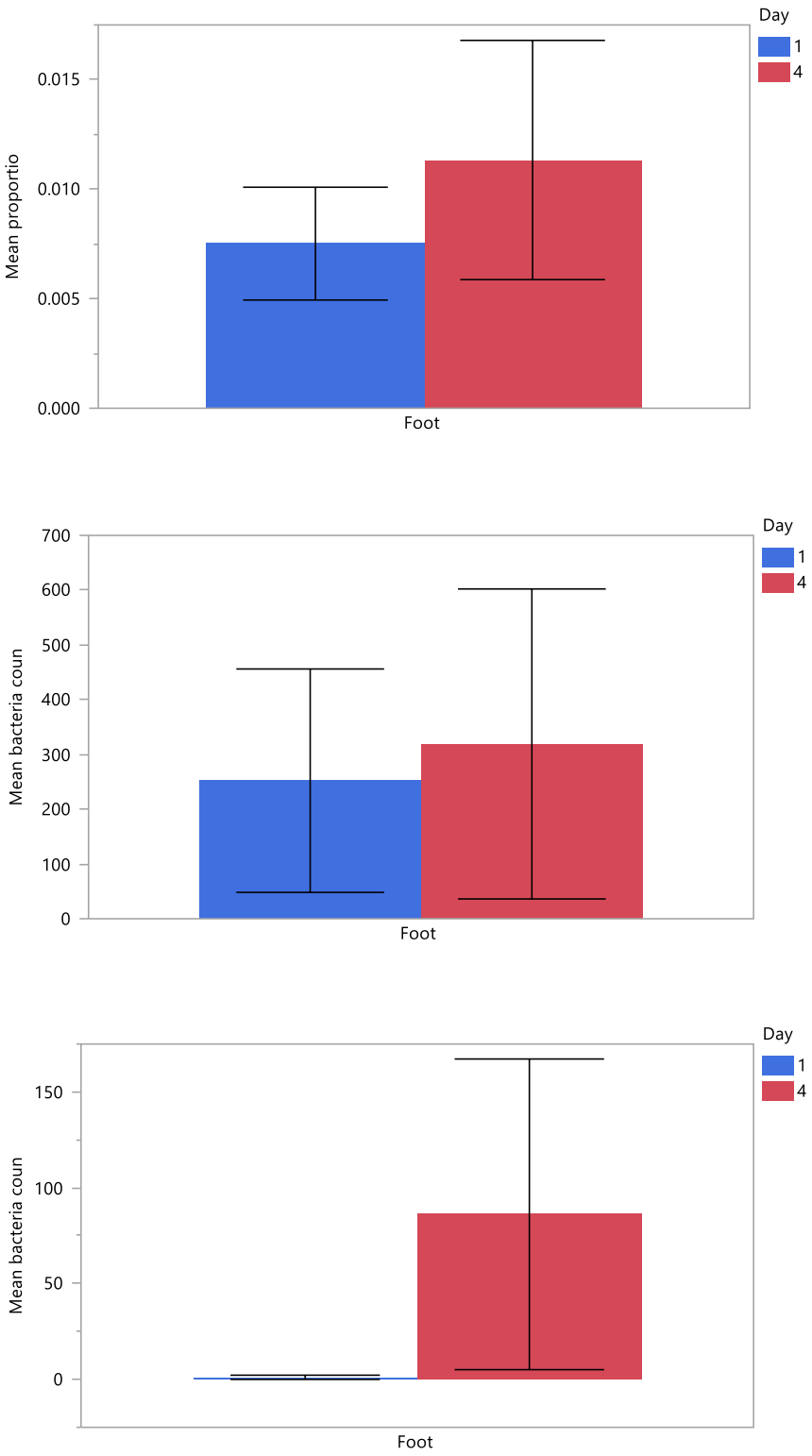


Figure 5-2. Top graph shows the mean amount of compound RI 977 between foot day 1 and day 4. Middle graph shows the mean of *Ralstonia* bacteria counts, bottom graph shows the mean of *Tubieribacillus* bacteria counts.

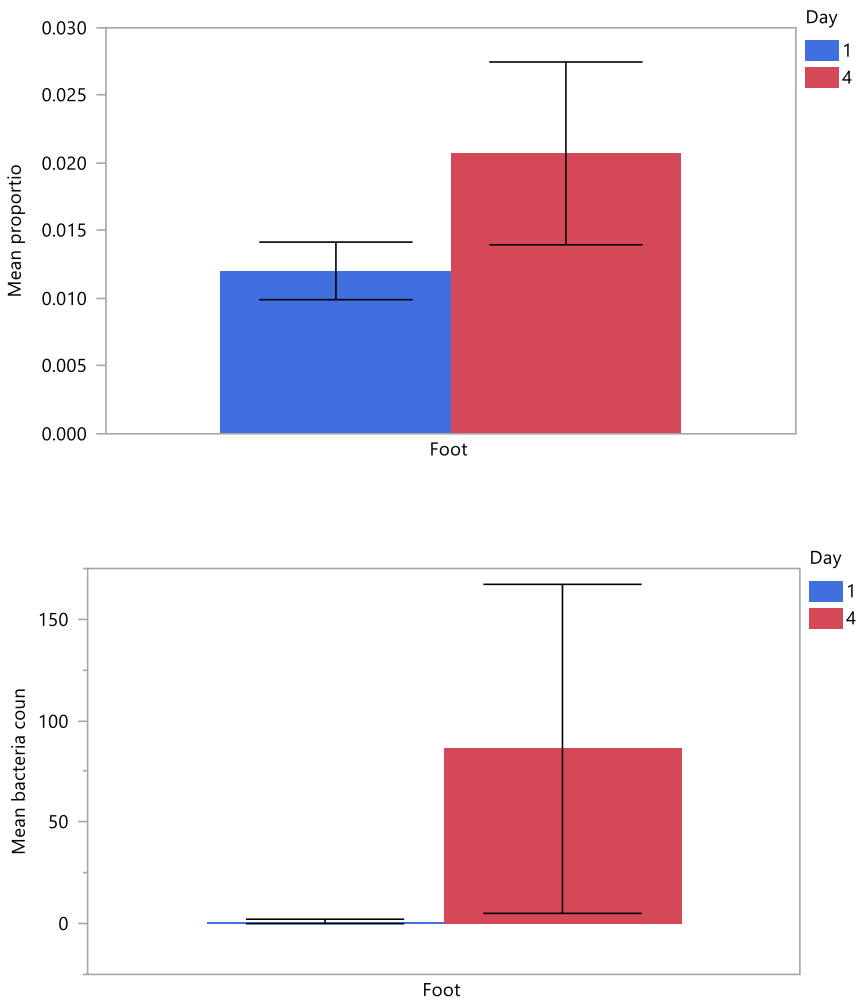


Figure 5-3. Top graph shows the mean amount of compound RI 1172 between foot day 1 and day 4. The bottom graph shows mean *Tuberibacillus* bacteria counts between foot day 1 and day 4.

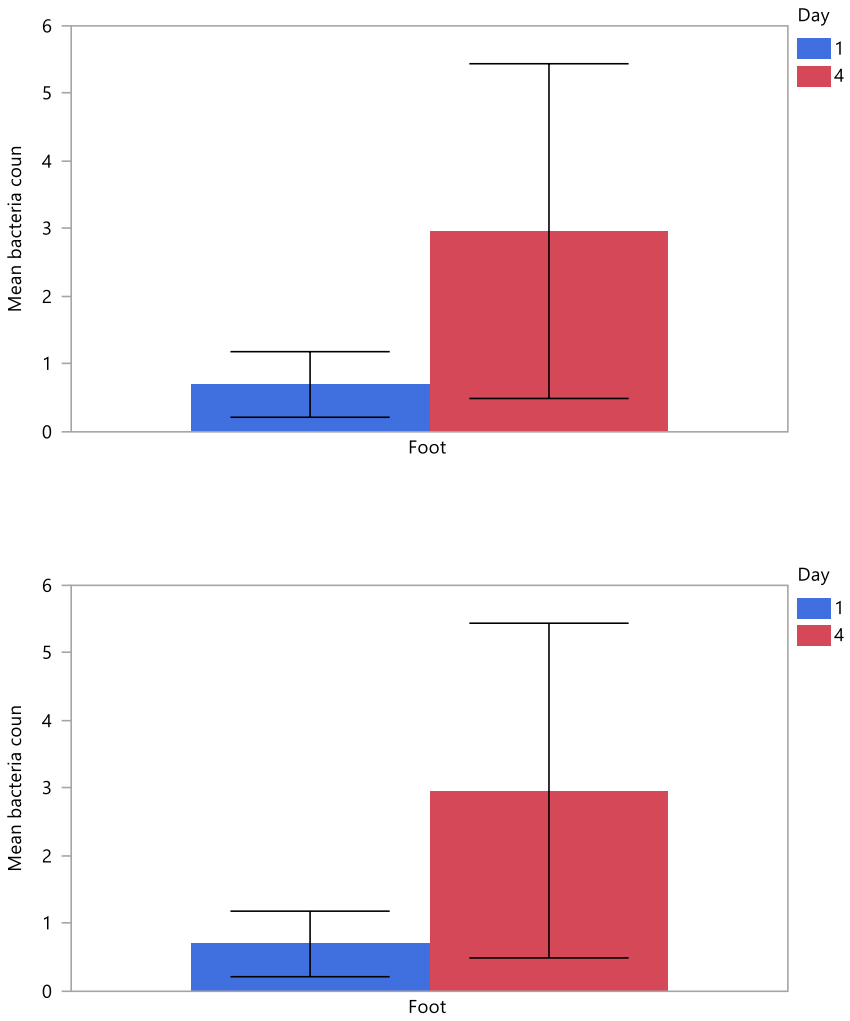


Figure 5-4. Top graph shows the mean amount of compound RI 1232 between foot day 1 and day 4. The bottom graph shows mean *Megasphaera* bacteria counts between foot day 1 and day 4.

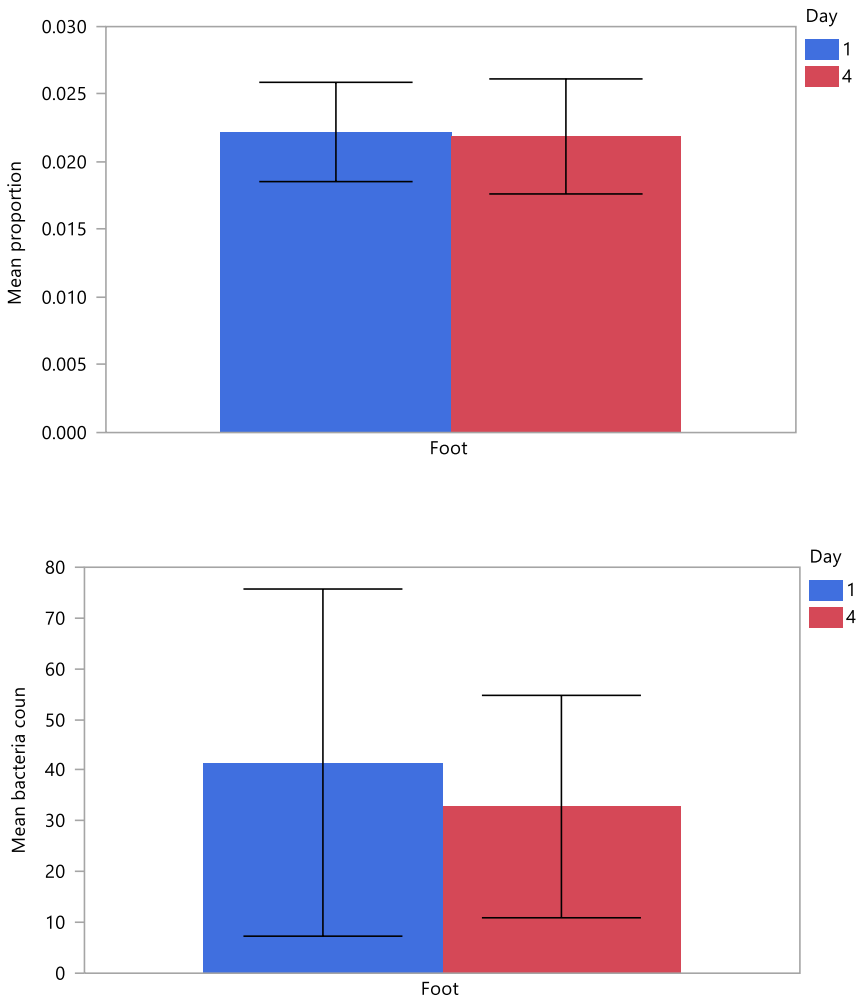


Figure 5-5. Top graph shows the mean amount of compound RI 1711 between foot day 1 and day 4. The bottom graph shows mean *Peptococcus* bacteria counts between foot day 1 and day 4.

Positive correlations

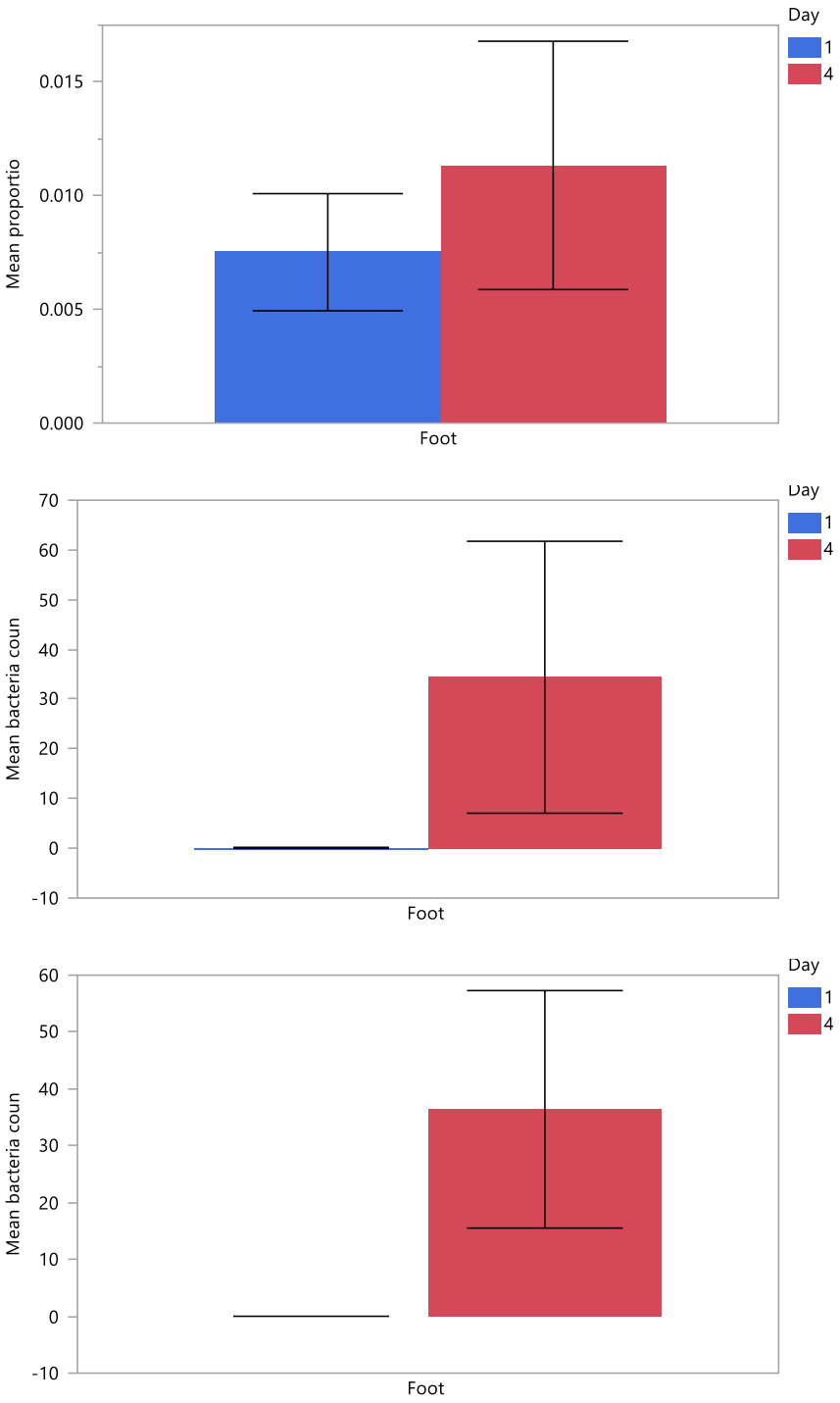


Figure 5-6. Top graph shows the mean amount of compound RI 841 between foot day 1 and day 4. The middle graph shows mean *Phascolarctobacterium* bacteria counts between foot day 1 and day 4. The bottom graph shows mean *Tyzerella* bacteria counts between foot day 1 and day 4.

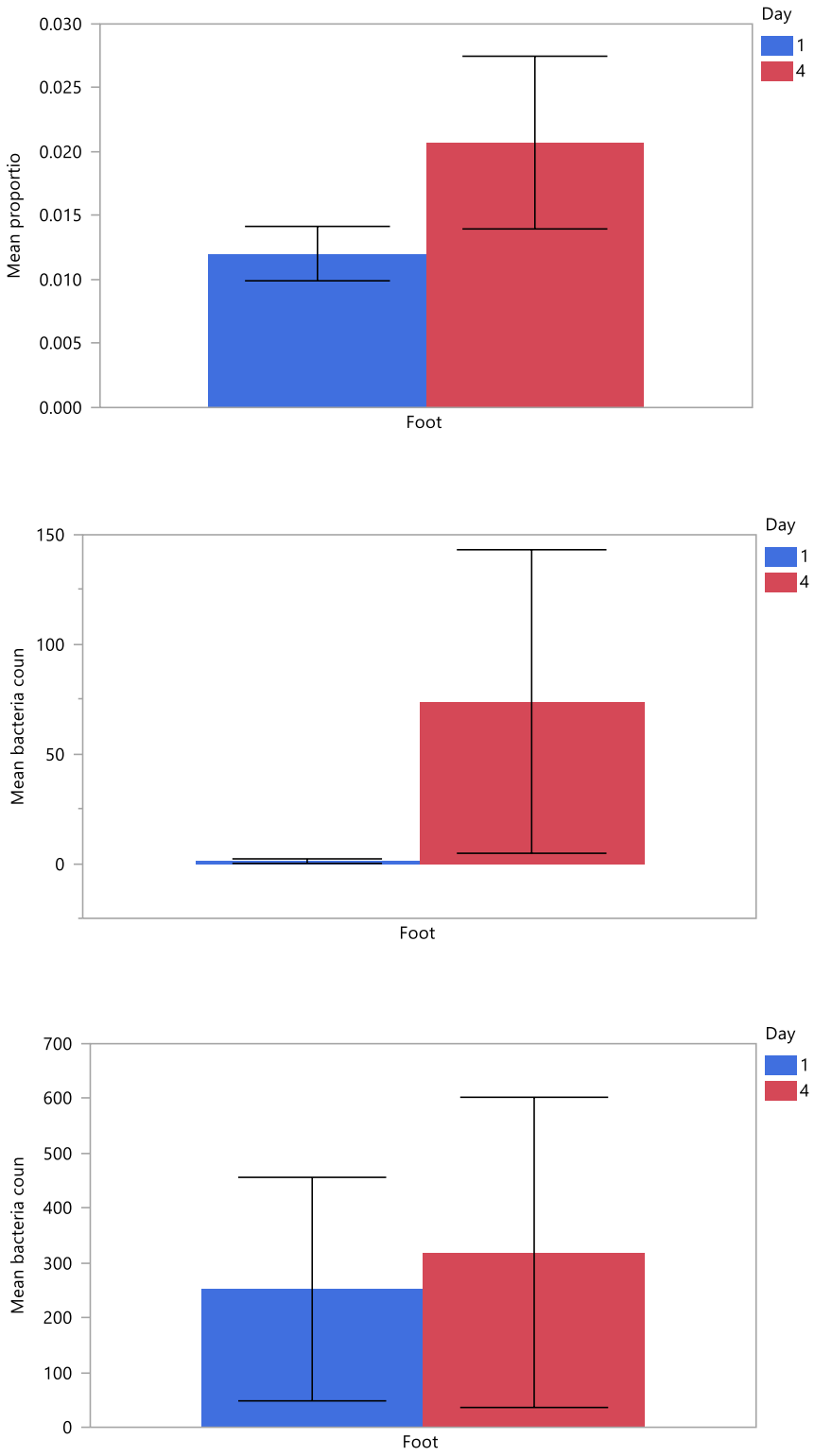


Figure 5-7. Top graph shows the mean amount of compound RI 1172 between foot day 1 and day 4. The middle graph shows mean *Paludibacter* bacteria counts between foot day 1 and day 4. The bottom graph shows mean *Ralstonia* bacteria counts between foot day 1 and day 4.

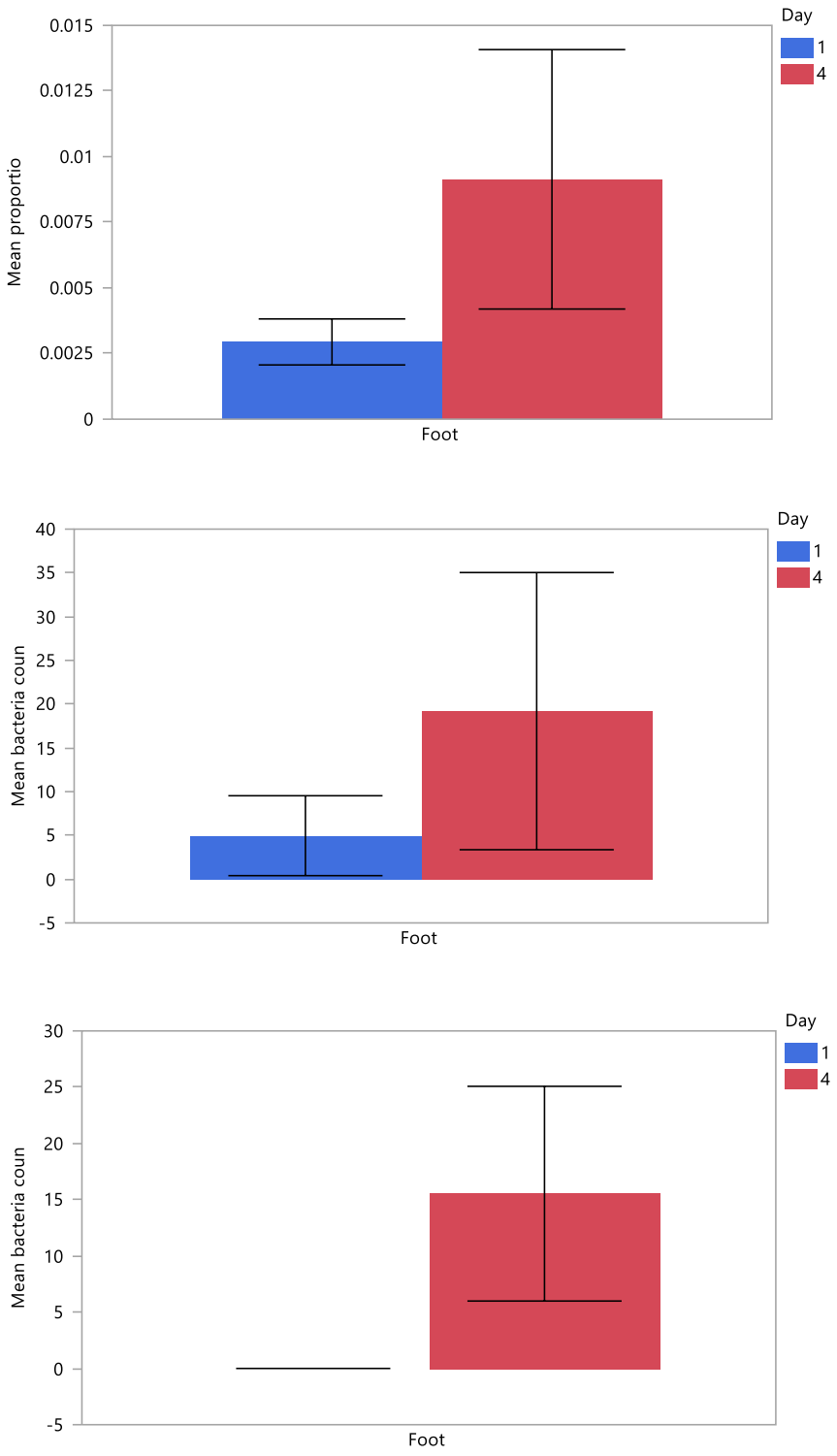


Figure 5-8. Top graph shows the mean amount of compound RI 1232 between foot day 1 and day 4. The middle graph shows mean *Oryzihumus* bacteria counts between foot day 1 and day 4. The bottom graph shows mean *Parabacteroides* bacteria counts between foot day 1 and day 4.

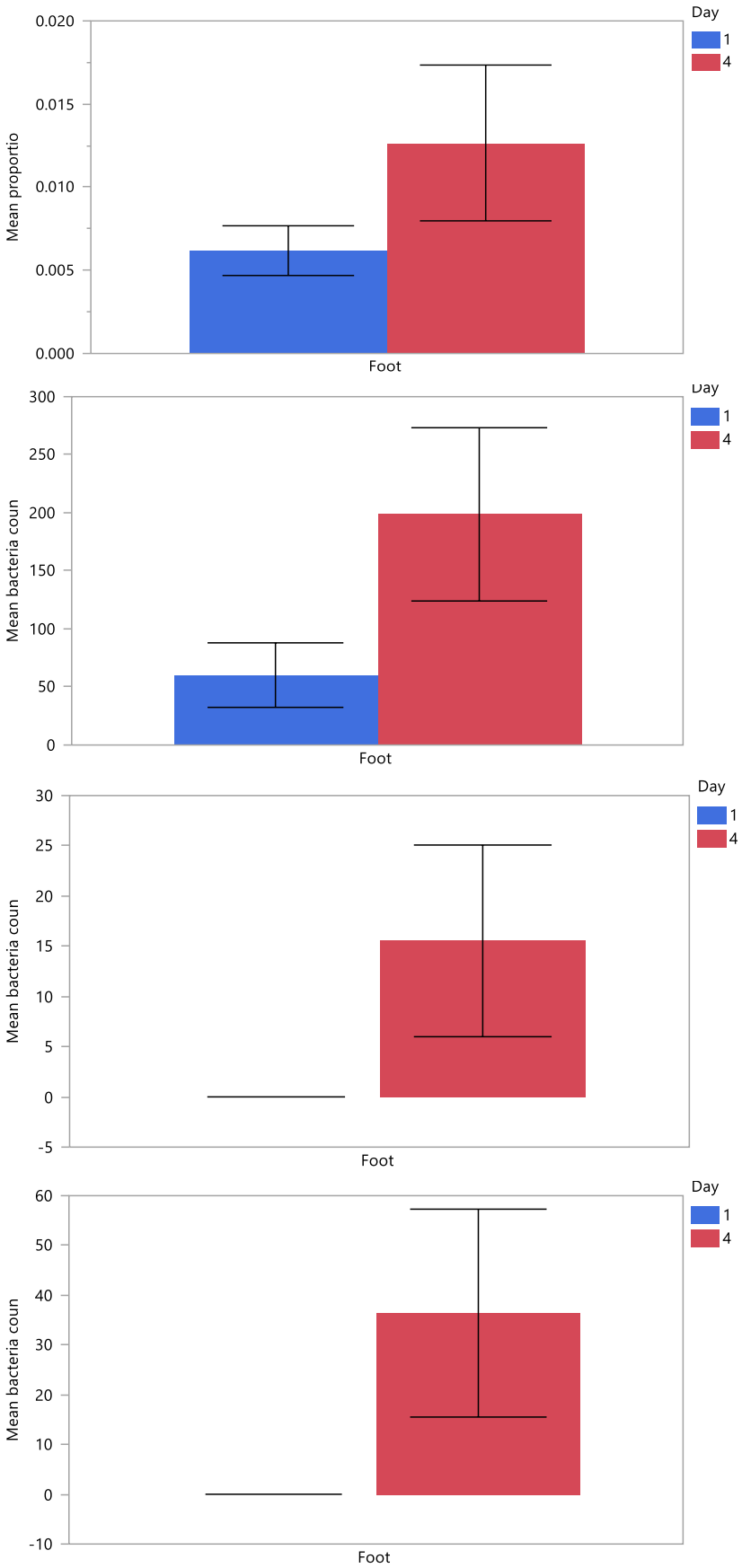


Figure 5-9. Top graph shows the mean amount of compound RI 1250 between foot day 1 and day 4. The second graph shows mean *Nakamurella* bacteria counts between foot day 1 and day 4. The third graph shows mean *Parabacteroides* bacteria counts between foot day 1 and day 4. The bottom graph shows mean *Tyzzerella* bacteria counts between foot day 1 and day 4.

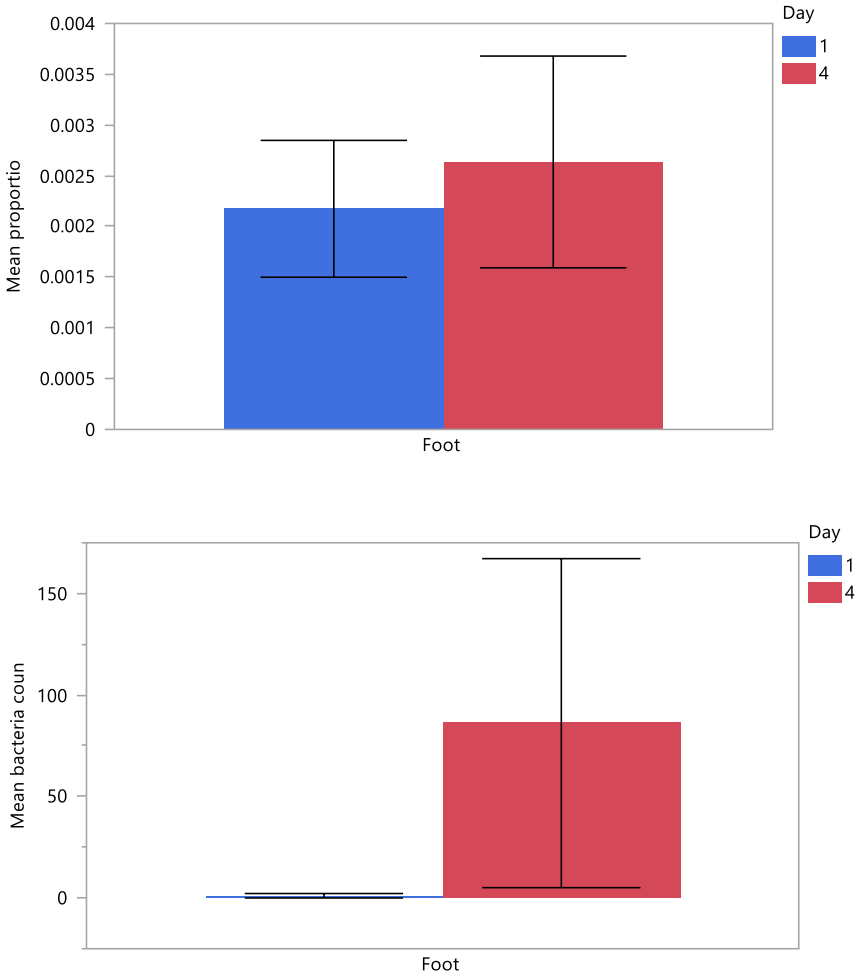


Figure 5-10. Top graph shows the mean amount of compound RI 1348 between foot day 1 and day 4. The bottom graph shows mean *Tuberibacillus* bacteria counts between foot day 1 and day 4.

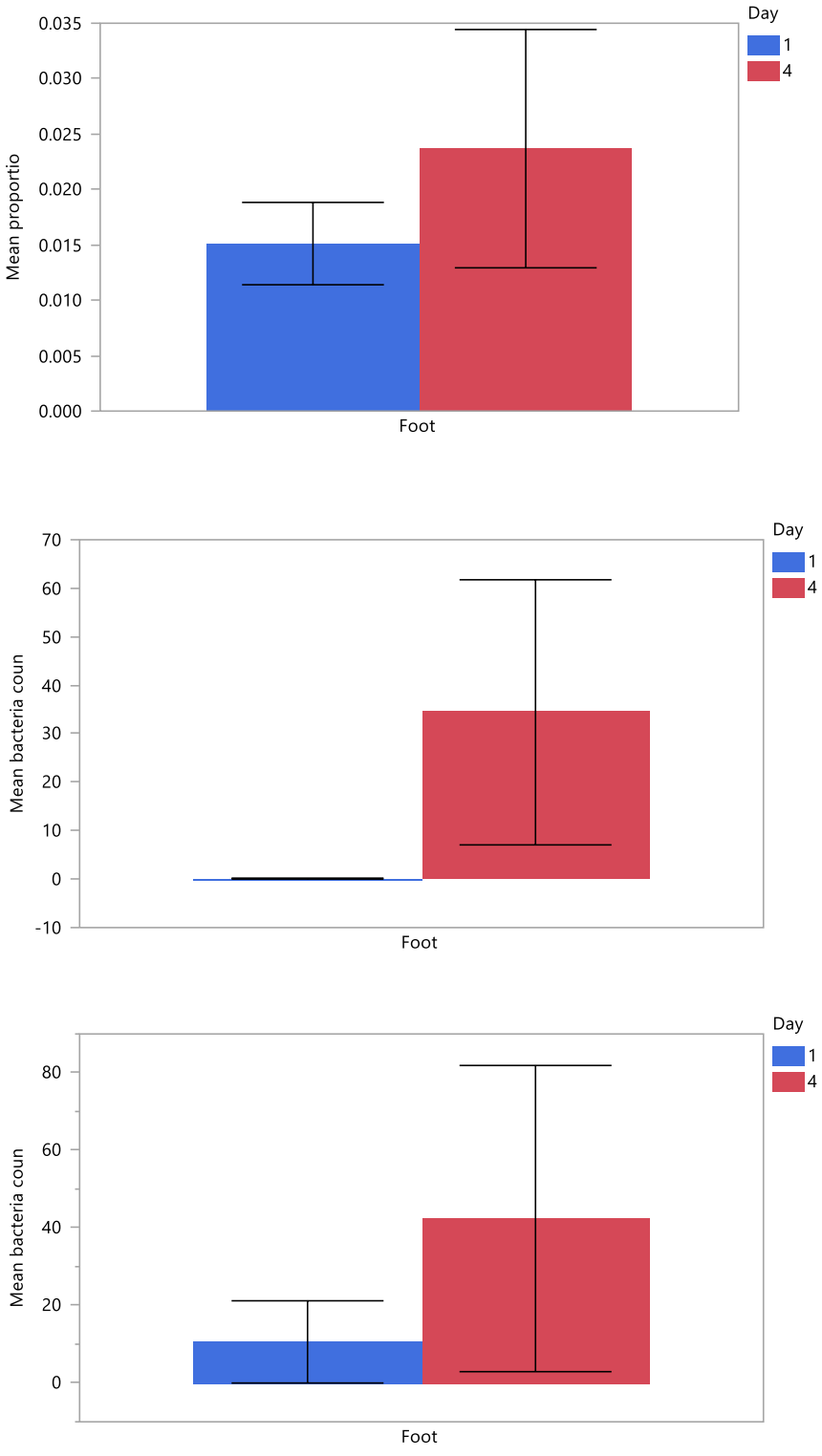


Figure 5-11. Top graph shows the mean amount of compound RI 1489 between foot day 1 and day 4. The middle graph shows mean *Phascolartobacterium* bacteria counts between foot day 1 and day 4. The bottom graph shows mean *Sutterella* bacteria counts between foot day 1 and day 4.

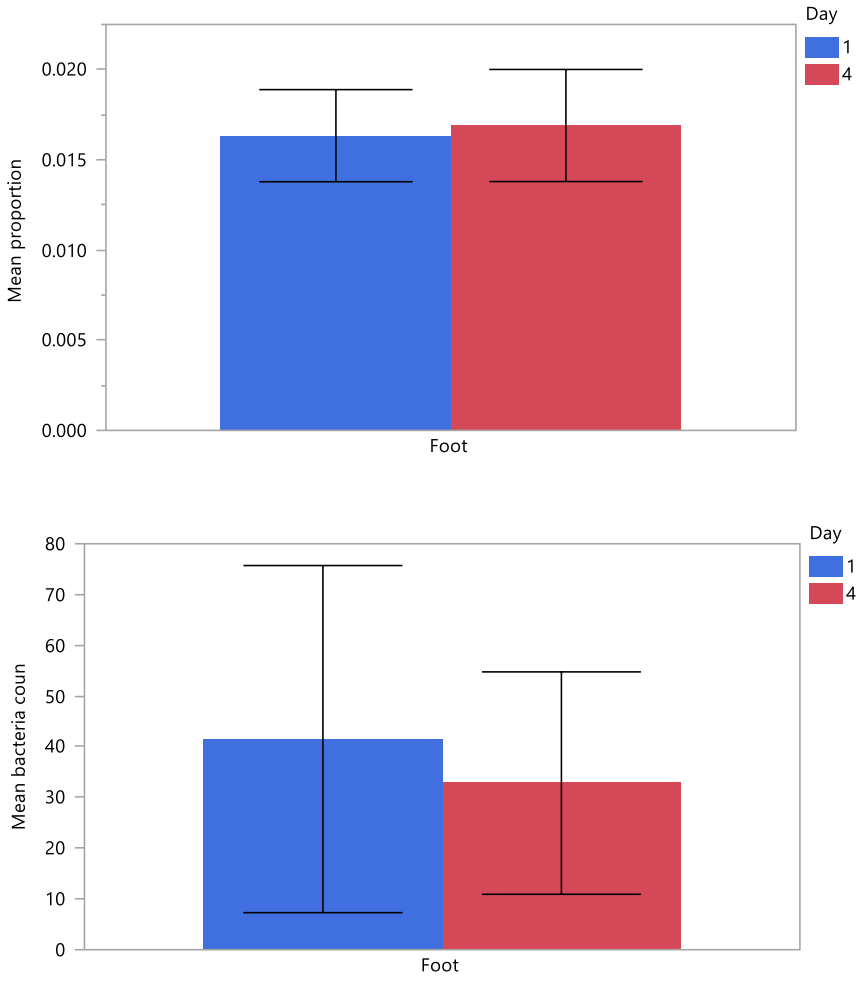


Figure 5-12. Top graph shows the mean amount of compound RI 1601 between foot day 1 and day 4. The bottom graph shows mean *Peptococcus* bacteria counts between foot day 1 and day 4.

Strong negative correlations

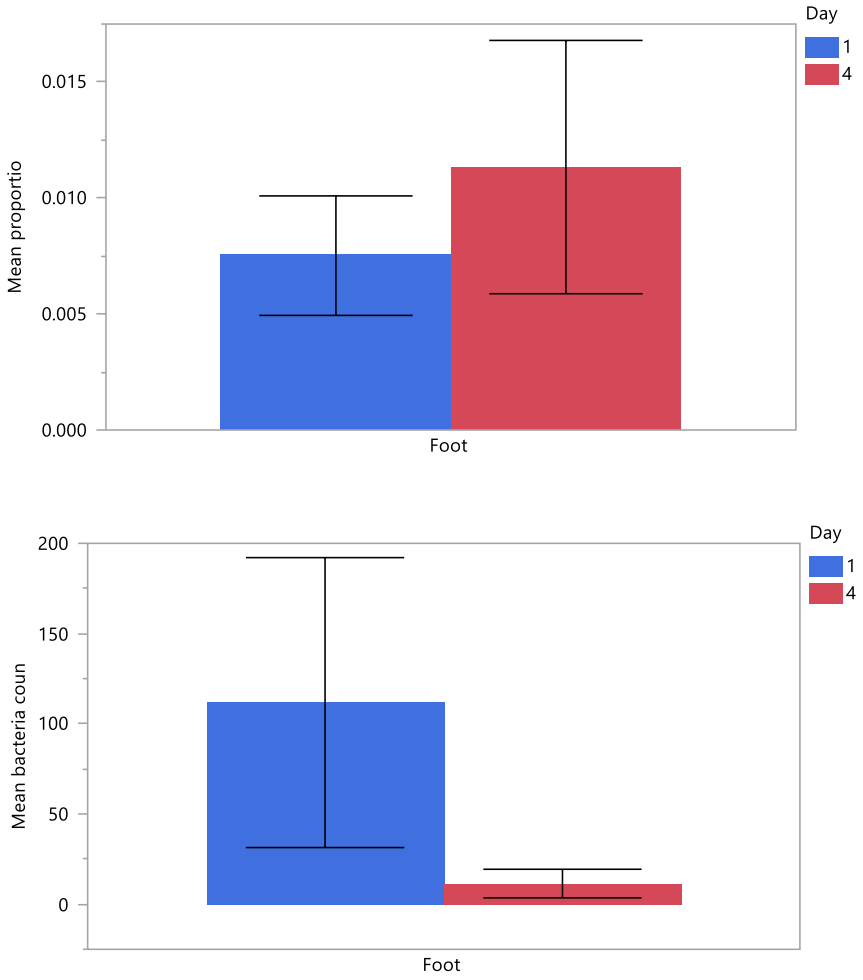


Figure 5-13. Top graph shows the mean amount of compound RI 1134 between foot day 1 and day 4. The bottom graph shows mean *Turicella* bacteria counts between foot day 1 and day 4.

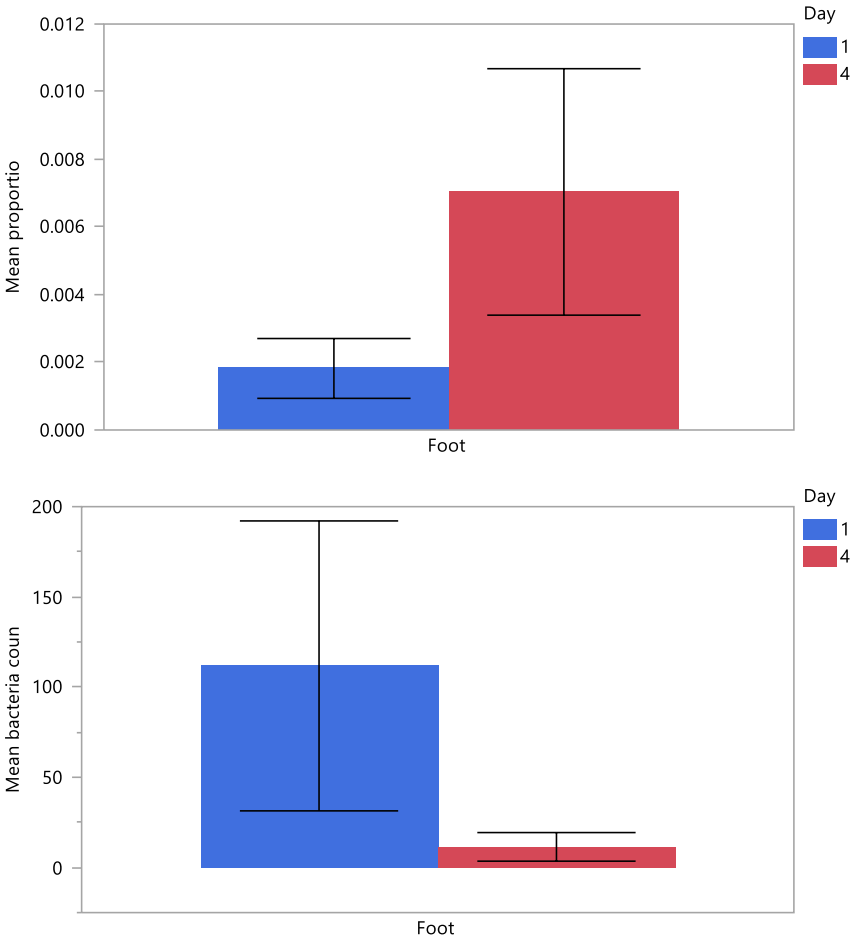


Figure 5-14. Top graph shows the mean amount of compound RI 1148 between foot day 1 and day 4. The bottom graph shows mean *Turicella* bacteria counts between foot day 1 and day 4.

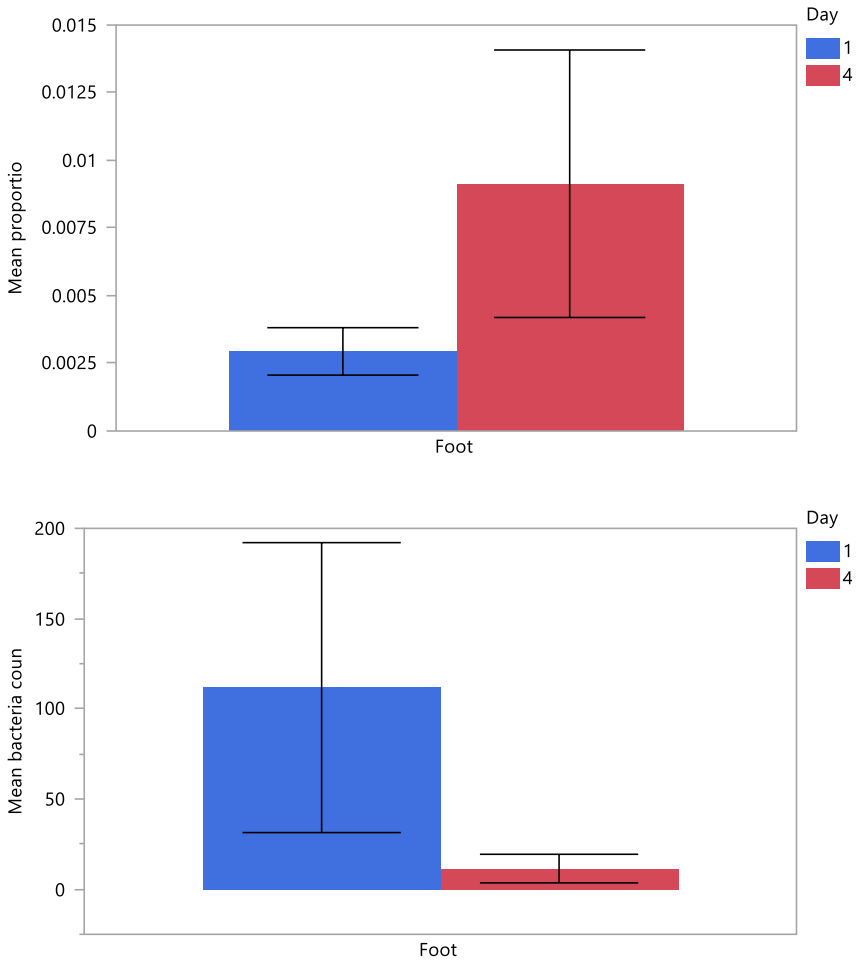


Figure 5-15. Top graph shows the mean amount of compound RI 1232 between foot day 1 and day 4. The bottom graph shows mean *Turicella* bacteria counts between foot day 1 and day 4.

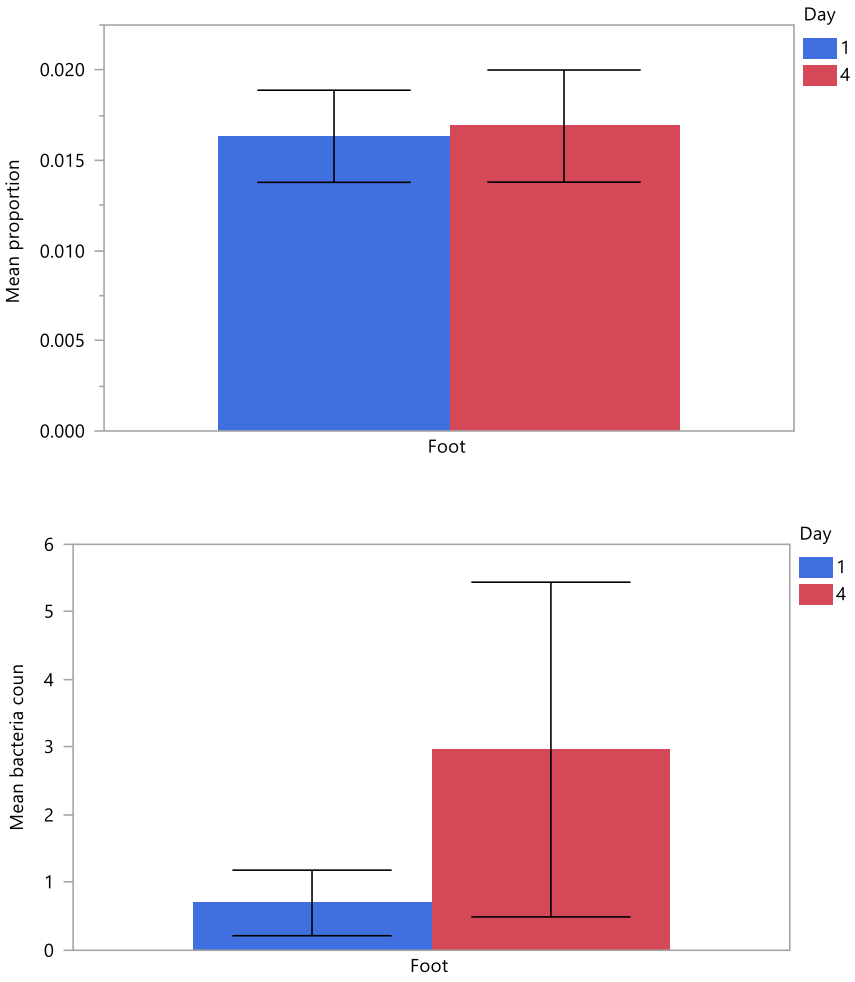


Figure 5-16. Top graph shows the mean amount of compound RI 1494 between foot day 1 and day 4. The bottom graph shows mean *Megasphera* bacteria counts between foot day 1 and day 4.

Negative correlations

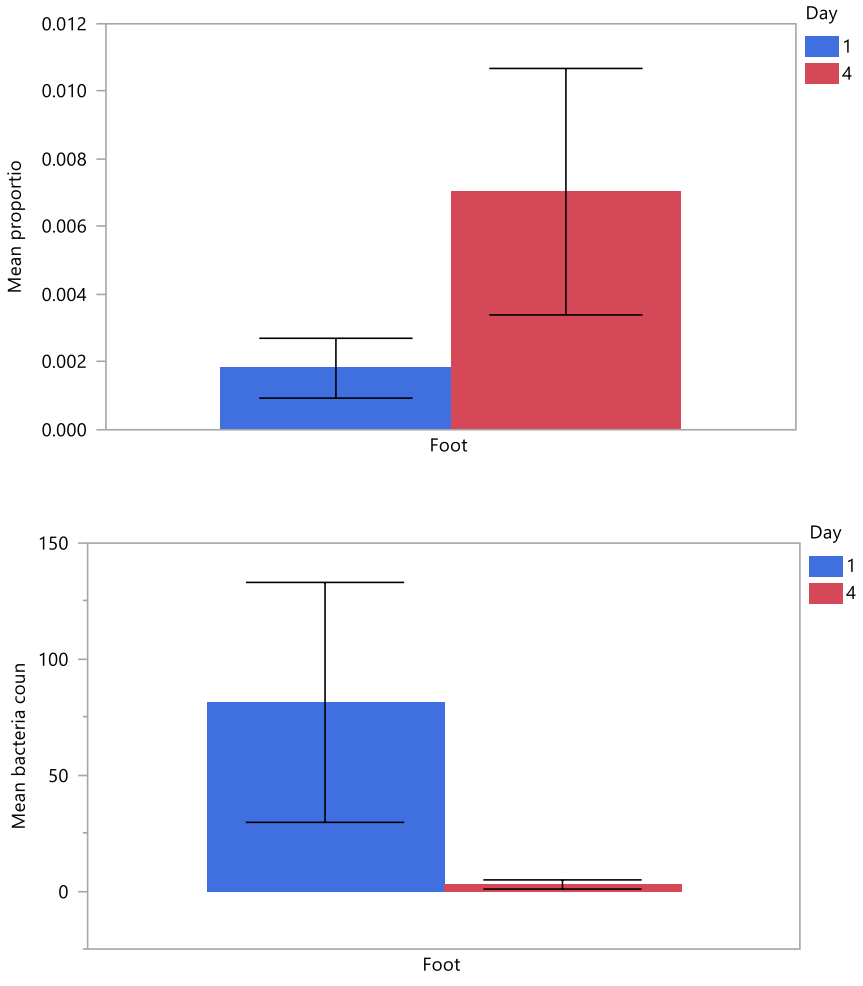


Figure 5-17. Top graph shows the mean amount of compound RI 1148 between foot day 1 and day 4. The bottom graph shows mean *Sphingopxis* bacteria counts between foot day 1 and day 4.

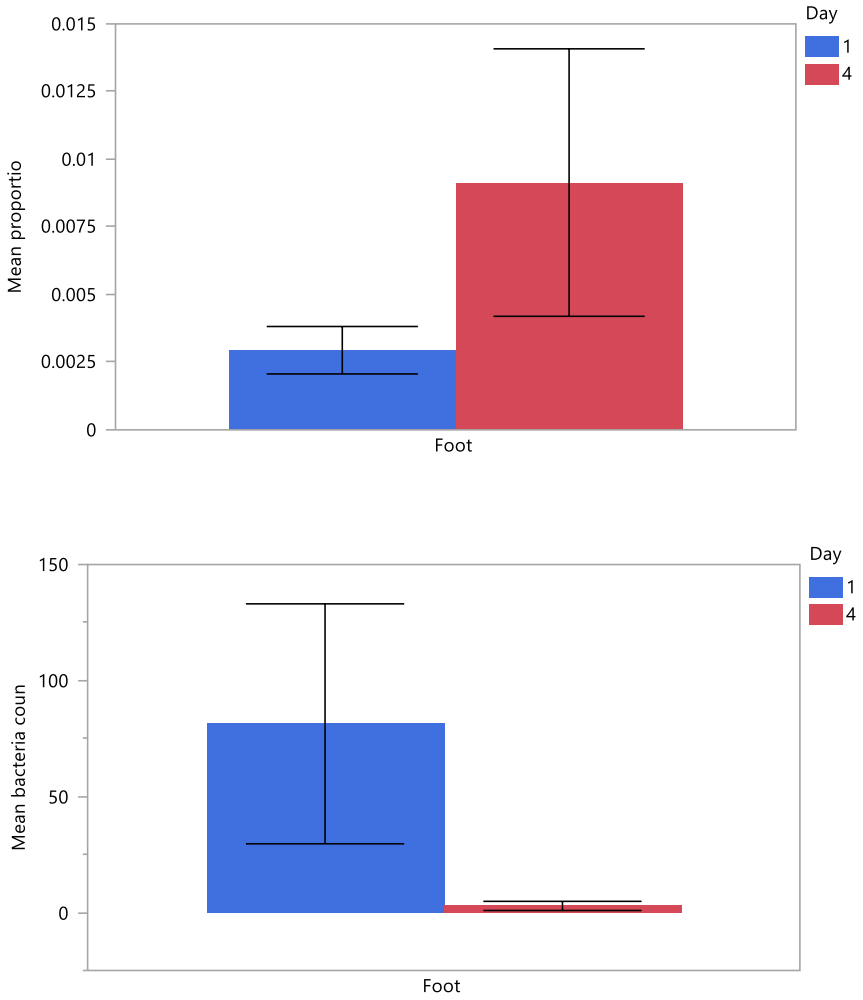


Figure 5-18. Top graph shows the mean amount of compound RI 1232 between foot day 1 and day 4. The bottom graph shows mean *Sphingopxis* bacteria counts between foot day 1 and day 4.

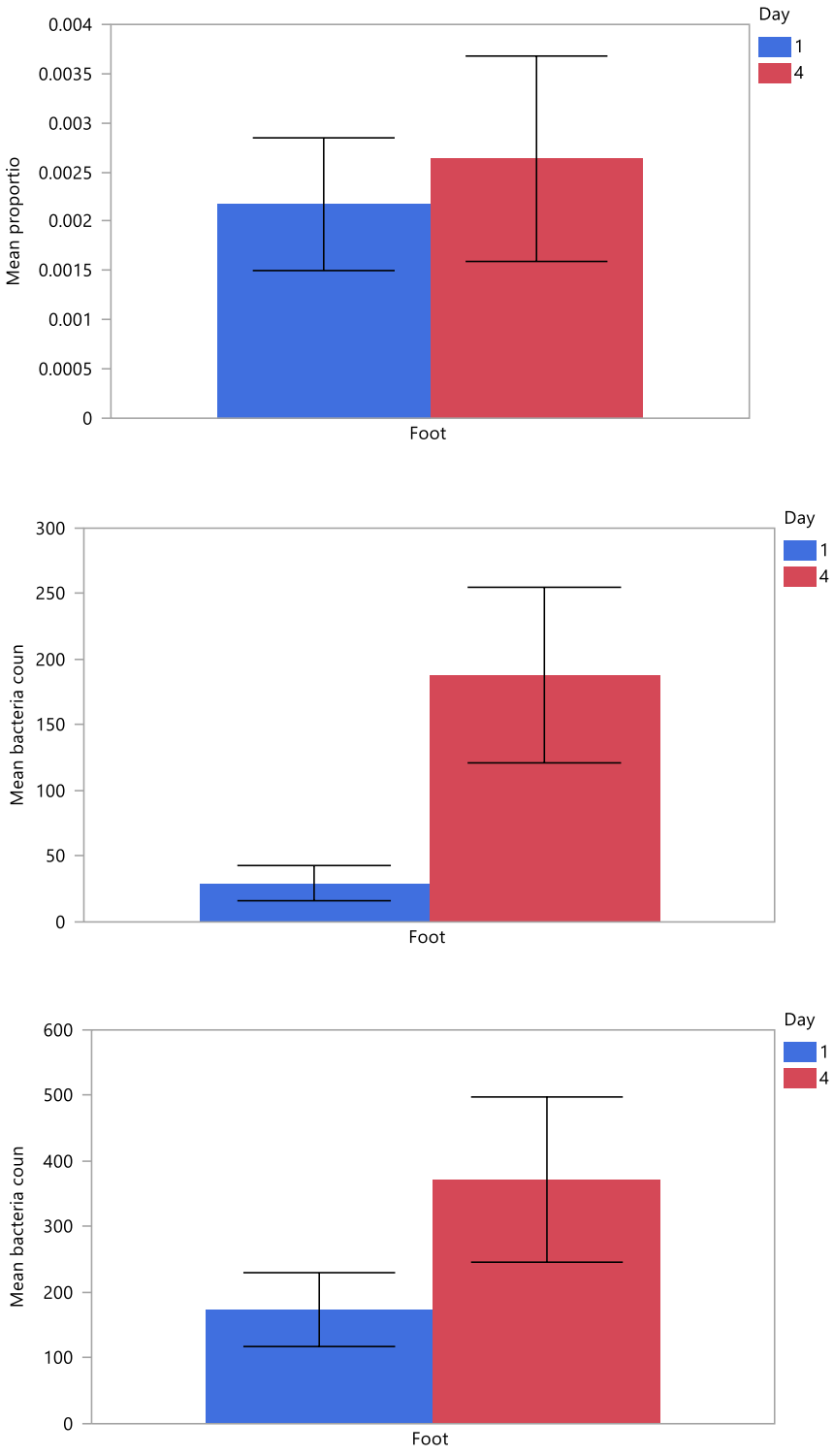


Figure 5-19. Top graph shows the mean amount of compound RI 1348 between foot day 1 and day 4. The middle graph shows mean *Pseudohodoferax* bacteria counts between foot day 1 and day 4. The bottom graph shows mean *Terrabacter* bacteria counts between foot day 1 and day 4.

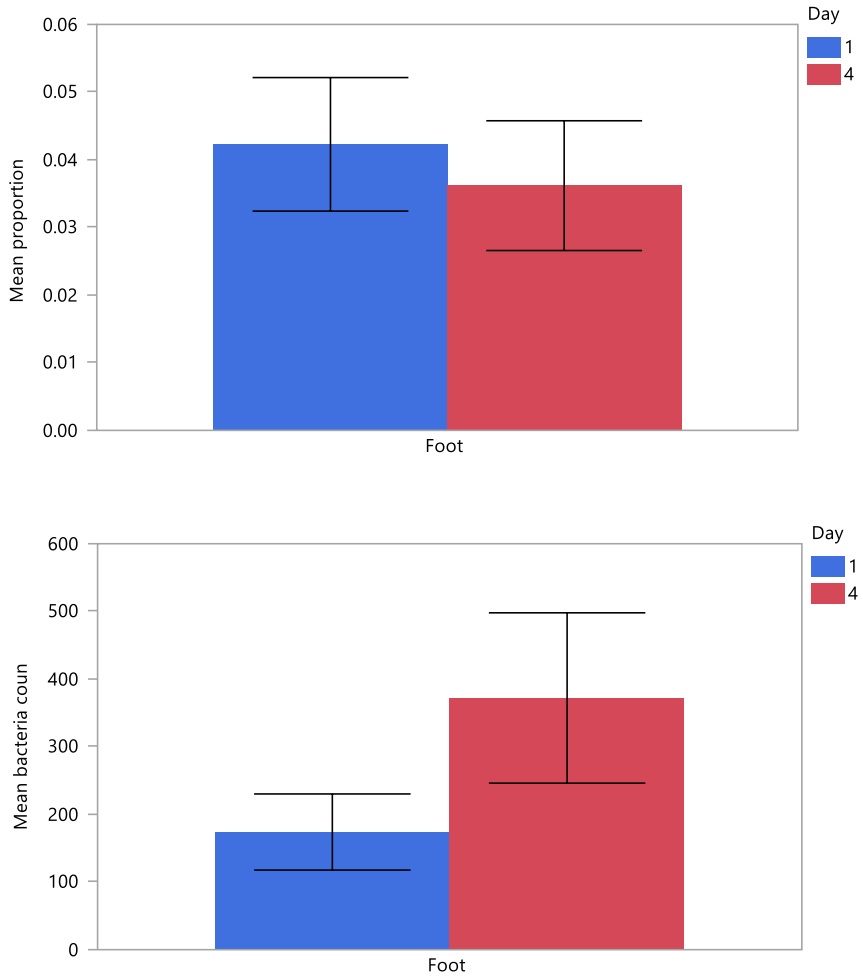


Figure 5-20. Top graph shows the mean amount of compound RI 1812 between foot day 1 and day 4. The bottom graph shows mean *Schlegelella* bacteria counts between foot day 1 and day 4.

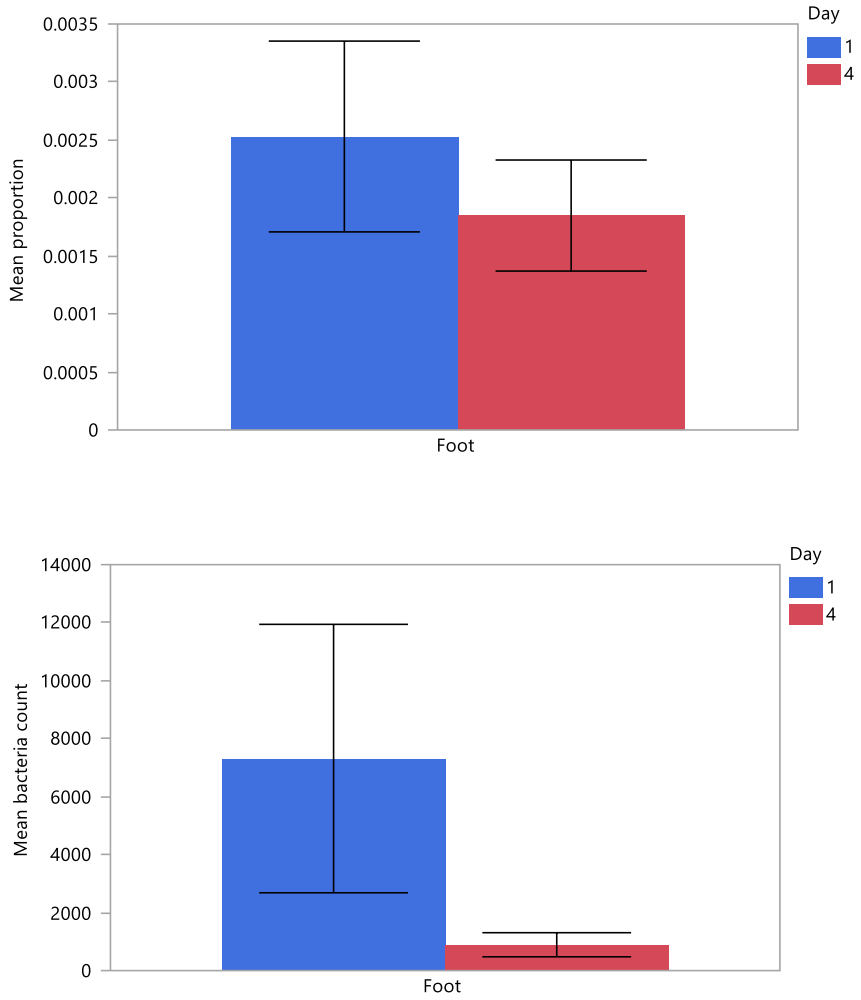


Figure 5-21. Top graph shows the mean amount of compound RI 2010 between foot day 1 and day 4. The bottom graph shows mean *Rhodococcus* bacteria counts between foot day 1 and day 4.

5.7 Discussion

In Chapter 2, the behavioural response of mosquitoes to “fresh” foot odour (day 1) and ‘incubated’ foot odour (day 4) was investigated and revealed that mosquitoes are more attracted to feet after four days of not washing. Not washing is likely to affect the type and amount of bacteria present on the feet. By identifying and quantifying the bacteria present on day 1 and day 4, it could be possible to determine which bacteria are associated with the production or presence of volatiles that attract mosquitoes.

This study found 414 bacteria genera associated with feet. The ten most abundant bacteria were *Staphylococcus, Corynebacterium, Acinetobacter, Brevibacillus, Anaerococcus, Propionibacterium, Paracoccocus, Aerococcus, Pseudomonas* and *Brevibacterium*. These bacteria were consistently the most abundant on day 1 and day 4. However, the relative amount of each bacteria changed between days. On day 1, 61.9% of the total bacteria were *Staphylococcus*, followed by *Corynebacterium* (16.17%), *Acinetobacte*r (3.41%) and *Anaerococcus* (2.1%). On day 4, Staphylococcus decreased to 52.73%, whereas *Corynebacterium* increased to 22.54%, this was followed by *Brevibacillus* (4.17%) and *Acinetobacte*r (2.14%). This is similar to a study by Costello *et al.*, (2009), who collected microbiota from the sole of the foot, and found the most dominant bacteria to be *Staphylococcus* (over 50%), followed by *Corynebacterineae* (less than 20%), followed by other microbiota including *Micrococcinaea* and *Propionibacterineae*. Similarly, Stevens *et al.* (2015) collected samples from the foot plantar and foot dorsal, however, they found that 98.6% of the total skin microbiome of the foot plantar was *Staphylococcus.* The difference in microbiota diversity could be explained due to the stringent criteria used, in the Stevens *et al.* (2015) study, volunteers had their feet washed 24 hours before sampling, and were given supplied socks to wear for the duration of the study. In this study, the volunteers were not provided with socks as they were allowed to wear their own, which could explain why the most abundant bacteria is more similar to the Costello *et al*. (2009) study, furthermore, the shoes and socks were not controlled in the Costelllo *et al.* (2009) study. This could suggest that the difference in bacteria diversity observed between the two studies could be due to a difference in

environment created by shoes, and that the conditions in which bacteria are collected affect the bacteria present. In this study, it was important to collect bacteria that reflected an everyday state for the feet, therefore foot wear was not controlled for.

5.7.1 Differences in bacteria between washed and unwashed feet

In this study, there were 68 genera that significantly increased in abundance between day 1 and day 4, whereas there were only 5 genera that significantly decreased in abundance. This suggests that the longer an individual doesn't wash, a number of bacteria incubate on the skin and therefore increase. There have not been any studies that have investigated the effects of skin microbiota after not washing for a number of days. However, Fierer *et al.* (2008) collected microbiota samples from volunteer's hands every 2 hours over a 6 hour period after washing their hands. The authors found that time since last handwashing had a significant effect on the composition of some bacteria, specifically *Proprionibacteria, Neisseriales, Burkholderiales* and *Pasteurellaceae* taxa were relatively more abundant with time. These bacteria were not found in this study, which is not surprising as hand microbiota differs from feet microbiota.

Furthermore, 107 bacteria had either no value or too few values on day 1 to compare with day 4. Similarly, 6 bacteria had either no value or too few values on day 4 to compare with day 1. In many cases, these bacteria were only present in a few individuals. This could suggest that they are not a part of the generic skin microbiota and could be contamination from clothing and, therefore, they could be transient microbiota. However, it could also suggest that they are part of the normal skin microbiota, and that current modern washing regimes frequently remove and modify resident microbiota.

Most of the bacteria present in this study did not significantly change in abundance between day 1 and day 4, this was observed particularly with the most abundant bacteria including *Staphylococcus* and C*orynebacteirum*. However, this was observed at genus level and further differences may occur at species level. Grice *et al*. (2009) investigated the temporal variation of skin microbiota by collecting skin samples with follow up samples at 4 and 6 months after the initial visit. They found that skin microbiota are relatively stable over time. However, volunteers were allowed to

continue their normal washing regimen, which means their results cannot be directly compared with this study. They also found that more occluded areas such as the external ear canal or nasal cavity are more stable than other areas such as the buttock or the foot plantar. This suggests that there are differences between different parts of the body, which was also seen in this study and will be discussed further in Chapter 6.

The bacteria that did change between day 1 and day 4 may contribute to the differences in odorants measured and the enhanced mosquito attraction at day 4. Both attractant and repellent odorant compounds have been observed on skin previously but their origin is unknown; For example natural repellents have been shown to decrease attractiveness of individuals to mosquitoes (Logan *et al.* 2008). To confirm this, an analysis to determine the correlation between bacteria and the compounds was done.

5.7.2 Correlation with bacteria and compounds

The correlation between bacteria and compounds was predominantly positive, where an increase of bacteria was associated with an increase in the amount of compound, or a decrease in bacteria was associated with the decrease in compound. This suggests that the bacteria could be responsible for the production of compounds they are correlated to. Additionally, in most cases, one compound was correlated to more than one genera of bacteria. For example, peak number 5 (ethyl-cyclohexane; RI 841), which increased on day 4, was correlated with *Pascolarctobacterium, Tyzerella* and *Sutterella,* which all also increased on day 4. It is likely that certain compounds may be produced by more than one type of bacteria and that may be the case here. Furthermore, some bacteria genera were associated with more than one compound. For example, *Turicella* was negatively correlated with compounds peak number 27 (2-nonanal; RI 1130), RI 1148 and peak number 33 (unidentified; RI 1232). This suggests that a single bacteria may produce more than one compound. Indeed, Kai *et al.* (2009) reported that some bacteria strains can produce up to 60 volatile compounds.

The correlation analysis performed in this study had significant power to detect associations between bacteria and compounds. However, when analysing differences between day 1 and 4, the power was significantly reduced, meaning only 3 bacteria genera (*Sutterella, Nakamurella* and *Oryzihumus*) were found to be significantly

different between day 1 and day 4. Data for both bacteria and volatiles was varied as many samples had few values, therefore the comparison between day 1 and day 4 had little power.

5.7.3 EAG active compounds

The results from this chapter demonstrate the compounds and bacteria that are correlated with each other, however, not all the compounds identified are likely to act as kairomones for mosquitoes. Chapter 3 revealed the compounds that *An. stephensi* responded to electrophysiologically. The following compounds were found to be correlated with bacteria and, importantly, they also elicited an electrophysiological response in *An. stephensi*: peak number 5 (Ethyl-cyclohexane; RI 841), peak number 27 (2-nonanal; RI 1130), peak number 7 (unidentified; RI 1232), Peak number 28 (menthol; RI 1172), and peaks numbers 46 and 48 (both unidentified RI 1712; RI 1812).

Positive correlations were observed with ethyl-cyclohexane (RI 849) and *Phascolarctobacterium, Tyzzerella* and *Sutterella* bacteria. Both the bacteria and the compound increased from day 1 to day 4. Interestingly, *Phascolarctobacterium* and *Tyzzerella* were only present on day 4. Surprisingly the EAG response to this compound was only found on day 1, and not on day 4 despite there being an increase in volatile on that day (Chapter 3). This could be due to the compound being present in an amount that was above a threshold response. Or this could also be due to a fault with the EAG system. These bacteria are not commonly associated with the skin. *Phascolarctobacterim, Sutterella* and *Tyzzerella* have been isolated or are abundant in the human gastrointestinal tract (GIT), but have not been associated with human skin (Wu *et al.* 2017; Watanabe *et al.* 2012; Yutin & Galperin 2013; Hiippala *et al.* 2016).

Peak number 33 (unidentified 7; RI 1232) was positively correlated with *Oryzihumus, Parabacteroides, Megasphaera* and negatively correlated with *Sphingopyxis* and *Turicella*. This suggests that the compound could be produced by the bacteria it is positively correlated to, and these bacteria may directly compete with the bacteria that it is negatively correlated. *Parabacteroides, Megasphaera* and *Turicella* have human origins, however they have been associated with human faeces, human blood cultures, vaginal flora and an abscess located in the ear (Sakamoto *et al.* 2007; Zozaya-Hinchliffe *et al.* 2008; Reynolds *et al.* 2001). Whereas *Sphingopyxis* and *Oryzihumus*

have been isolated from the environment, including seawater, freshwater and soil (Kim *et al.* 2008; Kageyama *et al.* 2005).

Peak number 27 (2-nonenal; RI 1130) was negatively correlated with *Turicella*, therefore, the decrease of *Turicella* between day 1 and day 4 was associated with the increase of 2-nonenal. This could suggest that this is not responsible for the production of this compound, but it may affect the presence of other bacteria and therefore the amount of the compound produced, furthermore, *Turicella* was shown to be negatively correlated with other compounds. Therefore, if it is there in greater amounts, other bacteria that produce this compound may be negatively affected perhaps through competition for resources. It may also be that the compound is not produced by the bacteria itself, but the presence of the bacteria prevents its release from the skin surface, perhaps through metabolism of the compound by the bacteria itself. Haze *et al.* (2001) associated 2-nonenal with older people, it is produced by when unsaturated fatty acids degrade and skin surface lipids oxidatively decompose. It is unlikely that the presence of this compound is due to older volunteers as the majority of volunteers were aged between 18-25.

Peak number 28 (Menthol; RI 1172) was positively correlated with *Paludibacter, Ralstonia* and *Tuberibaccilus*, and both the bacteria and compound increased from day 1 to day 4. *Tuberibacillus* and *Ralstonia* were only present in day 4, which could suggest that these bacteria independently produce menthol. To our knowledge, menthol has not been associated with skin microbiota in previous studies. However, the presence of menthol could also be due to the residual fragrances from fabric washing. Because menthol was EAG active with most samples, further research is needed to investigate this. To our knowledge, no studies have tested menthol behaviourally with mosquitoes.

The bacteria that were correlated with the volatile organic compounds in this study were not the most abundant in the samples. Bacteria such as *Staphylococcus* or *Corynebacterium* were not correlated with any compounds that changed in abundance between day 1 and day 4. This could mean that these bacteria are not involved in making someone more or less attractive to mosquitoes, due to not washing. However, it is possible that there are changes seen at species level, and not genus. Previous

studies have investigated the attractiveness *of Staphylococcus* to mosquitoes. Verhulst *et al.* (2009) cultured *Staphylococcus epidermidis* on agar plates and have found it to be attractive to *An. gambiae*, which suggests *Staphylococcus* produces attractants. The agar plates in that study were entrained and the authors detected various compounds (1-butanol, 2,3-butanedione, 2-methyl-1-butanol, 2-methylbutanal, 2-methylbutanoic acid, 3-hydroxy-2-butanone, 3-methyl-1-butanol, 3-methylbutanal 3-methylbutanoic acid and Benzeneethanol), however, the same compounds were not tentatively identified with GC-MS based on the results from chapter 3. It is possible that they were present in the samples but not identified. Similarly, (Frei *et al.* 2017) tested the response of sweat individually incubated with *Staphylococcus epidermidis, Staphylococcus haemolyticus* and *Corynebacterium jeikeium* in a dual-choice olfactometer and found the incubated sweat was attractive to *An. gambiae* over sterile sweat, however *An. stephensi* may respond differently. The bacteria were selected as they produce specific compounds ((*R*)/(*S*)-3-hydroxy-3-methylhexanoic acid and (*R*/*S*)-3-methyl-3-sulfanylhexan-1-ol), however, when the compounds were tested on their own they were not attractive. This suggests that the bacteria tested produce complex volatile profiles, where more than one compound is responsible for the attraction to mosquitoes. It may also suggest that although these bacteria may be associated with compounds that are responsible for the general attraction of mosquitoes to human beings, they are not responsible for changes in attractiveness.

In the present study, only males were used. This could have impacted the results as previous studies have shown a difference in microbiota between men and women, as male skin tends to be more acidic which may affect the bacteria present (Fierer *et al.* 2008). Interestingly, Frei *et al.* (2017) found that male sweat incubated with bacteria was more attractive than female sweat incubated with the same bacteria.

5.8 Conclusion

The correlation revealed associations between bacteria and volatiles, and some of these volatiles were found to be EAG active in chapter 3: peak number5 (ethyl-cyclohexane; RI 841), peak number 27 (2-nonanal; RI 1130), and peak number 33 (unidentified; RI 1232), peak number 28 (menthol; RI 1172), peak number 46 (unidentified; RI 1711) and peak number 48 (unidentified, RI 1817). These compounds were associated with *Phascolarctobacterium, Tyzzerella, Sutterella, Turicella, Schlegelella, Oryzihumus, Parabacteroides, Megasphaera, Shingopxis, Paludibacter, Ralstonia, Tuberibacillus* and *Peptococcus*.

From these results, it is possible that these correlations show a casual relationship between bacteria and volatiles, however this is only the first step in determining the relationship between specific compounds and bacteria. Furthermore, some of the compounds that were correlated were also found to be EAG active (chapter 3), which could suggest that these bacteria may be responsible for the production of mosquito important compounds. In future studies, these bacteria could be targeted as a novel intervention to reduce mosquito attractiveness. Furthermore, these compounds may act as attractants or repellents, however, more research involving behavioural experiments are needed to determine this. The results show that the associations between volatiles and bacteria is complex and it is likely that there is a lot of interaction between various compounds and bacteria, as well as a highly complex interaction between compound concentrations and ratios. Future research that focuses to prove whether the bacteria have a role in the production of the compounds is needed. Therefore, further research that investigates the effect of specific bacteria, and compounds, found in this study on mosquito attractiveness.

CHAPTER 6

6 Associations of bacteria and compounds for axilla, upper back and forearm

6.1 Introduction

Chapter 5 focused on the correlation between bacteria and volatiles for feet only, based on the differential behavioural observed to foot odour in Chapter 2. This chapter will focus on the correlation between bacteria and volatiles on the axilla, back and forearm. Although mosquitoes did not behave differently to odour from these areas, there was still a significant response to the odours and, therefore, an investigation is still warranted.

In the human body, the axilla has one of the greatest densities of glands, including sebaceous, apocrine and eccrine glands. As a result, the axilla also has a high microbiota density (Smallegange *et al.* 2011). The axillary microflora has been characterised and is dominated with *Staphylococcu*s and *Corynebacterium, Micrococci* and *Propionibacteria* (Taylor *et al.* 2003). Subjects that have higher levels of axillary malodour have a greater presence of aerobic coryneforms as well as *Micrococcaceae* and *Propionibacteria* (Leyden *et al.* 1981) which appear to be associated with a pungent odour.

Fewer studies have investigated bacteria and volatiles found on the upper back and forearms, presumably due to their lack of malodour. The upper back has a high density of sebaceous glands, few eccrine and no apocrine glands. Sites with sebaceous glands are less diverse than other sites, the upper back is dominated by *Propionibacteriaceae*, however, other bacteria such as *Betaproteobacteria* and *Flavobacteriales* are also present (Smallegange *et al.* 2011; Grice *et al.* 2009). The forearm has few sebaceous and eccrine glands, and no apocrine glands (Smallegange *et al.* 2011). Grice *et al.*, (2009) collected skin microbiota from 20 different skin sites and found that the forearm was the site with the greatest number of Operational Taxonomic Unit (OTUs), the most abundant bacteria on the forearm were *Proteobacteria* and *Bacteroidetes*.

6.1.1 Compounds found on axilla, forearm and back

With axilla, most of the research that investigates the volatiles produced focusses on malodour as the cosmetic industry aims to sell cosmetic products that reduce malodour. Malodour is caused in part by short (C2-C5) and medium (C6-C11) chain volatile fatty acids, 16-androstene steroids and thioalcohols that are produced via various metabolic routes, it is believed these originate from the apocrine gland (James *et al*. 2013). Specifically, saturated and unsaturated branched acids with (E)-3-methyl-2-hexenoic acid (3M2H) and 3-hydroxylhexanoic acid (HMHA; 6) have been found to be a major component of malodour in human beings and they have been linked with axilla isolates of *Corynebacteria* (Zeng *et al.* 1996; Natsch *et al.* 2005). Penn *et al.* (2007) collected axillary sweat from 197 adults over 10 weeks using Twister polydimethylsiloxane (PDMS) coated stir bars and found 373 compounds that were consistent over time. Furthermore, they identified marker compounds characteristic of individuals including 2-phenylethanol, 1-tridecanol, undecanal, lilial and diphenyl ether, which have all been identified in previous studies. Gender markers were also identified including pentadecanoic acid, hexadecanoic acid, heptadecanoic acid, methylhexadecanoic acid and docosane, which have also been identified in previous studies (Zeng *et al.* 1991). However, the compounds that are associated with malodour are not necessarily the compounds that play a role in mosquito attractiveness

Curran *et al.* (2005) collected volatiles from axillary sweat samples using solid phase microextraction and analysed by GC-MS. They found a wide range of volatiles including organic fatty acids, ketones, aldehydes, esters and alcohols. Some of the compounds they detected included: (E)-2-nonenal, benzaldehyde, decanal, hexanal, nonanal, octanal, undecanal, dodecanoic acid, tetradecanoic acid, 6,10-dimethyl-5-,9-undecandien-2-one, 6-methyl-5-hepten-2-one, 2-furanmethanol, benzyl alcohol, phenol, dodecane, heptadecane, hexadecane, naphthalene, nonane, tetradecane, toluene, undecane, 7-hexadecenoic acid-methyl ester, hexadecanoic acid-methyl ester, hexanedioic acid-dimethyl ester, nonanoic acid-methyl ester, tridecanoic acid-methyl ester and pyridine, some of which were also detected by Penn *et al.* (2007).

Gallagher *et al.* (2008) investigated the volatile profile of the forearm and the upper back with 25 volunteers using SPME extraction and GC-MS. Fewer than 100 compounds were identified, and both body sites had similar types of compounds. However, there were some differences. Nine compounds were observed only in upper back samples, and there were significant differences in the levels of dimethylsulphone, hexyl salicylate and α-hexyl cinnamaldehyde between the upper back and the forearm. These differences could be due to a difference in glands present at the two sites, or due to cosmetics. Furthermore, three compounds were found significantly in higher quantities in older volunteers (dimethylsulphone, benziothiazole and nonanal). Although the authors remarked that these differences could be due to cosmetic use, it could be due to genuine differences in compounds produced at different body sites.

6.1.2 Associations of bacteria and volatile organic compounds

One study analysed microbiota and volatiles from the axilla of 200 volunteers. Microbiota was collected using denaturing gradient gel electrophoresis and volatiles were collected using a stir bar sampling device and GC-MS. The authors found no correlation between volatiles and bacteria overall. However, when the authors did the analysis by grouping individuals based on four different behaviours (axilla washing between specific hours of the day; deodorant usage less than 48 hours before sampling, t shirts had to be worn before sampling and a suggested soap had to be used for axilla washing) there was a significant correlation. The authors therefore concluded that the correlation observed was due to behavioural patterns. However, the correlation was done overall and no specific bacteria or compounds were correlated with one another (Xu *et al.* 2007). To our knowledge, there are no studies that have investigated the associations of bacteria and volatiles for the upper back and the forearm. Furthermore, there are no studies that have investigated these associations in relation to mosquito attractiveness.

6.2 Aims and objectives

The first aim of this study was to investigate the bacteria and volatiles found on the axilla, forearm and upper back of volunteers and to determine whether there is an association between the bacteria and odours. The second aim was to determine whether there were any differences between day 1 (washed participants) and day 4 (unwashed participants).

Objectives:

1. To collect bacteria samples using swabs and buffer from the axilla, forearm and back of volunteers and, using 16S rRNA analysis, identify, and quantify, the bacteria species present
2. To collect odour samples using air entrainment, from the axilla, forearm and back of volunteers and identify, and, using gas chromatography (GC) to quantify the volatile compounds present.
3. To determine whether there are qualitative or quantitative differences in bacteria and/or volatiles between day 1 and day 4.
4. To perform statistical analysis to determine whether there is an association between bacteria presence/absence or quantity and volatiles between day 1 and day 4.

6.3 Methods

The methods are described fully in Chapter 5, page 109, section 5.3. Volatiles collected from different body sites were analysed by GC. Bacteria were collected from the same sites and were analysed using 16S rRNA sequencing.

6.4 Results

6.4.1 Bacteria found on axilla, arm and back

The analysis from the 16S rRNA sequencing revealed 414 different bacteria collected from the volunteers across all body sites. Table 6-1 shows the mean bacteria counts for axilla, forearm and back and the different visits. The bacteria *Staphylococcus, Corynebacterium, Acinetobacter, Anaerococcus, Paracoccus, Propionibacterium and Aerococcus* dominated the samples and were found in all volunteers. Many other bacteria were found but these were in lower quantities and were not consistently found in all volunteers.

6.4.2 Volatiles found on axilla, arm and back

The GC analysis revealed the presence of 117 different compounds across all bodysites. Table 6-2 shows the mean proportion for each compound, for the different body sites, and the different visits. The major compounds found were RI 1583 RI 1657, RI 1812, RI 1489 for axilla and forearm, and RI 1015, RI 1023 (indene, 3-methylphenylacetone or p-ethylnytoluene), RI 1583 and RI 1172 (menthol) for upper back. These dominated the samples and were found in all volunteers. Many other volatiles were found but these were in lower quantities and were not consistently found in all volunteers.

6.4.3 Comparison of bacteria between day 1 and day 4

There was a significant increase between day 1 and day 4 for: *Nesterenkonia* (p=0.005), *Anaerococcus* (0.029), *Pelomonas* (0.038) on the forearm, and *Helcococcus* (p=0.040) on the upper back. There was a significant decrease between day 1 and day 4 for *Moraxella* (p=0.026) on the axilla, and *Jeotgalicocccus* (p=0.005), *Solirubrobacter*

(p=0.006), *Brachybacterium* (p=0.010), *Moraxella* (p=0.013), *Novosphingobium* (p=0.016), *Shewanella* (p=0.025), *Dorea* (p=0.032), *Paracoccus* (p=0.034) on the forearm.

6.4.4 Comparison of volatiles between day 1 and day 4:

There was a significant increase between day 1 and day 4 for the compounds RI 962 (p=0.007), RI 1867 (p=0.01), RI 1667 (p=0.024), RI 977 (p=0.026), KI 1120 (p=0.048) on the axilla, and RI 1568 (p=0.046) on the forearm. There was a significant decrease between day 1 and day 4 for the compounds RI 1618 (p=0.002), RI 1562 (p=0.037) on the axilla; RI 1404 (p=0.048) on the forearm; RI 1470 (p=0.004), RI 1618 (p=0.027), RI 1591 (p=0.028) and RI 1389 (p=0.039) on the upper back.

6.4.5 Correlation between bacteria and volatiles for axilla

The correlation between compounds and bacteria for axilla can be seen in Table 6-3.

6.4.6 Strong positive correlation for axilla

Compound RI 1348 was correlated with *Thermomicrobium* (0.785); compound RI 1494 was correlated with *Kineosporia* (0.720), *Ramilbacter* (0.725), and *Rickettsiella* (0.726). Compound RI 1526 was correlated with *Thermomicrobium* (0.837); Compound 1790 was correlated with *Modestobacter* (0.802), *Pedomicrobium* (0.825) and Solobacterium (0.799).

6.4.7 Positive correlation for axilla:

Compound RI 1172 was correlated with *Legionella*-like (0.695) and *Nitrospira* (0.653); compound RI 1200 was correlated with bacteria *Kineosporia* (0.654) and *Legionella*-like (0.640); compound RI 1287 was correlated with *Thermomicrobium* (0.673); compound RI 1301 was correlated with *Paracraurococcus* (0.69), *Pedomicrobium* (0.630) and *Photobacterium* (0.656); compound RI 1501 was correlated with *Thermomicrobium* (0.637); compound RI 1526 was correlated with *Rhodoplanes* (0.64); compound RI

1790 was correlated with *Helcobacillus* (0.651), *Oribacterium* (0.685), *Paracraurococcus* (0.685), *Photobacterium* (0.661), *Psychrobacter* 90.689) and *Rathayibacter* (0.653); compound RI 1935 was correlated with *Sporichthya* (0.659).

6.4.8 Strong negative correlation for axilla

Compound RI 1287 was correlated with *Helcobacillus* (-0.702), *Modestobacter* (-0.753), *Pedomicrobium* (-0.751) and *Polynucleobacter* (-0.816); compound RI 1300 was correlated with *Modestobacter* (-0.802), *Pedomicrobium* (-0.821) and *Solobacterium* (-0.755); compound RI 1348 was correlated with *Helcobacillus* (-0.714), *Modestobacter* (-0.919), *Paracraurococcus* (-0.810) and *Pedomicrobium* (-0.922); compound RI 1404 was correlated with *Helcobacillus* (-0.716) and *Pedomicrobium* (-0.704); compound RI 1451 was correlated with *Helcococcus* (-0.781) and *Ketogulonicigenium* (-0.727); compound RI 1501 was correlated with *Helcobacillus* (-0.712) and *Photobacterium* (-0.738); compound RI 1526 was correlated with *Helcobacillus* (-0.76), *Modestobacter* (-0.956), *Oribacterium* (-0.768), *Paracraurococcus* (-0.872), *Pedomicrobium* (-0.963) and *Solobacterium* (-0.751).

6.4.9 Negative correlation for axilla

Compound RI 1022 was correlated with *Nitrospira* (-0.642), *Rheinheimera* (-0.670), *Ruminococcus* (-0.645) and *Sutterella* (-0.685); compound RI 1287 was correlated with *Paracraurococcus* (-0.639) and *Photobacterium* (-0.659); compound RI 1300 was correlated with *Helcobacillus* (-0.639), *Oribacterium* (-0.631), *Paracraurococcus* (-0.664) and *Thermomicrobium* (-0.662); compound RI 1348 was correlated with *Helcococcus* (-0.695), *Iamia* (-0.664), *Photobacterium* (-0.644) and *Rathayibacter* (0.692), compound RI 1404 was correlated with *Hymenobacter* (-0.679), *Iamia* (-0.679), *Lautropia* (-0.660), *Modestobacter* (-0.645), *Nocardioides* (-0.655), *Psychrobacter* (-0.635), *Rathayibacter* (-0.635), *Rhodobacter* (-0.672) and *Solobacterium* (-0.638); compound RI 1451 was correlated with *Rhodocytophaga* (-0.682); compound RI 1501 was correlated with *Lautropia* (-0.674), *Modestobacter* (-0.696), *Oribacterium* (-0.650), *Paracraurococcus* (-0.635), *Pedomicrobium* (-0.696) and *Psychrobacter* (-0.650);

compound RI 1526 was correlated with *Helcococcus* (-0.686), *Photobacterium* (-0.679), *Polynucleobacter* (-0.684), *Rathayibacter* (-0.682); compound RI 1790 was correlated with *Thermomicrobium* (-0.677).

6.4.10 Correlation between bacteria and volatiles for forearm

The correlation between compounds and bacteria for forearm can be seen in **Error! Reference source not found.**.

6.4.10.1 Strong positive correlation for forearm

Compound RI 1200 was correlated with *Nocardia* (0.737), *Rhodanobacter* (0.743) and *Saccharopolyspora* (0.907); compound RI 1336 was correlated with *Schlegelella* (0.7830, compound RI 1494 was correlated with *Nocardia* (0.789), *Rhodanobacte*r (0.823) and *Saccharopolyspora* (0.933).

6.4.10.2 Positive correlation for forearm

Compound RI 1180 was correlated with *Schlegelella* (0.659); compound RI 1354 was correlated with *Schlegelella* (0.664), compound RI 1494 was correlated with *Kaistibacter* (0.689) and *Sporocytophaga* (0.675); compound RI 1830 was correlated with *Nocardia* (0.640) and *Rhodanobacter* (0.659).

6.4.10.3 Strong negative correlation for forearm

Compound RI 1180 was correlated with *Nocardia* (-0.718), *Rhodanobacter* (-0.752) and *Saccharopolyspora* (-0.847); compound RI 1200 was correlated with *Schlegelella* (-0.711); compound RI 1232 was correlated with *Kaistibacter* (-0.745), *Nocardia* (-0.788), *Rhodanobacter* (-0.836), *Saccharopolyspora* (-0.934), *Sporocytophaga* (-0.717); compound RI 1354 was correlated with *Saccharopolyspora* (-0.827), compound 1381 was correlated with *Selenomonas* (-0.725); compound RI 1494 was correlated with *Schlegelella* (-0.718).

6.4.10.4 Negative correlation for forearm

Compound RI 851 was correlated with *Schlegelella* (-0.689); compound RI 1180 was correlated with *Isotericola* (-0.669); compound RI 1232 was correlated with *Solobacterium* (-0.657); compound RI 1553 was correlated with *Sphingobium* (-0.697).

6.4.11 Correlation betwe en bacteria and volatile for upper back

The correlation between compounds and bacteria for upper back can be seen in table 6-5.

6.4.11.1 Positive correlations

Compound RI 1200 was correlated with *Thermus* (0.75) and *Nitrospira* (0.616); compound RI 1494 was correlated with *Thermu*s (0.652).

6.4.11.2 Negative correlations

Selomonas was correlated with compound RI 1232 (0.754), compound RI 1331 (-0.866) and compound RI 1381 (-0.810); *Treponema* was correlated with compound RI 1232 (-0.647) and compound RI 1381(-0.65).

Table 6-1. Mean amount of bacteria collected from the axilla, arm and upper back (P-values indicate significant differences between day 1 and day 4). Bacteria in bold had significant changes between day 1 and day 4.

Bacteria	**Axilla**			**Forearm**			**Upper back**		
	Day 1	**Day 4**	**P value**	**Day 1**	**Day 4**	**P value**	**Day 1**	**Day 4**	**P value**
Abiotrophia	0.00	9.83	n/a	0.17	0.00	n/a	0.00	0.00	n/a
Acetivibrio	0.00	57.23	n/a	0.00	0.00	n/a	0.00	0.00	n/a
Acetobacter	12.27	16.60	0.54	0.03	19.83	0.19	1.03	1.10	0.92
Achromobacter	6.60	1.83	0.78	4.90	12.60	0.38	12.27	3.87	0.72
Acidipila	0.50	0.00	n/a	47.60	0.00	n/a	0.03	0.00	n/a
Acidisphaera	67.27	126.03	0.76	47.10	7.07	0.03	741.63	130.27	0.14
Aciditerrimonas	4.47	7.90	0.77	3.33	71.97	0.24	59.63	12.97	0.81
Acidobacterium	0.03	16.63	n/a	0.97	0.00	n/a	0.90	0.03	0.44
Acidothermus	0.27	33.13	n/a	4.80	0.43	0.68	7.67	0.40	0.43
Acidovorax	20.03	16.13	0.32	9.70	53.23	0.84	61.63	41.33	0.51
Acinetobacter	10069.23	17074.23	0.96	33153.63	42507.17	0.72	27120.17	34258.53	0.58
Actinobaculum	0.07	18.13	n/a	11.43	71.17	0.07	5.33	10.80	0.48
Actinomadura	10.30	5.30	0.92	85.93	0.73	0.63	0.03	0.33	0.50
Actinomyces	1590.30	410.97	0.17	1893.13	2869.63	0.71	807.80	582.77	0.89
Actinomycetospora	20.47	27.73	0.94	22.77	7.30	0.43	75.63	75.97	0.31
Actinoplanes	1.03	25.73	0.12	72.67	6.47	0.58	8.67	5.70	0.55
Actinotignum	6.77	0.00	n/a	65.90	66.13	0.40	18.37	54.90	0.15
Adhaeribacter	0.77	1.53	0.38	88.70	0.47	0.25	0.07	0.63	0.32
Advenella	12.10	0.27	n/a	0.00	0.00	n/a	0.00	1.40	n/a
Aerococcus	904.70	390.27	0.10	18125.63	5033.87	0.81	3728.10	1507.90	0.68
Aeromicrobium	287.57	101.37	0.98	192.83	289.67	0.92	364.80	109.63	0.31
Aeromonas	275.87	1219.43	0.56	1714.73	1985.50	0.85	572.93	1667.40	0.45
Aetherobacter	0.00	0.03	n/a	43.43	0.60	n/a	1.57	0.00	n/a
Agaricicola	1.23	0.33	n/a	0.00	0.00	n/a	41.97	0.90	0.29
Aggregatibacter	3.67	37.67	0.15	66.00	241.80	0.11	6.80	78.40	0.56
Agromyces	13.20	0.00	n/a	0.00	0.00	n/a	0.00	0.00	n/a
Akkermansia	0.00	0.00	n/a	0.00	0.00	n/a	0.00	0.00	n/a
Alcaligenes	0.03	1.03	n/a	19.90	15.23	0.86	8.53	15.57	0.58
Alcanivorax	0.00	1.83	n/a	0.00	0.00	n/a	0.00	6.40	n/a
Algiphilus	0.00	0.03	n/a	0.00	2.10	n/a	0.00	0.00	n/a
Algoriphagus	7.00	0.57	0.41	10.70	46.90	0.77	0.00	3.10	n/a
Aliihoeflea	15.07	0.07	0.11	3.07	1.33	0.28	0.83	16.30	0.17
Alistipes	0.00	0.00	n/a	14.50	0.00	n/a	0.00	0.23	n/a
Alkaliphilus	0.07	0.03	n/a	32.00	0.03	0.38	0.00	0.10	n/a
Alkanindiges	3.90	58.67	0.52	48.57	18.73	0.97	89.47	102.30	0.94
Allisonella	0.00	0.00	n/a	9.67	0.00	n/a	0.00	0.60	n/a
Allocatelliglobosispora	0.00	0.00	n/a	0.00	0.00	n/a	0.00	0.00	n/a
Alloiococcus	662.37	310.03	0.69	49.03	222.07	0.80	56.97	133.20	0.30

Bacteria	Axilla			Forearm			Upper back		
	Day 1	Day 4	P value	Day 1	Day 4	P value	Day 1	Day 4	P value
Amaricoccus	197.10	55.40	0.34	202.87	1846.67	0.81	3658.90	606.37	0.13
Aminobacter	43.67	54.23	0.64	63.40	27.13	0.10	114.43	69.77	0.62
Anaerococcus	50413.43	53662.50	0.70	15861.13	26850.37	0.03	15650.47	46801.97	0.78
Anaeroglobus	0.00	0.20	n/a	1.33	13.77	0.75	0.00	0.00	n/a
Anaeromyxobacter	0.00	0.00	n/a	12.87	0.00	n/a	0.00	0.00	n/a
Ancylobacter	19.67	10.93	0.74	15.90	52.23	0.61	33.30	9.23	0.68
Aquamicrobium	5.37	2.30	0.70	10.27	10.43	0.86	16.37	7.10	0.79
Aquaspirillum	0.00	31.07	n/a	0.00	0.00	n/a	0.00	0.00	n/a
Aquicella	0.00	0.00	n/a	0.30	0.00	n/a	35.27	0.00	n/a
Aquihabitans	14.77	57.57	0.57	4.43	10.30	0.44	70.87	22.97	0.34
Arcicella	0.00	0.73	n/a	0.00	9.63	n/a	0.00	0.00	n/a
Arcobacter	1.90	22.57	0.27	5.67	5.10	0.81	173.47	0.10	0.15
Arenimonas	1.77	7.33	0.65	6.30	14.03	0.94	0.50	1.70	0.53
Arsenicicoccus	2.27	9.87	0.17	1.43	5.33	0.65	26.73	9.10	0.93
Arthrobacter	107.40	183.57	0.96	250.33	158.97	0.44	301.00	329.07	0.21
Asaia	1.23	67.53	0.45	34.47	3.83	0.80	4.53	38.17	0.55
Asticcacaulis	0.33	0.60	0.63	0.00	1.90	n/a	0.00	0.13	n/a
Atopobium	40.10	19.90	0.28	168.50	129.73	0.90	112.47	36.20	0.05
Atopostipes	3.23	0.17	0.36	0.00	18.90	n/a	5.93	0.33	0.55
Azonexus	0.23	0.00	n/a	0.50	4.07	0.80	21.43	0.00	n/a
Azorhizobium	5.60	0.93	0.58	18.73	1.60	0.41	11.17	9.03	0.71
Azospira	1.43	2.20	0.85	22.43	5.37	0.62	8.20	0.13	0.55
Azospirillum	0.53	2.23	0.29	39.60	75.57	0.56	22.17	16.13	0.56
Azovibrio	0.03	0.00	n/a	0.00	0.00	n/a	0.77	2.33	0.60
Bacillus	2127.87	451.87	0.56	4439.57	1501.10	0.55	513.10	644.30	0.21
Bacteriovorax	17.30	2.30	0.30	8.50	94.37	0.06	39.50	66.23	0.20
Bacteroides	0.23	21.73	0.09	53.93	6.40	0.15	23.83	4.97	0.13
Bavariicoccus	0.00	0.00	n/a	0.00	0.00	n/a	0.00	19.03	n/a
Bdellovibrio	5.97	44.47	0.73	63.87	46.23	0.62	165.60	8.60	0.29
Bergeyella	5.20	30.10	0.46	23.87	62.17	0.65	21.27	66.73	0.31
Bifidobacterium	10.50	26.03	0.74	78.90	42.87	0.96	39.03	95.57	0.16
Blastococcus	211.07	1.03	0.08	25.90	12.50	0.70	26.93	7.90	0.67
Blautia	28.80	29.87	0.85	231.63	154.93	0.26	62.50	34.23	0.86
Bombiscardovia	0.00	0.00	n/a	0.00	0.00	n/a	0.00	0.27	n/a
Brachybacterium	2920.77	1237.63	0.60	4571.00	3001.57	0.01	1848.90	2015.70	0.77
Brachymonas	0.27	0.00	n/a	0.00	5.97	n/a	0.00	25.90	n/a
Branchiibius	1.30	0.30	0.48	0.00	53.20	n/a	17.77	45.43	0.82
Brevibacillus	39.27	11192.43	0.41	427.77	234.90	0.96	662.00	2332.50	0.71
Brevibacterium	4950.80	2372.80	0.87	9010.33	12052.40	0.17	5574.07	9900.90	0.90
Brevundimonas	1568.20	110.03	0.46	487.00	830.60	0.57	352.70	2086.47	0.91
Buchnera	3.37	6.60	0.58	0.00	31.57	n/a	47.17	0.33	0.18
Bulleidia	0.00	4.67	n/a	0.00	1.57	n/a	0.00	7.13	n/a
Burkholderia	3.43	36.93	0.92	14.40	39.60	0.41	57.57	11.90	0.96

Bacteria	Axilla			Forearm			Upper back		
	Day 1	Day 4	P value	Day 1	Day 4	P value	Day 1	Day 4	P value
Camelimonas	30.27	0.97	0.36	33.47	4.67	0.59	13.93	32.17	0.37
Campylobacter	137.87	28.87	0.10	193.07	265.63	0.43	120.27	45.57	0.24
Candidatus	24.13	14.77	0.56	161.80	16.47	0.41	31.40	73.30	0.84
Capnocytophaga	0.33	37.57	n/a	110.73	86.50	0.20	127.83	27.67	0.28
Cardiobacterium	0.07	37.23	n/a	115.50	131.50	0.80	13.60	45.10	0.85
Carnobacterium	131.60	12.70	0.79	6.00	54.97	0.99	1.60	0.97	0.86
Catonella	0.17	22.83	n/a	27.27	0.13	n/a	3.40	7.47	0.95
Caulobacter	1.90	1.57	0.91	15.67	3.57	0.18	47.13	5.73	0.79
Cellvibrio	12.77	34.30	0.39	141.90	64.80	0.90	66.33	19.63	0.95
Cetobacterium	0.07	0.00	n/a	0.00	0.00	n/a	0.00	0.00	n/a
Chelatococcus	1.53	2.10	0.86	2.30	0.00	n/a	27.23	7.87	0.39
Chiayiivirga	0.13	10.23	0.14	14.13	21.20	0.44	0.00	7.03	n/a
Chitinimonas	0.00	0.07	n/a	0.87	0.00	n/a	0.00	0.00	n/a
Chondromyces	0.17	1.03	0.68	0.20	17.60	0.26	12.33	0.13	0.53
Christensenella	0.00	0.00	n/a	0.00	0.00	n/a	0.00	0.00	n/a
Chryseobacterium	72.50	70.17	0.68	278.07	351.77	0.39	153.50	153.23	0.80
Clostridium	141.23	161.00	0.69	246.17	239.70	0.78	120.13	137.00	0.72
Cohnella	0.00	3.17	n/a	0.13	60.13	n/a	0.00	0.07	n/a
Collinsella	0.47	0.67	0.97	17.00	25.40	0.98	11.93	14.53	0.88
Comamonas	159.13	530.47	0.51	506.63	2109.40	0.95	140.20	330.37	0.73
Conchiformibius	28.00	0.30	n/a	0.10	1.00	n/a	0.00	0.00	n/a
Conexibacter	5.30	42.37	0.38	5.13	16.13	0.60	10.80	7.47	0.85
Coprococcus	0.00	0.00	n/a	31.50	12.10	0.73	6.40	0.00	n/a
Corynebacterium	246977.53	252742.20	0.76	237485.23	197046.60	0.72	144944.90	206557.33	0.85
Coxiella	0.00	0.00	n/a	0.00	8.80	n/a	0.00	0.00	n/a
Craurococcus	28.23	96.33	0.67	34.20	29.10	0.86	154.10	101.97	0.81
Cryptosporangium	0.00	0.00	n/a	0.00	0.00	n/a	0.00	0.00	n/a
Curtobacterium	0.43	2.60	0.97	0.67	5.70	0.38	22.00	8.23	0.30
Curvibacter	3.90	0.97	0.71	4.53	0.30	0.46	17.93	4.10	0.13
Cystobacter	0.30	0.30	1.00	6.20	13.93	0.49	33.17	0.73	0.43
Cytophaga	2.77	0.07	0.34	0.00	13.03	n/a	52.30	0.00	n/a
Dechloromonas	1.33	0.63	0.55	3.33	11.93	0.87	50.37	0.83	0.33
Defluviicoccus	1.20	7.83	0.48	0.13	1.83	n/a	3.27	21.73	0.66
Deinococcus	709.00	505.30	0.94	379.07	533.77	0.92	4515.83	406.13	0.58
Delftia	43.80	53.63	0.42	255.40	90.27	0.30	286.67	23.40	0.20
Denitratisoma	0.03	0.00	n/a	0.00	0.00	n/a	0.77	0.00	n/a
Dermabacter	1703.23	1306.17	0.83	1866.77	3750.93	0.40	1439.10	1007.93	0.26
Desulfosporosinus	0.00	0.00	n/a	9.57	0.13	0.43	0.00	10.13	n/a
Desulfotomaculum	0.00	0.00	n/a	0.00	0.00	n/a	0.00	8.33	n/a
Desulfovibrio	0.03	0.00	n/a	0.00	22.27	n/a	0.00	0.00	n/a
Devosia	71.80	44.00	0.53	126.47	64.77	0.16	77.97	57.23	0.68
Dialister	65.47	136.90	0.38	384.13	457.27	0.75	429.93	244.27	0.85
Dickeya	0.30	0.30	1.00	0.00	0.00	n/a	4.97	0.00	n/a

Bacteria	Axilla			Forearm			Upper back		
	Day 1	Day 4	P value	Day 1	Day 4	P value	Day 1	Day 4	P value
Dolosigranulum	363.07	102.27	0.82	220.57	830.63	0.67	501.63	477.87	0.69
Dorea	48.13	0.73	0.43	79.07	5.90	0.03	61.70	19.97	0.67
Duganella	0.03	0.03	n/a	0.00	0.00	n/a	0.00	2.20	n/a
Dyadobacter	47.30	18.57	0.53	66.23	23.20	0.52	87.77	22.70	0.67
Eggerthella	0.00	0.00	n/a	0.00	0.00	n/a	0.00	0.00	n/a
Eikenella	0.30	0.03	n/a	167.33	50.53	0.34	2.73	4.70	0.92
Emticicia	0.00	0.03	n/a	9.20	0.00	n/a	10.83	0.03	0.39
Enterococcus	21.13	69.07	0.65	227.80	87.10	0.76	34.50	141.27	0.46
Eremococcus	387.53	702.90	0.87	601.70	2888.40	0.54	559.23	537.63	0.86
Erysipelatoclostridium	0.00	0.00	n/a	0.00	0.00	n/a	0.00	0.00	n/a
Erythrobacter	4.80	1.93	0.95	1.50	0.97	0.35	20.10	0.73	0.46
Erythromicrobium	41.20	2.33	0.11	30.63	36.00	0.75	2.93	14.77	0.31
Eubacterium	35.67	4.77	0.39	154.77	115.97	0.43	108.53	88.03	0.54
Facklamia	212.27	518.47	0.95	880.13	420.37	0.08	567.60	839.57	0.38
Faecalibacterium	183.80	22.50	0.71	417.90	207.33	0.32	124.30	171.17	0.94
Fastidiosipila	20.73	2.33	0.91	119.83	40.13	0.47	3.33	6.17	1.00
Ferrimicrobium	9.33	3.40	0.88	0.17	0.47	n/a	3.97	2.43	0.57
Ferruginibacter	15.40	13.37	0.83	6.60	4.70	0.92	11.73	9.10	0.75
Fibrella	0.07	0.67	n/a	0.00	0.63	n/a	0.00	0.37	n/a
Filifactor	1.30	0.87	0.77	7.70	31.53	0.87	26.43	0.00	n/a
Fimbriimonas	0.00	0.00	n/a	0.57	1.37	0.93	1.07	0.20	0.62
Finegoldia	6321.83	5050.07	0.55	6281.97	14174.03	0.07	7835.27	10238.50	0.47
Flaviflexus	1.97	0.97	0.49	5.30	0.00	n/a	0.30	12.23	n/a
Flavihumibacter	3.07	0.03	n/a	12.37	3.33	0.33	30.40	0.97	0.23
Flavisolibacter	14.87	2.27	0.52	21.27	29.23	0.12	8.93	18.90	0.83
Flavitalea	8.50	1.50	0.97	3.40	1.53	0.78	23.30	10.30	0.84
Flavobacterium	61.60	14.40	0.49	36.13	64.03	0.86	33.03	53.13	0.23
Flectobacillus	0.00	0.07	n/a	12.70	0.03	0.39	1.27	0.00	n/a
Flexithrix	0.00	0.00	n/a	0.00	0.00	n/a	0.00	0.00	n/a
Flexivirga	4.53	1.20	0.83	0.00	0.00	n/a	0.00	0.00	n/a
Fluviicoccus	0.00	0.00	n/a	0.00	0.00	n/a	0.00	0.00	n/a
Fluviicola	0.57	0.47	0.50	69.57	0.00	n/a	47.33	0.07	0.34
Fontimonas	0.03	0.00	n/a	0.03	0.03	n/a	0.00	0.00	n/a
Frankia	1.00	0.00	n/a	0.00	0.03	n/a	0.37	0.97	0.49
Frondihabitans	0.13	0.00	n/a	0.33	2.30	0.53	0.00	0.30	n/a
Fusobacterium	50.17	399.90	0.34	1012.27	1430.80	0.17	234.07	565.53	0.89
Gaiella	18.27	37.90	0.94	93.13	115.93	0.59	49.23	46.20	0.75
Gallionella	0.00	0.00	n/a	0.00	0.00	n/a	14.80	4.07	0.87
Gardnerella	19.37	7.50	0.54	782.47	287.30	0.71	204.27	278.10	0.52
Gelidibacter	0.03	1.43	0.43	0.03	0.00	n/a	0.00	0.00	n/a
Gelria	0.00	0.00	n/a	6.33	0.00	n/a	0.00	0.00	n/a
Gemmatimonas	0.13	42.90	0.17	3.63	15.27	0.92	12.77	4.47	n/a
Gemmatirosa	0.93	1.17	0.76	9.03	0.00	n/a	10.10	1.17	0.60

Bacteria	Axilla			Forearm			Upper back		
	Day 1	Day 4	P value	Day 1	Day 4	P value	Day 1	Day 4	P value
Geobacillus	0.00	0.00	n/a	0.00	0.00	n/a	16.63	0.00	n/a
Geobacter	0.03	0.43	n/a	0.00	0.50	n/a	8.73	0.03	n/a
Geodermatophilus	0.00	4.47	n/a	0.03	25.60	0.38	0.03	0.00	n/a
Georgenia	15.57	1.07	0.99	23.30	26.60	0.40	5.23	1.80	0.68
Globicatella	2.23	0.73	0.74	36.33	42.50	0.52	0.00	2.77	n/a
Gluconobacter	3.43	4.37	0.85	10.17	2.40	0.52	1.17	8.83	0.26
Glycomyces	0.00	0.00	n/a	0.00	0.00	n/a	8.37	0.00	n/a
Gordonia	204.57	4953.20	0.48	2558.97	462.53	0.45	1170.53	1574.07	0.63
Granulicatella	52.77	67.40	0.98	358.70	549.27	0.64	31.20	492.53	0.94
Granulicella	0.23	0.00	n/a	0.00	0.00	n/a	34.60	0.17	0.48
Gulbenkiania	0.73	15.47	n/a	159.77	0.00	n/a	209.73	10.63	0.94
Haematobacter	195.87	1573.37	0.95	837.30	382.57	0.63	205.73	1274.40	0.66
Haemophilus	184.83	960.93	0.65	2331.73	4908.50	0.73	630.83	1621.43	0.94
Hahella	0.00	0.10	n/a	0.00	11.23	n/a	0.00	0.00	n/a
Haliscomenobacter	0.03	0.00	n/a	0.00	0.00	n/a	0.00	0.00	n/a
Halochromatium	0.03	0.03	n/a	188.20	0.00	n/a	518.47	49.38	n/a
Haloechinothrix	2.80	4.27	n/a	0.20	0.00	n/a	0.00	3.43	n/a
Halomonas	15.67	2.30	0.14	230.73	158.63	0.43	1397.57	142.60	0.84
Helcococcus	0.63	51.55	0.24	131.73	106.07	0.40	8.47	34.66	0.04
Herbaspirillum	0.33	1.66	0.79	9.00	8.00	0.81	12.33	1.59	0.95
Herminiimonas	2.77	0.76	0.75	0.30	0.20	n/a	12.10	20.07	0.63
Hydrocarboniphaga	0.37	0.59	0.29	0.00	206.20	n/a	0.00	6.10	n/a
Hydrogenophaga	8.73	1.31	0.70	11.57	5.70	0.66	44.00	6.72	0.56
Hydrogenophilus	27.43	9.83	0.77	46.63	11.13	0.44	31.67	9.55	0.41
Hymenobacter	199.87	169.00	0.88	387.13	969.10	0.63	939.10	399.00	0.71
Hyphomicrobium	248.40	232.45	0.19	109.23	218.47	0.91	323.63	56.72	0.93
Hyphomonas	0.00	0.00	n/a	1.00	0.00	n/a	130.30	0.00	n/a
Iamia	18.43	58.69	0.53	138.43	10.87	0.06	1986.57	24.55	0.32
Ignavigranum	4.90	2.41	0.62	5.47	32.73	0.53	3.50	17.62	0.35
Ilumatobacter	38.33	37.59	0.36	44.53	140.57	0.62	63.17	72.00	0.92
Isoptericola	39.30	28.00	0.88	29.83	50.93	0.26	899.47	38.07	0.41
Jeotgalicoccus	411.50	1266.41	0.72	1223.10	644.50	0.01	322.17	381.90	0.31
Jiangella	1.17	29.07	n/a	0.00	0.07	n/a	0.00	1.48	n/a
Jonquetella	0.07	0.00	n/a	51.10	0.37	n/a	1.27	0.00	n/a
Kaistia	1.33	0.62	0.70	17.57	1.80	n/a	22.07	1.38	0.56
Kaistibacter	1.03	0.83	0.18	1.10	12.50	0.17	17.87	2.69	0.48
Ketogulonicigenium	1.77	2.14	0.08	10.00	8.83	0.53	51.17	16.55	0.76
Kineococcus	43.87	18.45	0.44	8.67	33.03	0.56	36.73	26.28	0.80
Kineosporia	1.77	0.48	0.20	0.47	7.43	0.26	13.27	98.55	0.79
Kingella	0.47	5.69	0.46	94.93	193.80	0.24	43.63	12.34	0.36
Kribbella	6.27	0.07	0.48	0.00	1.20	n/a	0.00	0.21	n/a
Kurthia	0.13	0.00	n/a	13.30	3.03	0.65	0.00	0.24	n/a
Kytococcus	1040.77	641.66	0.68	995.73	1117.60	0.09	1795.37	660.45	0.96

Bacteria	Axilla			Forearm			Upper back		
	Day 1	Day 4	P value	Day 1	Day 4	P value	Day 1	Day 4	P value
Lactococcus	53.83	293.97	0.94	677.63	999.20	0.76	88.30	269.79	0.43
Lautropia	23.87	344.72	0.77	988.27	3333.77	0.66	644.67	1144.69	0.25
Legionella	19.30	11.21	0.80	56.30	67.40	0.95	43.97	24.14	0.93
Legionella-like	0.47	6.90	0.10	13.93	20.33	0.90	0.63	0.76	n/a
Leptotrichia	28.30	86.24	0.62	504.53	315.03	0.35	89.33	63.69	0.61
Leucobacter	29.53	31.10	0.75	3.30	8.77	0.44	15.70	25.00	0.68
Leuconostoc	4.87	31.83	0.19	46.90	27.50	0.53	49.33	34.83	0.95
Lewinella	0.30	0.45	n/a	0.00	0.00	n/a	0.00	0.86	n/a
Luedemannella	0.13	0.03	n/a	0.03	0.00	n/a	2.80	0.21	n/a
Luteimonas	238.47	59.97	0.37	393.00	57.10	0.31	1101.53	88.24	0.83
Luteolibacter	4.37	1.52	0.45	6.03	1.37	0.17	1.67	11.86	0.19
Lysobacter	148.07	59.97	0.56	269.30	149.07	0.19	344.70	532.52	0.55
Marinococcus	30.50	74.55	0.33	43.97	230.63	0.09	40.93	3.34	1.00
Marinomonas	0.00	0.00	n/a	0.00	0.00	n/a	43.83	0.00	n/a
Marmoricola	51.87	98.45	0.88	210.80	85.07	0.72	223.87	211.03	0.98
Massilia	49.77	95.55	0.97	153.53	520.23	0.82	220.97	338.79	0.28
Megasphaera	5.13	0.21	0.68	19.37	23.57	0.97	98.97	6.55	0.35
Meiothermus	0.03	0.10	n/a	77.57	0.23	n/a	200.43	1.90	0.53
Mesorhizobium	36.80	78.10	0.26	13.90	102.37	0.48	41.37	69.07	0.31
Methylobacillus	55.20	0.79	n/a	0.03	0.00	n/a	0.00	0.00	n/a
Methylobacterium	168.90	299.90	0.81	709.60	402.67	0.10	1139.80	974.72	0.70
Methylocystis	1.53	13.34	0.14	7.17	0.03	0.23	6.83	11.93	0.95
Methylophilus	0.53	0.00	n/a	0.00	7.33	n/a	25.80	1.28	0.99
Methylotenera	0.50	0.31	n/a	16.53	0.60	0.34	0.00	8.59	n/a
Micromonospora	16.77	0.00	n/a	0.00	22.93	n/a	0.00	0.00	n/a
Mobiluncus	29.37	34.79	0.39	38.80	48.50	0.20	45.80	5.38	0.20
Modestobacter	0.00	5.17	n/a	3.50	0.23	0.35	0.10	0.17	n/a
Moraxella	3848.47	1475.79	0.03	1669.17	884.03	0.01	214.20	574.24	0.76
Morococcus	25.70	524.90	0.77	1054.33	762.70	0.84	158.57	251.17	0.56
Moryella	0.00	19.28	n/a	0.00	21.90	n/a	1.70	20.10	n/a
Mucilaginibacter	0.03	0.00	n/a	0.07	0.00	n/a	15.60	1.03	0.68
Mycobacterium	1393.37	699.03	0.96	2023.40	2498.00	0.70	3362.13	1073.03	0.69
Nakamurella	19.53	16.10	0.65	15.70	31.63	0.95	68.63	98.14	0.61
Nannocystis	0.00	0.00	n/a	0.00	0.03	n/a	2.40	5.79	n/a
Negativicoccus	175.27	34.66	0.41	101.13	267.77	0.97	162.17	121.62	0.23
Neisseria	15.37	1058.00	0.36	2960.37	2735.47	0.62	382.20	535.03	0.69
Nesterenkonia	587.03	3591.59	0.55	489.87	503.23	0.01	277.87	145.97	0.39
Nitrosomonas	143.70	153.14	0.92	177.67	91.57	0.88	727.33	175.52	0.06
Nitrosospira	6.20	0.00	n/a	0.00	0.23	n/a	0.67	27.24	0.44
Nitrospira	0.17	55.93	0.25	0.83	0.40	n/a	15.67	8.76	0.96
Nocardia	9.97	0.00	n/a	0.73	8.17	0.53	5.90	4.21	0.44
Nocardioides	240.67	218.66	0.92	223.53	308.67	0.31	777.63	367.76	0.78
Nocardiopsis	13.93	111.59	0.41	61.13	0.67	0.27	0.37	16.93	0.28

Bacteria	Axilla			Forearm			Upper back		
	Day 1	Day 4	P value	Day 1	Day 4	P value	Day 1	Day 4	P value
Novispirillum	0.50	0.90	0.89	32.87	0.00	n/a	0.17	6.03	n/a
Novosphingobium	46.07	245.83	0.25	170.87	64.07	0.02	197.40	216.17	0.43
Ohtaekwangia	0.97	7.41	n/a	0.03	2.80	n/a	0.27	1.03	0.30
Oligella	12.73	0.00	n/a	50.10	29.33	0.33	8.80	8.07	0.98
Oribacterium	0.67	79.07	0.09	257.90	50.53	0.33	8.03	6.69	0.64
Ornithinimicrobium	38.27	21.59	0.91	266.60	49.73	0.59	11.37	35.48	0.47
Oryzihumus	0.13	0.07	n/a	0.30	0.00	n/a	0.00	0.28	n/a
Paenibacillus	29.23	93.03	0.46	31.40	140.30	0.76	88.10	192.14	0.93
Paludibacter	0.03	0.21	n/a	15.47	0.00	n/a	0.00	0.07	n/a
Panacagrimonas	0.10	0.03	n/a	0.00	0.00	n/a	18.83	0.03	n/a
Pandoraea	0.00	0.00	n/a	69.30	0.00	n/a	0.00	0.00	n/a
Pantoea	2499.03	1530.28	0.92	1889.60	2900.77	0.58	4418.47	1556.76	0.66
Parabacteroides	0.03	0.00	n/a	17.27	1.10	n/a	10.87	0.45	0.22
Paracoccus	5229.53	6440.83	0.64	8941.23	2704.43	0.03	7964.67	9415.97	0.74
Paracraurococcus	0.43	12.62	0.27	0.10	159.83	n/a	18.40	3.31	0.84
Parasegetibacter	0.07	16.41	n/a	0.03	3.67	n/a	0.00	0.03	n/a
Parvimonas	3.73	44.86	0.36	142.90	140.27	0.56	126.77	14.34	0.26
Parvularcula	0.00	0.00	n/a	0.00	0.00	n/a	48.20	0.00	n/a
Patulibacter	5.83	0.24	0.40	13.30	15.27	0.34	145.67	3.69	0.32
Pedobacter	21.97	69.41	0.68	71.37	32.27	0.41	80.67	272.45	0.25
Pedomicrobium	0.70	37.14	0.34	0.00	0.60	n/a	24.97	0.52	0.41
Pelistega	1.40	116.07	0.38	256.77	498.53	0.62	143.87	626.45	0.58
Pelomonas	16.73	2.90	0.54	3.87	40.03	0.04	60.93	31.21	0.61
Peptococcus	2.93	23.21	0.46	39.77	69.20	0.82	70.90	64.52	0.97
Peptoniphilus	3240.20	3152.24	0.50	3774.77	7602.30	0.18	3458.30	6182.07	0.16
Peptostreptococcus	42.30	58.86	0.25	486.53	327.83	0.16	195.93	179.24	0.70
Perlucidibaca	95.63	45.93	0.65	119.63	161.87	0.96	55.20	48.48	0.25
Phascolarctobacterium	0.03	0.00	n/a	3.57	0.00	n/a	0.00	0.31	n/a
Phaselicystis	0.57	0.14	n/a	3.00	5.03	0.88	19.03	1.00	0.96
Phenylobacterium	46.43	10.17	0.24	78.07	315.20	0.16	174.73	154.10	0.99
Photobacterium	2.20	66.52	0.17	26.47	367.87	0.92	15.53	7.90	0.76
Piscicoccus	67.40	1.83	0.74	909.20	30.80	0.31	19.63	94.21	0.57
Planifilum	0.10	0.00	n/a	0.03	0.00	n/a	0.00	0.10	n/a
Planococcus	14.37	0.97	0.65	31.47	76.67	0.88	16.77	54.83	0.33
Polaromonas	0.40	0.62	0.08	2.13	1.13	0.82	4.80	0.66	0.73
Polynucleobacter	4.00	2.76	0.92	0.00	0.00	n/a	18.37	4.24	0.61
Pontibacter	0.00	0.03	n/a	0.00	103.93	n/a	0.00	0.00	n/a
Porphyromonas	17.93	195.79	0.41	446.30	423.83	0.97	209.87	156.52	0.70
Prevotella	96.03	494.55	0.70	1179.30	1452.17	0.59	715.40	354.48	0.57
Promicromonospora	0.40	0.10	n/a	20.43	2.67	n/a	0.00	0.83	n/a
Propionibacterium	5015.93	14187.48	0.59	524.87	15644.53	0.06	17717.03	31303.34	0.27
Pseudoalteromonas	54.87	0.97	n/a	0.00	0.00	n/a	0.00	0.00	n/a
Pseudoclavibacter	121.47	126.55	0.25	390.70	516.17	0.37	388.70	319.76	0.25

Bacteria	Axilla			Forearm			Upper back		
	Day 1	Day 4	P value	Day 1	Day 4	P value	Day 1	Day 4	P value
Pseudonocardia	23.53	107.41	0.98	567.93	906.93	0.12	66.47	161.38	0.89
Pseudorhodobacter	3.83	4.97	0.33	59.67	3.00	0.64	81.17	13.97	0.81
Pseudorhodoferax	18.20	37.90	0.92	44.53	12.03	0.56	32.13	37.79	0.25
Pseudoxanthobacter	0.00	3.72	n/a	0.07	0.00	n/a	0.97	0.00	n/a
Pseudoxanthomonas	29.73	38.72	0.86	48.67	186.43	0.36	497.97	127.97	0.90
Psychrobacter	59.40	362.93	0.31	347.03	245.23	0.75	174.00	217.76	0.71
Ralstonia	4.83	50.62	0.35	192.53	119.60	0.53	92.43	8.07	0.31
Ramlibacter	1.50	0.41	0.39	4.83	12.93	0.76	20.13	2.14	0.74
Rathayibacter	2.10	45.24	0.26	18.83	19.27	0.73	78.67	10.38	0.81
Rheinheimera	173.20	728.31	0.73	829.10	411.27	0.56	271.47	5252.69	0.19
Rhizobium	199.63	112.69	0.65	34.90	96.47	0.93	97.57	59.83	0.61
Rhodanobacter	26.87	3.17	0.86	0.90	9.40	0.14	12.97	0.03	0.20
Rhodobacter	47.60	40.55	0.57	28.27	1943.17	0.66	3705.80	104.79	0.96
Rhodoblastus	1.93	19.90	n/a	0.07	4.37	n/a	0.43	0.00	n/a
Rhodococcus	775.00	749.59	0.99	2795.33	514.27	0.52	10433.70	446.59	0.21
Rhodocytophaga	0.90	0.59	0.50	35.07	0.00	n/a	4.13	1.59	1.00
Rhodoferax	69.90	191.14	0.96	57.77	44.97	0.62	443.87	225.76	0.23
Rhodoplanes	1.13	0.14	0.47	0.23	11.00	0.33	19.60	0.83	0.84
Rhodopseudomonas	15.97	13.45	0.87	27.50	28.40	0.50	62.60	19.41	0.73
Rhodovulum	92.83	62.55	0.82	67.90	686.13	0.76	2911.03	1649.03	0.47
Rickettsiella	0.23	0.55	0.21	0.93	3.27	0.27	44.30	17.93	0.75
Roseburia	0.53	6.45	0.31	57.20	48.87	0.31	90.73	0.79	0.15
Roseiarcus	1.10	0.00	n/a	30.73	0.00	n/a	0.17	0.00	n/a
Roseococcus	17.03	60.97	0.29	39.87	23.80	0.78	104.33	19.17	0.32
Roseomonas	7913.50	1579.62	0.61	1825.63	2028.40	0.38	1162.77	833.72	0.94
Rothia	211.13	236.07	0.63	1018.37	3019.47	0.34	609.47	1446.59	0.73
Rubellimicrobium	178.77	318.03	0.77	497.30	209.80	0.21	818.17	501.48	0.71
Rubrobacter	3.37	17.79	0.17	21.27	0.87	0.22	717.37	32.03	0.64
Rufibacter	0.00	11.28	n/a	0.00	26.90	n/a	0.00	2.69	n/a
Ruminococcus	1.63	83.17	0.13	130.60	80.80	0.46	155.93	46.00	0.28
Saccharopolyspora	0.43	0.10	n/a	0.17	1.73	0.42	7.33	5.34	0.73
Salinicoccus	151.47	4104.90	0.49	1131.83	1297.27	0.31	566.63	268.07	0.27
Salmonella	21.97	19.03	0.71	80.63	20.73	0.23	56.47	28.55	0.11
Sandaracinobacter	5.87	0.14	0.33	0.07	2.10	n/a	5.87	35.07	0.21
Sandaracinus	0.03	0.00	n/a	0.27	15.70	n/a	0.03	4.69	0.18
Sanguibacter	5.53	2.41	0.89	56.30	6.37	0.94	64.83	34.52	0.99
Schlegelella	0.00	4.62	n/a	173.53	0.10	0.14	159.63	0.14	0.21
Sediminibacterium	0.23	0.00	n/a	35.43	0.03	n/a	5.60	0.00	n/a
Selenomonas	0.20	19.97	n/a	1.03	68.70	0.16	34.00	0.86	0.42
Serratia	143.03	46.93	0.76	108.13	138.97	0.73	37.67	57.38	0.71
Shewanella	26.20	120.93	0.19	324.47	152.10	0.03	371.07	733.24	0.13
Simonsiella	0.03	0.00	n/a	31.83	0.00	n/a	0.00	5.34	n/a
Skermanella	340.67	191.38	0.42	193.33	401.83	0.76	278.57	234.55	0.58

Bacteria	Axilla			Forearm			Upper back		
	Day 1	Day 4	P value	Day 1	Day 4	P value	Day 1	Day 4	P value
Solirubrobacter	87.87	49.48	0.91	150.23	58.03	0.01	39.53	29.10	0.30
Solobacterium	0.87	44.55	0.26	72.23	55.80	0.60	5.03	8.07	0.76
Sphaerobacter	0.80	0.00	n/a	0.00	0.20	n/a	1.17	0.72	0.75
Sphingobacterium	125.63	178.69	0.89	50.43	118.10	0.32	89.10	119.00	0.77
Sphingobium	2.50	0.59	0.26	39.93	1.33	0.36	97.90	13.41	0.87
Sphingomicrobium	0.00	0.00	n/a	0.00	0.00	n/a	0.00	0.00	n/a
Sphingomonas	1125.60	1102.38	0.80	1686.43	1549.47	0.28	3796.90	1538.07	0.25
Sphingopyxis	1.10	1.97	0.83	77.93	34.57	0.99	5.53	107.69	0.20
Spirosoma	4.47	6.00	0.81	2.67	6.57	0.14	45.80	54.86	0.27
Sporichthya	45.70	83.38	0.24	210.97	2.37	0.28	24.17	53.86	0.17
Sporocytophaga	2.60	4.79	0.89	10.50	23.13	0.69	0.00	1.45	n/a
Stackebrandtia	0.43	7.38	n/a	7.37	0.23	n/a	0.43	0.69	n/a
Staphylococcus	590843.40	534387.60	0.08	600757.57	697506.50	0.66	720455.00	673332.03	0.70
Stenotrophomonas	2402.57	740.86	0.44	1894.23	1373.40	0.38	1046.37	794.97	0.46
Steroidobacter	3.87	0.03	n/a	0.03	0.23	n/a	0.03	0.83	0.26
Streptococcus	9087.73	3648.24	0.26	14597.80	24557.67	0.80	5782.30	12267.14	0.89
Streptomyces	23.70	5.24	0.54	42.10	52.10	0.37	178.17	47.34	0.43
Subtercola	0.03	0.03	n/a	18.70	0.03	n/a	55.00	0.00	n/a
Succinivibrio	0.00	1.10	n/a	51.57	0.00	n/a	5.37	0.00	n/a
Sulfuricurvum	0.27	0.14	n/a	11.87	80.27	0.86	0.00	0.62	n/a
Sulfurimonas	21.50	24.86	0.63	264.80	506.00	0.77	48.67	30.55	0.85
Sutterella	0.00	5.69	n/a	0.03	4.83	n/a	12.17	0.93	n/a
Tahibacter	3.37	54.41	n/a	0.00	0.13	n/a	1.93	0.07	n/a
Tannerella	0.10	2.17	n/a	15.87	12.33	0.03	8.13	10.14	1.00
Tepidiphilus	2.10	0.38	0.31	6.43	9.87	0.77	3.03	1.31	0.59
Terrabacter	177.73	93.03	0.95	238.20	230.87	0.21	122.17	134.97	0.27
Terriglobus	0.03	0.00	n/a	0.00	0.00	n/a	0.00	46.72	n/a
Terrimonas	7.17	18.03	0.46	21.10	40.47	0.47	9.07	14.62	0.25
Thauera	4.33	0.93	n/a	0.33	50.37	0.12	98.60	37.14	0.68
Thermoactinomyces	2.60	0.17	0.40	1.57	11.57	0.09	7.37	2.28	0.80
Thermomicrobium	1.03	0.00	n/a	52.90	0.67	0.46	13.50	0.97	0.41
Thermomonas	0.67	9.07	0.19	0.20	1.13	0.60	57.67	1.17	0.41
Thermomonospora	0.00	32.97	n/a	0.00	0.00	n/a	0.00	0.00	n/a
Thermovum	0.90	0.03	0.26	8.13	0.20	0.41	0.00	0.34	n/a
Thermus	4.17	0.07	n/a	1.63	10.87	0.81	0.13	3.24	0.30
Thiobacillus	0.07	0.00	n/a	0.00	0.00	n/a	31.43	0.28	n/a
Thioprofundum	1.33	8.72	0.68	22.57	23.37	0.61	437.67	78.93	0.50
Toxopsis	0.23	0.28	0.50	0.00	1.60	n/a	6.13	9.93	0.62
Treponema	0.03	1.34	n/a	1.07	12.17	0.35	9.63	15.72	0.67
Trichococcus	0.47	0.62	0.69	0.70	0.00	n/a	32.63	36.86	0.74
Truepera	52.43	11.72	0.74	59.90	16.30	0.31	96.43	150.86	0.76
Tsukamurella	6.73	16.72	0.40	74.50	197.30	0.05	21.80	19.38	0.70
Tuberibacillus	0.00	0.07	n/a	0.07	0.80	n/a	0.03	0.76	n/a

Bacteria	Axilla			Forearm			Upper back		
	Day 1	Day 4	P value	Day 1	Day 4	P value	Day 1	Day 4	P value
Turicibacter	1.50	0.69	0.39	35.03	18.70	0.36	0.97	0.93	n/a
Tyzzerella	0.00	0.00	n/a	0.00	0.03	n/a	0.00	0.00	n/a
Uliginosibacterium	0.00	0.00	n/a	0.00	0.00	n/a	0.00	0.03	n/a
Variovorax	2841.37	113.45	0.82	283.30	118.50	0.03	257.40	387.14	0.37
Veillonella	761.30	197.10	0.76	697.17	905.67	0.83	251.70	168.00	0.79
Vibrio	15.43	5.38	0.90	171.70	235.50	1.00	107.40	56.86	0.90
Virgibacillus	0.27	0.14	n/a	0.00	35.10	n/a	0.00	0.21	n/a
Weissella	23.93	23.83	0.86	30.73	13.83	0.17	13.30	27.38	0.98
Williamsia	237.57	57.76	0.98	80.10	79.63	n/a	257.70	105.76	0.21
Wolbachia	0.07	5.31	0.26	55.37	0.03	n/a	0.03	16.48	0.23
Xanthomonas	8.80	1.38	0.47	16.93	40.73	0.97	3.00	46.90	0.11

Table 6-2. Mean amount of volatile compounds expressed as percetages collected from each body site on day 1 and day 4. Compounds in bold indicate significant differences between day 1 and day 4.

RI	Axilla			Forearm			Upper back		
	Day 1	Day 4	P value	Day 1	Day 4	P value	Day 1	Day 4	P value
810	0.060	0.050	0.105	0.060	0.050	0.435	0.050	0.030	0.802
820	0.060	0.120	0.753	0.060	0.120	0.222	0.220	0.260	0.521
841	0.930	1.460	0.095	0.930	1.460	0.201	1.000	1.570	0.145
851	1.080	2.020	0.079	1.080	2.020	0.176	1.400	1.740	0.333
865	0.160	0.070	0.897	0.160	0.070	0.324	0.200	0.070	0.233
870	0.760	0.580	0.082	0.760	0.580	0.447	0.520	0.700	0.824
879	0.290	0.450	0.320	0.290	0.450	0.366	0.460	0.450	0.745
880	0.230	0.170	0.773	0.230	0.170	0.645	0.250	0.090	0.112
889	0.030	0.080	0.260	0.030	0.080	0.157	0.150	0.090	0.236
910	0.230	0.220	0.354	0.230	0.220	0.750	0.260	0.260	0.941
930	0.040	0.050	0.085	0.040	0.050	0.717	0.460	0.500	0.918
933	2.580	3.020	0.729	2.580	3.020	0.611	3.630	2.620	0.473
943	0.030	0.020	0.394	0.030	0.020	0.539	0.160	0.080	0.270
953	0.070	0.100	0.354	0.070	0.100	0.538	0.330	0.300	0.889
958	0.020	0.030	0.420	0.020	0.030	0.518	0.140	0.100	0.455
962	0.660	0.800	0.007	0.660	0.800	0.706	0.640	0.430	0.142
965	1.630	2.150	0.128	1.630	2.150	0.262	1.410	1.160	0.462
972	0.290	0.350	0.431	0.290	0.350	0.701	1.100	1.030	0.766
974	0.000	0.000	n/a	0.000	0.000	n/a	1.070	1.460	0.158
977	0.010	0.040	0.026	0.010	0.040	0.087	0.240	0.310	0.763
982	0.820	1.030	0.452	0.820	1.030	0.529	1.190	1.070	0.665
990	0.030	0.140	-	0.030	0.140	-	0.020	0.700	-
995	0.750	0.500	0.334	0.750	0.500	0.316	0.330	0.400	0.593
1000	0.100	0.210	0.282	0.100	0.210	0.160	0.350	0.340	0.971
1005	1.620	1.870	0.063	1.620	1.870	0.397	2.790	2.710	0.779
1015	0.470	0.520	0.223	0.470	0.520	0.845	18.010	21.710	0.468
1022	2.460	2.750	0.735	2.460	2.750	0.771	4.800	4.370	0.545
1033	0.090	0.130	0.237	0.090	0.130	0.708	0.320	0.330	0.801
1052	0.000	0.020	0.512	0.000	0.020	n/a	0.080	0.070	0.867
1060	0.200	0.230	0.581	0.200	0.230	0.781	0.290	0.140	0.066
1063	0.020	0.100	0.478	0.020	0.100	0.444	0.010	0.100	0.252
1073	0.020	0.030	0.228	0.020	0.030	0.970	0.060	0.030	0.764
1085	2.500	2.650	0.264	2.500	2.650	0.687	2.520	2.030	0.540
1086	0.000	0.000	0.110	0.000	0.000	n/a	0.030	0.040	0.325
1102	0.390	0.480	0.339	0.390	0.480	0.704	0.460	0.450	0.906
1120	0.110	0.120	0.048	0.110	0.120	0.880	0.160	0.100	0.202
1130	1.060	0.750	0.698	1.060	0.750	0.111	1.710	2.410	0.130
1148	0.530	0.260	0.616	0.530	0.260	0.123	0.260	0.440	0.535
1154	0.300	0.320	0.080	0.300	0.320	0.859	0.100	0.220	0.339
1159	0.280	0.170	0.762	0.280	0.170	0.359	0.230	0.280	0.687

RI	Axilla			Forearm			Upper back		
	Day 1	Day 4	P value	Day 1	Day 4	P value	Day 1	Day 4	P value
1164	0.100	0.050	0.349	0.100	0.050	0.338	0.120	0.080	0.321
1172	2.130	2.890	0.250	2.130	2.890	0.170	3.980	4.060	0.610
1180	0.020	0.010	0.175	0.020	0.010	0.247	0.030	0.060	0.358
1187	2.230	2.510	0.397	2.230	2.510	0.773	0.850	0.650	0.272
1200	0.110	0.210	0.605	0.110	0.210	0.243	0.200	0.220	0.754
1213	0.030	0.020	0.184	0.030	0.020	0.815	1.440	1.920	0.119
1217	0.030	0.040	n/a	0.030	0.040	0.721	0.020	0.010	0.585
1225	0.000	0.010	n/a	0.000	0.010	n/a	0.290	0.450	0.100
1232	0.980	0.500	0.962	0.980	0.500	0.118	0.340	0.870	0.181
1250	1.160	1.180	0.684	1.160	1.180	0.661	0.480	0.900	0.173
1259	0.100	0.170	0.755	0.100	0.170	0.578	0.000	0.000	n/a
1269	0.250	0.250	0.464	0.250	0.250	0.759	0.060	0.110	0.130
1275	0.350	0.380	0.394	0.350	0.380	0.498	0.640	0.520	0.443
1282	0.030	0.090	0.453	0.030	0.090	0.510	0.200	0.140	0.383
1287	0.420	0.330	0.670	0.420	0.330	0.239	0.320	0.190	0.189
1301	0.050	0.070	0.508	0.050	0.070	0.618	0.120	0.070	0.242
1305	0.670	0.600	0.813	0.670	0.600	0.943	0.620	0.720	0.407
1317	0.330	0.240	0.053	0.330	0.240	0.549	0.160	0.320	0.188
1331	0.230	0.170	0.968	0.230	0.170	0.419	0.120	0.350	0.277
1336	1.130	0.830	0.702	1.130	0.830	0.267	0.710	0.660	0.862
1348	0.550	0.430	0.382	0.550	0.430	0.229	0.860	0.810	0.823
1354	1.740	1.680	0.213	1.740	1.680	0.532	1.130	0.950	0.437
1374	0.070	0.080	0.064	0.070	0.080	0.404	0.100	0.070	0.996
1381	0.270	0.090	0.498	0.270	0.090	0.078	0.080	0.200	0.272
1389	0.190	0.270	0.578	0.190	0.270	0.740	0.120	0.040	0.039
1396	0.160	0.110	0.066	0.160	0.110	0.462	0.250	0.130	0.472
1404	0.850	0.580	0.814	0.850	0.580	0.048	0.480	0.430	0.922
1422	0.080	0.070	0.175	0.080	0.070	0.917	0.070	0.090	0.745
1432	2.490	2.710	0.761	2.490	2.710	0.682	1.010	0.740	0.308
1451	0.070	0.080	0.370	0.070	0.080	0.882	0.070	0.070	0.962
1458	0.130	0.200	0.757	0.130	0.200	0.225	0.070	0.070	0.407
1462	0.360	0.450	0.176	0.360	0.450	0.756	0.290	0.250	0.432
1470	2.210	1.810	0.756	2.210	1.810	0.683	1.020	0.650	0.004
1489	2.870	2.610	0.587	2.870	2.610	0.655	2.000	1.910	0.907
1494	2.360	4.430	0.318	2.360	4.430	0.366	1.740	4.420	0.182
1499	1.310	1.160	0.635	1.310	1.160	0.600	0.590	0.470	0.224
1519	0.320	0.280	0.424	0.320	0.280	0.469	0.300	0.300	0.132
1539	0.910	1.090	0.911	0.910	1.090	0.652	0.500	0.310	0.217
1553	1.430	1.280	0.099	1.430	1.280	0.860	0.650	0.450	0.134
1562	0.240	0.100	0.037	0.240	0.100	0.296	0.080	0.080	0.461
1568	0.240	0.450	0.411	0.240	0.450	0.046	0.120	0.070	0.320
1574	0.110	0.090	0.126	0.110	0.090	0.566	0.080	0.030	0.128
1586	8.660	8.660	0.417	8.660	8.660	0.714	4.330	4.360	0.998

RI	Axilla			Forearm			Upper back		
	Day 1	Day 4	P value	Day 1	Day 4	P value	Day 1	Day 4	P value
1591	0.320	0.200	0.545	0.320	0.200	0.205	0.170	0.050	0.028
1601	1.810	1.530	0.269	1.810	1.530	0.463	1.010	0.850	0.226
1612	0.120	0.300	0.838	0.120	0.300	0.410	0.070	0.130	0.177
1618	0.900	0.720	0.002	0.900	0.720	0.695	0.500	0.190	0.027
1628	0.440	0.390	0.801	0.440	0.390	0.783	0.330	0.320	0.612
1640	0.170	0.190	0.120	0.170	0.190	0.167	0.140	0.100	0.258
1657	5.760	5.590	0.287	5.760	5.590	0.887	3.240	2.660	0.227
1667	0.490	0.600	0.024	0.490	0.600	0.737	0.290	0.210	0.211
1676	0.910	0.670	0.848	0.910	0.670	0.570	0.420	0.250	0.153
1684	0.730	0.510	0.264	0.730	0.510	0.056	0.410	0.340	0.585
1688	0.370	0.290	0.406	0.370	0.290	0.833	0.240	0.040	0.066
1693	0.280	0.210	0.343	0.280	0.210	0.930	0.160	0.110	0.534
1701	2.630	2.040	0.445	2.630	2.040	0.243	1.160	1.090	0.584
1711	1.500	1.200	0.513	1.500	1.200	0.354	0.710	0.650	0.463
1720	1.520	0.870	0.451	1.520	0.870	0.657	0.490	0.450	0.826
1725	1.530	1.420	0.900	1.530	1.420	0.600	0.940	0.610	0.071
1740	0.610	0.590	0.455	0.610	0.590	0.827	0.790	0.290	0.314
1747	0.290	0.350	0.845	0.290	0.350	0.263	0.230	0.150	0.199
1754	0.860	0.620	0.882	0.860	0.620	0.545	0.550	0.250	0.141
1761	1.640	1.520	0.986	1.640	1.520	0.673	0.860	0.710	0.411
1790	1.060	0.920	0.104	1.060	0.920	0.518	0.540	0.630	0.819
1801	1.590	1.350	0.794	1.590	1.350	0.763	0.770	0.750	0.769
1817	5.020	3.960	0.125	5.020	3.960	0.513	1.660	1.380	0.360
1830	2.010	1.840	0.107	2.010	1.840	0.812	0.970	1.250	0.270
1843	0.320	0.370	0.619	0.320	0.370	0.309	0.120	0.150	0.605
1867	0.180	0.300	0.010	0.180	0.300	0.197	0.040	0.080	0.173
1874	1.050	0.730	0.435	1.050	0.730	0.407	0.290	0.240	0.638
1880	0.050	0.150	0.143	0.050	0.150	0.411	0.090	0.080	0.401
1901	0.520	0.430	0.538	0.520	0.430	0.595	0.190	0.180	0.787
1909	0.170	0.330	0.231	0.170	0.330	0.223	0.240	0.230	0.475
1920	0.320	0.240	0.871	0.320	0.240	0.514	0.150	0.180	0.726
1935	0.530	0.600	0.764	0.530	0.600	0.521	0.240	0.250	0.981
2001	0.060	0.060	0.640	0.060	0.060	0.996	0.020	0.050	0.300
2010	0.190	0.260	0.283	0.190	0.260	0.102	0.080	0.100	0.602

Table 6-3. Correlation between bacteria and compounds between day 1 and day 4 for axilla at 0.0001% significance.

	Positive correlation		Strong positive correlation		Negative correlation		Strong negative correlation	
Compound (RI)	**Bacteria**	**Correlation**	**Bacteria**	**Correlation**	**Bacteria**	**Correlation**	**Bacteria**	**Correlation**
1022	-	-	-	-	*Nitrospira*	-0.642	-	-
	-	-	-	-	*Rheinheimera*	-0.670	-	-
	-	-	-	-	*Ruminococcus*	-0.645	-	-
	-	-	-	-	*Sutterella*	-0.685	-	-
1172	*Legionella-like*	0.695	-	-	-	-	-	-
	Nitrospira	0.653	-	-	-	-	-	-
1200	*Kineosporia*	0.654	-	-	-	-	-	-
	Legionella-like	0.64	-	-	-	-	-	-
1287	*Thermomicrobium*	0.673	-	-	*Paracraurococcus*	-0.639	*Helcobacillus*	-0.702
	-	-	-	-	*Photobacterium*	-0.659	*Modestobacter*	-0.753
	-	-	-	-	-	-	*Pedomicrobium*	-0.751
	-	-	-	-	-	-	*Polynucleobacter*	-0.816
1300	-	-	-	-	*Helcobacillus*	-0.639	*Modestobacter*	-0.802
	-	-	-	-	*Oribacterium*	-0.631	*Pedomicrobium*	-0.821
	-	-	-	-	*Paracraurococcus*	-0.664	*Solobacterium*	-0.755
	-	-	-	-	*Thermomicrobium*	0.662	-	-
1301	*Paracraurococcus*	0.69	-	-	-	-	-	-
	Pedomicrobium	0.63	-	-	-	-	-	-
	Photobacterium	0.656	-	-	-	-	-	-
1348	-	-	*Thermomicrobium*	0.785	*Helcococcus*	-0.695	*Helcobacillus*	-0.714
	-	-	-	-	*Iamia*	-0.664	*Modestobacter*	-0.919
	-	-	-	-	*Photobacterium*	-0.644	*Paracraurococcus*	-0.81
	-	-	-	-	*Rathayibacter*	-0.692	*Pedomicrobium*	-0.922
	-	-	-	-	-	-	*Solobacterium*	-0.829

1404	-	-	-	-	*Hymenobacter*	-0.679	*Helcobacillus*	-0.716
	-	-	-	-	*Iamia*	-0.679	*Pedomicrobium*	-0.704
	-	-	-	-	*Lautropia*	-0.66	-	-
	-	-	-	-	*Modestobacter*	-0.645	-	-
	-	-	-	-	*Nocardioides*	-0.655	-	-
	-	-	-	-	*Psychrobacter*	-0.635	-	-
	-	-	-	-	*Rathayibacter*	-0.635	-	-
	-	-	-	-	*Rhodobacter*	-0.672	-	-
	-	-	-	-	*Solobacterium*	-0.638	-	-
1451	-	-	-	-	*Rhodocytophaga*	-0.682	*Helcococcus*	-0.781
	-	-	-	-	-	-	*Ketogulonicigenium*	-0.727
1494	-	-	*Kineosporia*	0.72	-	-	-	-
	-	-	*Ramlibacter*	0.725	-	-	-	-
	-	-	*Rickettsiella*	0.726	-	-	-	-
1501	*Thermomicrobium*	0.637	-	-	*Lautropia*	-0.674	*Helcobacillus*	-0.712
	-	-	-	-	*Modestobacter*	-0.696	*Photobacterium*	-0.738
	-	-	-	-	*Oribacterium*	-0.65	-	-
	-	-	-	-	*Paracraurococcus*	-0.635	-	-
	-	-	-	-	*Pedomicrobium*	-0.696	-	-
	-	-	-	-	*Psychrobacter*	-0.65	-	-
1526	*Rhodoplanes*	0.64	*Thermomicrobium*	0.837	*Helcococcus*	-0.686	*Helcobacillus*	-0.76
	-	-	-	-	*Photobacterium*	-0.679	*Modestobacter*	-0.956
	-	-	-	-	*Polynucleobacter*	-0.684	*Oribacterium*	-0.768
	-	-	-	-	*Rathayibacter*	-0.682	*Paracraurococcus*	-0.872
	-	-	-	-	-	-	*Pedomicrobium*	-0.963
	-	-	-	-	-	-	*Solobacterium*	-0.751
1790	*Helcobacillus*	0.651	*Modestobacter*	0.802	*Thermomicrobium*	-0.677	-	-
	Oribacterium	0.685	*Pedomicrobium*	0.825	-	-	-	-
	Paracraurococcus	0.685	*Solobacterium*	0.799	-	-	-	-
	Photobacterium	0.661	-	-	-	-	-	-

	Psychrobacter	0.689	-	-	-	-	-	-
	Rathayibacter	0.653	-	-	-	-	-	-
1935	*Sporichthya*	0.659	-	-	-	-	-	-

Table 6-4. Correlation between bacteria and compounds between day 1 and day 4 for forearm at 0.001% significance.

	Positive correlation		Strong positive correlation		Negative correlation		Strong negative correlation	
Compound	**Bacteria**	**Correlation**	**Bacteria**	**Correlation**	**Bacteria**	**Correlation**	**Bacteria**	**Correlation**
851					*Schlegelella*	-0.689		
1180	*Schlegelella*	0.659			*Isoptericola*	-0.669	*Nocardia*	-0.718
							Rhodanobacter	-0.752
							Saccharopolyspora	-0.847
1200			*Nocardia*	0.737			*Schlegelella*	-0.711
			Rhodanobacter	0.743				
			Saccharopolyspora	0.907				
1232					*Solobacterium*	-0.657	*Selenomonas*	-0.746
1336			*Schlegelella*	0.783			*Kaistibacter*	-0.745
							Nocardia	-0.788
							Rhodanobacter	-0.836
							Saccharopolyspora	-0.934
							Sporocytophaga	-0.717
1354	*Schlegelella*	0.664					*Saccharopolyspora*	-0.827
1381							*Selenomonas*	-0.725
1494	*Kaistibacter*	0.689	*Nocardia*	0.789			*Schlegelella*	-0.718
	Sporocytophaga	0.675	*Rhodanobacter*	0.823				
			Saccharopolyspora	0.933				
1553					*Sphingobium*	-0.697		
1830	*Nocardia*	0.64						
	Rhodanobacter	0.659						

Table 6-5. Correlation between bacteria and compounds between day 1 and day 4 for upper back at 0.001% significance.

	Positive correlation		Strong positive correlation		Negative correlation		Strong negative correlation	
Compound	**Bacteria**	**Correlation**	**Bacteria**	**Correlation**	**Bacteria**	**Correlation**	**Bacteria**	**Correlation**
1200	*Nitrosospira*	0.616	*Thermus*	0.75	-	-	-	-
1232	-	-	-	-	*Treponema*	-0.647	*Selenomonas*	-0.754
	-	-	-	-	-	-	-	-
1331	-	-	-	-	-	-	*Selenomonas*	-0.866
1381	-	-	-	-	*Treponema*	-0.65	*Selenomonas*	-0.81
1494	*Thermus*	0.652	-	-	-	-	-	-

6.5 Discussion

In Chapter 5, the associations between volatiles and bacteria were discussed for the samples collected from feet only, based on the behavioural results from Chapter 2 which demonstrated that *An. stephensi* mosquitoes were more attracted to foot odour on day 4 than day 1. Although the samples collected from the axilla, upper back and forearm did not significantly change the behaviour of *An. stephensi* from day 1 to day 4, they did still elicit a significant behavioural response in mosquitoes. Therefore, volatile compounds were present that are used in host location and correlations between the volatiles and bacteria may indicate which bacteria are responsible for the production of certain host attractants. The results from this correlation analysis could also be important for the attractiveness of other mosquitoes species that have different preferential biting sites. Shirai *et al.*, (2002) tested the preferred body sites for *A. albopictus* and found that mosquitoes had a preference for feet, the second preferred landing site was the hand, followed by the face. Similarly, Self (1969) tested the preferred biting sites of *C. pipiens fatigans* and found that the lower leg was slightly more attractive than the forearm, however, other sites such as the back, abdomen, shoulder and chest also had considerable number of bites.

Out of the ten most abundant bacteria for all sites, *Staphylococcus, Corynebacterium, Acinetobacter, Streptococcus, Finegoldia, Anaerococcus* and *Paracoccus* were present in all sites. *Brevibacterium, Aerococcus* and *Pseudomonas* were among the ten most abundant for forearm and upper back only. This is consistent with other studies which have shown that the axilla is dominated by *Staphylococcos* and *Corynebacterium*. However, the axilla of some individuals may also be dominated with *Propionibacterium* (Taylor *et al.* 2003). For the forearm, one study found that *Proteobacteria* and *Bacteroidetes* (at phyla level) dominate, and the upper back is dominated by *Propionibacteriaceae*. Generally, few studies have been done to investigate bacteria on different body sites, therefore, it is difficult to compare the relative abundance from this study to the results of others (Grice & Segre 2011).

6.5.1 Differences in bacteria between day 1 and day 4

In this study, only *Moraxella* was significantly different between day 1 and day 4 on the axilla. For forearm, three bacteria significantly increased between day 1 and day 4, whereas 10 bacteria significantly decreased between day 1 and day 4. For the upper back, only one bacteria significantly increased between day 1 and day 4. It is surprising that there were few bacteria that significantly changed between day 1 and day 4 for axilla and forearm as the axilla is a particularly odourous area. The axilla is a partially occluded area that may be in touch with clothing everyday but is unlikely to be in contact with other objects, whereas the forearm has a higher probability of being in contact with objects other than clothing. Therefore it is likely that the small number of bacteria that significantly changed cannot be attributed to this. Interestingly, Fierer *et al.* (2010) sequenced residual skin bacteria left on surfaces, and showed that bacteria recovered from surfaces retain the community structure, even after two weeks. This could suggest that objects that the volunteers were in contact may pick up bacteria from other surfaces, however, this would likely be affected by washing regimes. Based on the results from Chapter 2, there was no difference in mosquito attractiveness between day 1 and day 4, which suggests the bacteria that do change have little effect on the production of compounds that modify mosquito behaviour.

6.5.2 Correlation with bacteria and compounds

The correlation of bacteria and compounds for axilla was predominately negative, however, the correlations were equally positive and negative for forearm and upper back. The axilla had the most correlations compared to the forearm and the upper back. This is an expected result due to axillae being extremely odorous because of the high density of glands, furthermore, all three types of glands are present.

For axilla, the following compounds had an EAG response and were also correlated with bacteria: peak number 18 (indene, 3-methylphenylacetylene or p-ehtynnytoluene; RI 1022), peak number 28 (menthol; RI 1172), peak number 30 (dodecane; RI 1200), peak number 34 (triacetin; RI 1301), peak number 37 (dodecanal; RI 1404), peak numbers 40, 52 (all of which are unidentified). For forearm, the following compounds had an EAG response and were also correlated with bacteria:

peak number 6 (ethylbenzene; RI 851), peak number 33 (unidentified; RI 1232), peak number 35 (unidentified; RI1354). For upper back, the following compounds had an EAG response and were also correlated with bacteria: peak number 30 (dodecane; RI 1200) and peak number 33 (unidentified; RI 1232). This suggests that these compounds are important for the general attractiveness of these body sites.

Peak number 30 (dodecane; RI 1200) was correlated for all sites, however, it was correlated with different bacteria, for axilla it was correlated with *Kineospora* and *Legionella*, for forearm it was correlated with *Schlegelella, Nocardia, Rhodanobacter* and *Saccharopolyspora* and for upper back it was correlated with *Nitrosospira* and *Thermus.* Similarly, peak number 33 (unidentified; RI 1232) was correlated for both forearm (correlated with *Solobacterium* and *Selenomona*s) and upper back (was correlated with *Treponema* and *Selenomonas*). It is surprising that the same compounds are not correlated with the same bacteria at different body sites, however, this could be further evidence that the relationship between compounds and bacteria is highly complex and that volatile production is likely caused by various different bacteria or groups of bacteria. It could also mean that there a is not a causal mechanisms between these bacteria and compounds.

Interestingly, in the forearm correlation, three bacteria were correlated together with various compounds. *Nocardia, Rhodanobacter* and *Saccharopolyspora* were positively correlated with compounds RI 1200 and RI 1494, and negatively correlated with compounds RI 1180 and RI 1336. This may suggest one or more of these three bacteria produce both compounds RI 1200 and RI 1494, however, because they are negatively correlated with RI 1180 and RI 1336, these three bacteria might be competing with other bacteria that produce those compounds, or they could utilise these compounds for growth. In fact, *Schlegelella* was correlated to the same compounds as the three of bacteria, for example, when the group of bacteria was correlated positively, *Schlegelella* was correlated negatively and vice versa. This suggests that these bacteria are in direct competition with each other which affect the compounds being produced. *Schlegelella* has not been associated with skin in previous study, one study isolated from a hot spring (Chou *et al.* 2007).

Some compounds that were EAG active and correlated with bacteria had been previously associated as human derived compounds. Peak number 28 (menthol, RI 1172) was found to be EAG active and was also correlated with bacteria in this study. Logan *et al.* (2008) collected it from whole body entrainments and was also found it to be EAG active to *A. aegypti*. Also, peak number 30 (dodecane; RI 1200) has been previously identified form axillary samples (Curran *et al.* 2005), however there have been no studies that have investigated the behavioural response to dodecane. Similarly, peak number 37 (dodecanal; RI 1404) has also been previously found from the hands, upper back, forearm and whole body entrainments. Dodecanal has been associoated with malaria infected individuals (De Boer *et al.* 2017) In this study, it was correlated with bacteria with axilla, forearm and upper back, which could suggest that it is an important compound for the overall attractiveness (Bernier *et al.* 2000; Dormont, Bessière & Cohuet 2013; de Lacy Costello *et al.* 2014). Other compounds including peak number 6 (ethylbenzene; RI 851), peak number 34 (triacetin; RI 1301) and peak number 18 (indene, 3-methylphenylacetylene or p-ehtynnytoluene; RI 1022) have either not been associated with human odour previously or have fragrance and flavour origins. They have also not been associated with mosquitoes. This therefore suggests that it could take more than 4 days to fully remove traces or cosmetic products, however, this is likely dependent on the compound (Fiume, 2003).

General attractiveness that is detected by mosquitoes from a distance is likely to be influenced by all body sites, it is therefore likely that when the mosquito is at a closer range they are able to distinguish between the sites. The results from this chapter demonstrate that the axilla, forearm and upper back produce important compounds for mosquitoes. This is the first time to our knowledge that the attractiveness of the forearm, the upper back and the axilla have been investigated in relation to skin bacteria.

6.6 Conclusion

The correlation identified associations between bacteria and volatiles, and some of the volatiles were found to be EAG active in chapter 3. For axilla, peak numbers 18 (indene, 3-methylphenylacetylene or p-ethynytoluene; RI 1022), peak number 28 (menthol; RI 1172), peak number 30 (dodecane; RI 1200), peak number 34 (triacetin; RI 1301) were EAG active and correlated with bacteria, for the forearm, compounds peak number 6 (ethylbenzene; RI 851), peak number 30 (dodecane; RI 1200), peak number 33 (unidenfied; RI 1232), and peak number 35 (unidenfied; RI 1354) were EAG active and correlated with bacteria and for the upper back, peak number 30 (dodecane; RI 1200) and peak number 33 (unidenfied; RI 1232) were also EAG active and correlated with bacteria.

While the attractiveness did not change for these body sites between day 1 and day 4 after washing, these associations are still important for the overall body attractiveness. This chapter further highlights the complex nature of the interactions between compounds and bacteria, where some certain bacteria influence the presence of other bacteria, and therefore the compounds being produced. The correlations investigated could be used for future work where a casual relationship between bacteria and volatiles is determined, specifically for compounds that are important for mosquito attractiveness. Furthermore, these associations could be important for other mosquitoes species that have a different biting preference sites, however, further research investigating this is needed.

CHAPTER 7

7 Discussion

The aim of this thesis was to identify the role of skin microbiota on mosquito attractiveness to human beings by investigating the correlation of bacteria and volatiles of various body sites in volunteers that were asked to comply with a washing regime. Volunteers' feet increased in attractiveness to *An. stephensi* between day 1 and day 4, however the other sites (axilla, forearm and upper back) did not. The volatile samples were pooled together according to the body site and visit and tested with GC-EAG, 52 compounds were found to be EAG active across all sites. The samples were then individually analysed with GC, and bacteria samples were sequenced with 16S rRna, and a correlation was done for each body site. Over 60 bacteria significantly changed between day 1 and day 4 for feet, however fewer bacteria significantly changed for the other sites (1 bacteria for axilla, 3 for forearm and 10 for the upper back). Furthermore, the correlations for feet revealed that the following compounds: Ethyl-cyclohexane (RI 849), 2-nonanal (RI 1134), and unidentified 7 (RI 1232), menthol (RI 1172) unidentified 19 (RI 1712) and unidentified 21 (RI 1812), were associated with *Phascolarctobacterium, Tyzzerella, Sutterella, Turicella, Schlegelella, Oryzihumus, Parabacteroides, Megasphaera, Shingopxis, Paludibacter, Ralstonia, Tuberibacillus* and *Peptococcus*.

Associations were found between bacteria and compounds, however, it is difficult to say at this stage if the bacteria are wholly responsible for the compounds found. To determine if the associations are true, the bacteria would have to be isolated and entrained to determine if they do emit the compounds present. In order to do this, bacteria would have to be also investigated at species level as compounds produced are likely different for different species in the same genus. Although compounds were associated with bacteria in this study, some could have different origins that may not necessarily be bacterial. This study found that 2-nonenal was correlated with bacteria, however Haze *et al.* (2001) associated it with lipid breakdown. It is probable that this is the case for other compounds that were associated in this study. It could also be

possible that the compound is produced by both bacteria and lipid breakdowns, which all contribute to overall human odour.

Some compounds identified in this study as EAG active have not been previously identified as human derived compounds. It is possible that these compounds are contaminants from clothing, the environment where the entrainments took place, cosmetic use, and contact with everyday object such as the floor or shoes. Some were seen on both visits, which suggests that cosmetic residues take longer to fade from the skin, this is likely dependent on the compound in question. Verhulst *et al.* (2016) found that deodorant compounds were not present in the axilla after 5 days of not washing This could be associated with the time it takes for epidermal turnover, which has been reported to be as long as 47 days and the turnover of the straum corneum upper layer which has been reported to be 14 days (Iizuka, 1994; Halprin, 1972; Hoath & Leahy 2003) However, it also possible that these compounds could be newly identified human derived compounds.

The following EAG active compounds were correlated with bacteria for foot but not for axilla, forearm and upper back: RI 841 (ethyl-cyclohexane), RI 1134 (2-nonenal or camphor), RI 1712 (unidentified) and RI 1812 (unidentified). This suggests that these compounds, and their correlated bacteria, may be responsible for the increased attraction between day 1 and day 4 after washing.

The differential attractiveness of different body sites has been investigated previously. Some studies have suggested that biting sites are influenced by convection currents rather than the skin bacteria present, rather than being influenced by bacteria (Dekker *et al.* 1998; Verhulst *et al.* 2016). This study supports the theory that the difference in biting sites is due to bacteria and the compounds produced as there was an increase in bacteria between day 1 and day 4. Furthermore, over 60 bacteria significantly changed between day 1 and day 4 for foot and this was much higher than the other body sites. This is likely to be associated with the behavioural results obtained in Chapter 2, where no behavioural difference was observed to volatiles from all the body sites except foot. It suggests that the bacteria that changed from day 1 and day 4 in axilla, back and forearm are not involved in the production of volatiles that change a person's attractiveness when they don't wash. Some compounds found in all sites were

correlated to different bacteria. This could be due to the different glands present at the different sites, which could cause complex interactions between various bacteria and compounds.

Previous studies have shown that bacteria produce compounds that are attractive to mosquitoes, the compounds associated with the attractive bacteria were not identified in this study (Verhulst *et al.* 2009). The bacteria were grown on agar plates and then entrained, however, agar plates do not support the growth of all bacteria found on the skin as they are not capable of supporting all the microorganisms in the sample due to the different growth conditions needed for different species (Gao *et al.* 2007). This could explain the difference in compounds found in both studies.

Skin microbiota remain relatively stable over time, where some sites are more stable than others (Smallegange *et al.* 2011). Studies that have tested this have allowed normal washing regimes where the samples were collected over a period of days or weeks (Costello *et al.* 2009). In this study, we found that the bacteria on the axilla, forearm and upper back did remain stable, even after a few days of not washing, furthermore the attractiveness did not increase. However, for feet, bacteria and attractiveness significantly increased between the sampling dates, which highlights the importance of maintaining clean feet to reduce the number of mosquito bites. It would be useful to investigate the effect of the use of old shoes and clothing on mosquito attractiveness, as based on the results from this study, mosquito attractiveness could be higher compared to a baseline level with clean shoes and clothing. This finding is important in areas where malaria is prevalent and transmitted by *An. stephensi* where foot washing practices would impact mosquito attractiveness. Living in an area with a warmer climate than London (where this study took place) might replicate and accelerate the increase in attractiveness. However, if people in warmer climates wear sandals more than occluded shoes, their mosquito attractiveness may not increase compared to someone wearing occluded shoes.

Anopheles stephensi are zoophilic mosquitoes, and they have been found in high densities in cattle sheds and they feed on cattle (Thomas *et al.* 2017; Edalat *et al.* 2015). The body odour of cattle and human beings is very different due to the difference in type, distribution and density of glands. Human beings have eccrine

glands all over the body, whereas in cattle, they are limited to the feet and tail. In human beings, apocrine glands are thought to play a role in pheromone production, but it cattle they are found over the entire body and play a role in evaporative cooling (Smallegange *et al.* 2011; Grice & Segre 2011). It is possible that some compounds are released by both humans and cattle, for example, 1-octen-3-ol has been identified in both human and cattle odour profiles and is an important cue for mosquitoes (Bernier *et al.* 2000). Further research that investigates compounds present in both human beings and cattle could be useful for trap development.

This study focussed on the associations between bacteria and volatiles, however, other elements of the skin microflora could also affect mosquito attractiveness such as yeasts and funguses which are often overlooked when associating bacteria with mosquito attractiveness. Eleven yeast species have been found on the skin of healthy humans, and are especially present in sebaceous sites (Wilson, 2008). Human opportunistic pathogenic yeasts have -been found to produce (E,E)-farnesol, and the fungus *Trichoderma* which is found in soil, has been associated with 1-octen-3-ol, a mosquito semiochemical. Although this fungus is not found on the human skin, this highlights the potential of compounds produced by funguses (Schmidt *et al.* 2015). Therefore, if they were to play a role in the production of compounds and mosquito attractiveness, this would likely affect the upper half of the torso which have the most sebaceous glands. The role of yeast and funguses needs to be further investigated in relation to mosquito attractiveness.

The human derived compounds that were EAG active for all body sites could be further investigated to develop a lure. This is particularly useful as this is the first time bacteria and compounds have been associated in relation to mosquito attractiveness with *An. stephensi*. Most studies have investigated the semiochemicals of *An. gambiae.* However, if a trap is attractive to mostly anthropophilic mosquitoes, other zoophilic mosquitoes may remain in the area as they are attracted to semiochemicals emitted by livestock. Therefore, the advantage of using a lure that is attractive to *An. stephensi* mosquitoes could reduce zoophilic mosquito species populations.

7.1 Study caveats

7.1.1 Volunteer participation

Volunteers were asked to follow a strict protocol when they were included in the study. They were asked to adhere to a washing regime and to limit certain foods, smoking and alcoholic drinks 24 hours before sampling. Because they were allowed to go home in between sampling days, this study relied on their honesty to adhere to the protocol, they may have not adhered to the protocol thus potentially affecting the results. However, 30 participants were recruited which would account for variation. Furthermore, this study only used men as participants. Men's skin is more acidic than women, which results in women having a higher microbiota diversity than men (Fierer *et al.* 2008). Certain compounds ((R)/(S)-3-Methyl-3-Sulfanylhexan-1-Ol and (R)/(S)-3-Hydroxy-3-Methyl-Hexanoic Acid) linked to malodour have been found to differ between men and women and this has been linked to skin microbiota (Troccaz *et al.* 2009). It is therefore likely that women could have different results from men, however it is unknown if this would change the attractiveness to mosquitoes. Studies have shown that men are more attractive than women to *Aedes aegpypti,* however this could be because men are larger than women, thus they have a larger surface, and *An. gambiae* do not discriminate between men and women. (Smallegange *et al.* 2011).

7.1.2 Volatile collection

The use of headspace entrainment allowed this study to collect many volatiles from the body sites. The samples were very complex, and many peaks were present in very small amounts, making identification difficult. Other studies have used different methods to collect volatiles from individuals including thermal desorption, solvent washing and SPME. Bernier *et al.* (2000) collected over 300 compounds using thermal desorption followed by GC-MS. However, their method could not be used for this study as the samples were needed multiple times for compound identification via GC and for GC-EAG and SPME samples are destroyed upon analysis. Studies have also used SPME to collect volatiles from individuals, however, similar to thermal desorption, the

sample can only be used once (Gallagher *et al.* 2008). Solvent extraction has also been previously used to extract volatiles, however, the use of solvents on direct skin would have likely affected the skin microbiota. Selecting different sites for solvent and bacteria collection could have been feasible for larger body sites such as the upper back or the forearm, however, this would have been difficult to achieve in smaller areas such as the axilla.

7.1.3 GC-EAG

The mosquitos' antennae are highly sensitive, indeed over 50 compounds were detected with GC-EAG, it is possible that this method was oversensitive and some compounds detected are not involved in host location, but were active as they could be important for other behaviours, such as ovipositioning. Furthermore, there was a notable absence of carboxylic acids detected with GC-EAG. Previous studies have found that they are important kairomones for *Anopheles* mosquitoes (Smallegange *et al.* 2010), it was therefore surprising that none were detected with EAG. Further analysis of the samples revealed that butanoic acid, hexanoic acid, pentanoic acid, dodecanoic acid and tetradecanoic acid were present in the samples, which have all been shown to be important mosquito kairomones (Smallegange *et al.* 2009). However, they were present in very small amounts and had lots of background, which may explain why they were not detected with GC-EAG. The type of column used could have also had an effect on the compounds detected with GC-EAG. Furthermore, the GC-EAG method was shorter than the method used to identify compounds (40 minutes vs 70 minutes). This was necessary to ensure the mosquito head was viable for the duration of the run. This meant that the compounds were being detected in closer proximity. For example, one compound that elicited a response could have caused habituation or hypersensitivity of the olfactory system by not allowing the olfactory organs to fully recover, consequently, another compound may have been undetected by the antennae. Furthermore, the shorter method used for GC-EAG could also explain why some compounds in the samples co-eluted. Other factors that could have also influenced the EAG responses observed include the concentration of the compound in the sample or the mosquitoes fitness. Some compounds that were detected by GC-

EAG, were unidentified with GC-MS. The quality of these compounds may have been in too small amounts and below detection thresholds with GC-MS, but still detectable with GC-EAG. The compounds may also have co-eluted with other compounds, making identification difficult. Further research is needed where the identified compounds from this study are tested individually with EAG.

7.2 Future research

The next steps would be to isolate the bacteria that have been associated with volatiles that are important for mosquitoes and test them, behaviourally in the laboratory and identify the volatiles they produce. Once identified, a novel intervention could be developed where specific bacteria could be targeted, therefore, reducing attractiveness to mosquitoes. Species-specific antimicrobials that work by targeting the peptide nucleic acid (PNA) have successfully targeted specific species in other studies, including *Bacillus subtilis, Salmonella typhimurium, E. coli, Klebsiella Pneumoniae* and *Staphylococcus aureus*, (Good & Nielsen 1998; Nekhotiaeva *et al.* 2004; Mondhe *et al.* 2014). Furthermore, this study correlated bacteria and volatiles at the genus level of bacteria only. The results revealed complex associations between compounds and bacteria, therefore, it is possible that by investigating these associations at species level, it could narrow down the associations.

Mosquito attractiveness has been shown to have a genetic component (Fernández-Grandon *et al.* 2015). Future research that investigates the skin microbiota of people who carry the 'attractive' or the 'unattractive' gene could be used in creating novel interventions. To further understand the metabolic pathways bacteria undergo to produce body odour, metatranscriptonomic sequencing could be used. This would also allow an understanding of the extent to which specific genes may play a role in skin microbiota compositions. However, at present, metatranscriptomic technology is not very developed and methods are expensive (Fredrich *et al.* 2013)

Some of the compounds detected in this study could be attractants or repellents for *Anopheles* mosquitoes, especially the compounds that have never been associated with mosquitoes before. The next stage for this research could be to test putative

kairomones in the field as odour baits in traps as a control method. It is important that experiments are carried out in non-laboratory conditions after they have been tested in the laboratory, as laboratory conditions may not reflect the true conditions found in the field. Laboratory mosquito strains may respond differently to wild mosquito populations and synthetic odour blends tested in the laboratory are limited to short-range behavioural responses. Effective odour-baits are dependent on the attractant used, the design of the trap, which mechanism it utilises and the feasibility of the cost involved (Njiru *et al.* 2006).

The use of synthetic odour-blend traps can have an advantage over other trapping methods by being cheaper and easy to reproduce. For the traps to be cost effective, the odour-blends must be released at a steady rate and be stable over time. Low density polyethylene (LDPE) material has been used previously in experiments, however nylon strips have been shown to be more effective at trapping mosquitoes, furthermore, they can last up to 40 nights after application (Mukabana *et al.* 2012). The addition of CO_2 improves the number of catches in odour-baited traps (Jawara *et al. 2011)*. Odour baits have been shown to be an effective novel tool for malaria control (Hiscox *et al.* 2012; Homan *et al.* 2016).

7.3 Conclusion

Previous studies have investigated the involvement of skin microbiota to mosquito attractiveness, however, studies that have investigated this, have selected abundant bacteria found on the skin and testing them behaviourally with mosquitoes. There are no studies that have investigated the overall associations of bacteria and volatiles across different sites in relation to mosquitoes. This thesis investigated this question by asking volunteers to comply with a washing regime and collecting bacterial and volatile samples on two separate days. Odours were also collected on stockings and tested behaviourally with *An. stephensi*. GC-EAG was then used to test the electrophysiological response of each body site and visit. Finally, the bacteria and compounds were sequenced and analysed with GC and a correlation was done. Mosquito attractiveness increased only for feet, and over 50 compounds were EAG active, some of which were identified with GC-MS. The correlation for foot revealed that six compounds (ethylcyclohexane, 2-nonanal, menthol and 3 unidentified) that were EAG active were correlated with bacteria (*Phascolarctobacterium, Tyzzerella, Sutterella, Turicella, Schlegelella, Oryzihumus, Parabacteroides, Megasphaera, Shingopxis, Paludibacter, Ralstonia, Tuberibacillus* and *Peptococcus)*.

8 References

Acree et al., 1968. L-lactic acid: a mosquito attractant isolated from humans. Science. 161(3848):1346-7.

Adams, S.E. et al., 2017. A randomised clinical study to determine the effect of a toothpaste containing enzymes and proteins on plaque oral microbiome ecology. *Scientific Reports*, 7.

Alam, M.T. et al., 2008. The type and mysorensis forms of the *Anopheles stephensi* (Diptera: Culicidae) in India exhibit identical ribosomal DNA ITS2 and domain-3 sequences. *Parasitology Research*, 103(1), pp.75–80.

Alphey, L. et al., 2010. Sterile-Insect Methods for Control. , 10(3).

Anjomruz, M. et al., 2014. ABO blood groups of residents and the ABO host choice of malaria vectors in southern Iran. *Experimental parasitology*, 136, pp.63–7.

Ansell, J. et al., 2002. Short-range mosquitoes attractiveness of pregnant women to *Anopheles gambiae. Transactions of the Royal Society of Tropical Medicine and Hygiene*, 96, pp.113–116.

Bass, C. et al., 2007. Detection of knockdown resistance (kdr) mutations in *Anopheles gambiae*: a comparison of two new high-throughput assays with existing methods. *Malaria journal*, 6, p.111.

Bernier, U.R. et al., 2000. Analysis of Human Skin Emanations by Gas Identification of Volatile Compounds That Are Candidate Attractants for the Yellow Fever Mosquito (*Aedes aegypti*) Anal Chem. 72 (4):747-56.

Bhatt, S. et al., 2012. The global distribution and burden of dengue. *NIH-PA Author Manuscript Nature*, 496(7446), pp.504–507.

De Boer, J.G. et al., 2017. Odours of Plasmodium falciparum-infected participants influence mosquito-host interactions. *Scientific Reports*, 7(1).

Braack, L. et al., 2015. Biting behaviour of African malaria vectors: 1. where do the main vector species bite on the human body? *Parasites & vectors*, 8(1), p.76. Available at:

Braks, M.A.H. & Takken, W., 1999. Incubated human sweat but not fresh sweat attracts the Malaria mosquito A*nopheles gambiae* sensu stricto. *Journal of Chemical Ecology*, 25, pp.663–672.

Bryan, J.H. & Smalley, M.E., 1978. The use of abo blood groups as markers for mosquito biting studies. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 72(4), pp.357–360.

Burton et al., 2011. The effect of handwashing with water or soap on bacterial contamination of hands. *International Journal of Environmental Research and Public Health*. 8(1): 97-104.

Cameron, M.M. & Lorenz, L.M., 2013. *Biological and Envrionmental Control of Disease Vectors* M. M. Cameron & L. M. Lorenz, eds., Wallingford: CABI.

Carvalho, D.O. et al., 2014. Mass production of genetically modified *Aedes aegypti* for field releases in Brazil. *Journal of visualized experiments : JoVE*, (83), p.e3579.

Chakravorty et al., 2007. A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. *Journal of Microbiology Methods*. 69(2), pp.330-9.

Chen-Hussey, V. et al., 2013. Can topical insect repellents reduce malaria? A cluster-randomised controlled trial of the insect repellent N,N-diethyl-m-toluamide (DEET) in Lao PDR. *PloS one*, 8(8), p.e70664.

Chou et al., 2007. *Schlegelella aquatica* sp nov., a novel thermophilic bacterium isolated from a hot spring. I*nternational Journal of Systematic and Evolutionary Microbiology.* 56(12):12793-7.

Clements, A., 1992. *The Biology of Mosquitoes: Development, Nutrition and Reproduction v.1*, CABI.

Cork, A. & Park, K.C., 1996. Identification of electrophysiologically-active compounds for the malaria mosquito, *Anopheles* gambiae, in human sweat extracts. *Medical and veterinary entomology*, 10, pp.269–276.

Cork, a & Park, K.C., 1996. Identification of electrophysiologically-active compounds for the malaria mosquito, *Anopheles gambiae,* in human sweat extracts. *Medical and veterinary entomology*, 10(3), pp.269–276.

Costantini, C. et al., 2001. Electroantennogram and behavioural responses of the malaria vector *Anopheles gambiae* to human-specific sweat components. *Medical and veterinary entomology*, 15(3), pp.259–66.

Costello, E.K. et al., 2009. Bacterial community variation in human body habitats across space and time. *Science (New York, N.Y.)*, 326(5960), pp.1694–7.

Cotter, C. et al., 2013. The changing epidemiology of malaria elimination: New strategies for new challenges. *The Lancet*, 382(9895), pp.900–911.

Curran, A.M. et al., 2005. Comparison of the Volatile Organic Compounds Present in Human Odor Using Spme-GC/MS. *Journal of Chemical Ecology*, 31(7), pp.1607–1619.

Daisy, B.H. et al., 2002. Naphthalene, an insect repellent, is produced by *Muscodor vitigenus,* a novel endophytic fungus. *Microbiology*, 148(11), pp.3737–3741.

Dekker, T. et al., 1998. Selection of biting sites on a human host by A*nopheles gambiae* s.s., An. arabiensis and An. quadriannulatus. *Entomologia Experimentalis et Applicata*, 87, pp.295–300.

Dekker, T., Steib, B. & Carde, R.T., 2002. Lactic acid: a human-signifying host cue for the anthropophilic mosquito *Anopheles* gambiae. , pp.91–98.

Dormont, L., Bessière, J.-M., McKey, D., et al., 2013. New methods for field collection

of human skin volatiles and perspectives for their application in the chemical ecology of human-pathogen-vector interactions. *The Journal of experimental biology*, 216(Pt 15), pp.2783–8.

Dormont, L., Bessière, J.-M. & Cohuet, A., 2013. Human skin volatiles: a review. *Journal of chemical ecology*, 39(5), pp.569–78. Available at:

Edalat, H. et al., 2015. Vectorial capacity and Age determination of *Anopheles Stephens Liston* (Diptera: Culicidae), during the malaria transmission in Southern Iran. *Journal of Entomology and Zoology Studies JEZS*, 256(31), pp.256–263.

Feldmann, AM & Ponnudarai, TE, 1989. Selection of *Anopheles stephensi* for refractoriness and susceptibility to Plasmodium falciparum. *Medical and Veterinary Entomology*, 3(1), pp.41–52.

Fernández-Grandon, G.M. et al., 2015. Heritability of attractiveness to mosquitoes. *PLoS ONE*, 10(4).

Fernstad, S.J. et al., 2011. Visual Exploration of Microbial Populations. *2011 IEEE Symposium on Biological Data Visualization (BioVis)*, pp.127–134.

Fierer, N. et al., 2010. Forensic identification using skin bacterial communities. *Proceedings of the National Academy of Sciences of the United States of America*, 107(14), pp.6477–81.

Fierer, N. et al., 2008. The influence of sex, handedness, and washing on the diversity of hand surface bacteria. *Proceedings of the National Academy of Sciences of the United States of America*, 105(46), pp.17994–9. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2584711&tool=pmcentrez&rendertype=abstract.

Fiume, M.Z., 2003. Final report on the safety assessment of triacetin. *International Journal Of Toxicology*, 22 Suppl 2, pp.1–10.

Fredrich, E. et al., 2013. Daily battle against body odor: towards the activity of the axillary microbiota. *Trends in microbiology*, 21, pp.305–12.

Frei, J. et al., 2017. Behavioral response of the malaria mosquito, *Anopheles* gambiae, to human sweat inoculated with axilla bacteria and to volatiles composing human axillary odor. *Chemical senses*, 42(2), pp.121–131.

Gallagher, M. et al., 2008. Analyses of volatile organic compounds from human skin. *British Journal of Dermatology*, 159(4), pp.780–791.

Gao, Z. et al., 2007. Molecular analysis of human forearm superficial skin bacterial biota. *Proceedings of the National Academy of Sciences of the United States of America*, 104, pp.2927–2932.

Geier et al., 1996. A search for components in human body odour that attracts females of *Aedes aegypti.* Olfaction in mosquito host interactions. *Ciba Foundation Symposium.* 200: 132-148.

Geier, M., Bosch, O.J. & Boeckh, J., 1999. Ammonia as an attractive component of host

odour for the yellow fever mosquito, *Aedes aegypti*. *Chemical senses*, 24, pp.647–653.

Gibson, G. & Torr, S.J., 1999. Visual and olfactory responses of haematophagous Diptera to host stimuli. *Medical and veterinary entomology*, 13(1), pp.2–23.

Githeko, A. et al., 1993. *Plasmodium falciparum* sporozoite and entomological inoculation rates at the Ahero rice irrigation scheme and the Miwani sugar-belt in western Kenya. *Ann Trop Med Parasit*, 87.

Good, L. & Nielsen, P.E., 1998. Inhibition of translation and bacterial growth by peptide nucleic acid targeted to ribosomal RNA. *Biochemistry*, 95, pp.2073–2076.

Grice, E.A. & Segre, J.A., 2011. The skin microbiome. *Nature reviews. Microbiology*, 9, pp.244–253.

Grice, E. a et al., 2009. Topographical and temporal diversity of the human skin microbiome. *Science (New York, N.Y.)*, 324(5931), pp.1190–2.

Guerenstein, P.G. & Lazzari, C.R., 2009. Host-seeking: How triatomines acquire and make use of information to find blood. *Acta tropica*, 110(2–3), pp.148–58.

Guidobaldi, F., May-Concha, I.J. & Guerenstein, P.G., 2014. Morphology and physiology of the olfactory system of blood-feeding insects. *Journal of physiology, Paris*.

Gutiérrez-García, A.G. et al., 2007. Urine from stressed rats increases immobility in receptor rats forced to swim: Role of 2-heptanone. *Physiology and Behavior*, 91(1), pp.166–172.

Halprin, K.M., 1972. Epidermal turnover time - a re-examination. *Br. J. Derm.*, 86, pp.14–19.

Haze, S. et al., 2001. 2-Nonenal newly found in human body odor tends to increase with aging. *Journal of Investigative Dermatology*, 116(4), pp.520–524.

Healy, T.P. et al., 2002. Landing responses of *Anopheles gambiae* elicited by oxocarboxylic acids. *Medical and veterinary entomology*, 16(2), pp.126–32.

Healy, T.P. & Copland, M.J., 2000. Human sweat and 2-oxopentanoic acid elicit a landing response from *Anopheles gambiae. Medical and veterinary entomology*, 14(2), pp.195–200.

Healy, T.P. & Copland, M.J.W., 1995. Activation of *Anopheles gambiae* mosquitoes by carbon dioxide and human breath. *Medical and Veterinary Entomology*, 9(3), pp.331–336.

Hiippala, K. et al., 2016. Mucosal prevalence and interactions with the epithelium indicate commensalism of *Sutterella* spp. *Frontiers in Microbiology*, 7(OCT).

Hill, N. et al., 2007. Plant based insect repellent and insecticide treated bed nets to protect against malaria in areas of early evening biting vectors: double blind randomised placebo controlled clinical trial in the Bolivian Amazon. *BMJ (Clinical research ed.)*, 335(7628), p.1023.

Hiscox, A. et al., 2012. The SolarMal Project: innovative mosquito trapping technology for malaria control. *Malaria Journal*, 11(Suppl 1), p.O45.

Hoath, S.B. & Leahy, D.G., 2003. The Organization of Human Epidermis: Functional Epidermal Units and Phi Proportionality. *Journal of Investigative Dermatology*, 121(6), pp.1440–1446.

Homan et al., 2016. The effect of mass mosquito trapping on malaria transmission and disease burden (SolarMal): a stepped-wedge cluster randomised trial. Lancet. 388(10050):1193-201.

Iizuka, H., 1994. Epidermal turnover time. *Journal of Dermatological Science*, 8(3), pp.215–217.

Isberg et al., 2016. Identification of cattle-derived volatiles that modulate the behavioural response of the biting midge *Culicoides nubeculosus*. *Journal of Chemical Ecology.* 42(1):24-32.

Ives, A.R. & Paskewitz, S.M., 2005. Testing vitamin B as a home remedy against mosquitoes. *Journal of the American Mosquito Control Association*, 21, pp.213–217.

James, A. et al., 2013. Microbiological and biochemical origins of human axillary odour. *FEMS microbiology ecology*, 83(3), pp.527–40.

James, A.G., Cox, D. & Worrall, K., 2013. Microbiological and biochemical origins of human foot malodour. *Flavour and Fragrance Journal*, 28(4), pp.231–237.

Januszkiewicz, J. et al., 2008. Optimization of headspace solid-phase microextraction for the analysis of specific flavors in enzyme modified and natural Cheddar cheese using factorial design and response surface methodology. *Journal of Chromatography A*, 1195(1–2), pp.16–24.

Jawara, M. et al., 2011. Field testing of different chemical combinations as odour baits for trapping wild mosquitoes in The Gambia. *PLoS ONE*, 6(5):e19676.

Jawara, M. et al., 2009. Optimizing odor-baited trap methods for collecting mosquitoes during the malaria season in The Gambia. *PloS one*, 4(12), p.e8167.

De Jong, R. & Knols, B.G.J., 1995. Selection of biting sites on man by two malaria mosquito species. *Experientia*, 51(1), pp.80–84.

Jordan, a M., 1995. Control of tsetse flies (Diptera: *Glossinidae*) with the aid of attractants. *Journal of the American Mosquito Control Association*, 11(2 Pt 2), pp.249–55.

Josling, G.A. & Llinás, M., 2015. Sexual development in *Plasmodium* parasites: Knowing when it's time to commit. *Nature Reviews Microbiology*, 13(9), pp.573–587.

Kageyama, A. et al., 2005. *Oryzihumus leptocrescens* gen. nov., sp. nov. *International Journal of Systematic and Evolutionary Microbiology*, 55(6), pp.2555–2559.

Kai, M. et al., 2009. Bacterial volatiles and their action potential. *Applied Microbiology and Biotechnology*, 81, pp.1001–1012.

Kim, B.S., Lim, Y.W. & Chun, J., 2008. *Sphingopyxis marina* sp. nov. and *Sphingopyxis litoris* sp. nov., isolated from seawater. *International Journal of Systematic and Evolutionary Microbiology*, 58(10), pp.2415–2419.

Kirk, K.M. et al., 2000. Twin study of adolescent genetic susceptibility to mosquito bites using ordinal and comparative rating data. *Genetic epidemiology*, 19(2), pp.178–90.

Kline, D.L. et al., 1990. Field studies on the potential of butanone, carbon dioxide, honey extract, l-octen-3-ol, L-lactic acid and phenols as attractants for mosquitoes. *Medical and Veterinary Entomology*, 4(4), pp.383–391.

Knols, B.G., de Jong, R. & Takken, W., 1994. Trapping system for testing olfactory responses of the malaria mosquito *Anopheles gambiae* in a wind tunnel. *Medical and veterinary entomology*, 8(4), pp.386–388.

Knols, B.G.J. & Meijerink, J., 1997. Mosquito Behavior. *Science and Medicine,* 4(5), pp.56–63.

de Lacy Costello, B. et al., 2014. A review of the volatiles from the healthy human body. *Journal of breath research*, 8(1), p.14001.

Leal, W.S. & Uchida, K., 1998. Application of GC-EAD to the Determination of Mosquito Repellents Derived from a Plant, *Cymbopogon citratus*. *Journal of Asia-Pacific Entomology*, 1(2), pp.217–221.

Lefèvre, T. et al., 2010. Beer consumption increases human attractiveness to malaria mosquitoes. *PLoS ONE*, 5(3):e9546.

Leyden, J.J. et al., 1981. The microbiology of the human axilla and its relationship to axillary odor. *The Journal of investigative dermatology*, 77, pp.413–416.

Lindsay, S. et al., 2000. Effect of pregnancy on exposure to malaria mosquitoes. The Lancet .3;355(9219):1972.

Logan, J.G. et al., 2008. Identification of human-derived volatile chemicals that interfere with attraction of *Aedes aegypti* mosquitoes. *Journal of chemical ecology*, 34(3), pp.308–22.

Logan, J.G. et al., 2009. Identification of human-derived volatile chemicals that interfere with attraction of the Scottish biting midge and their potential use as repellents. *Journal of medical entomology*, 46(2), pp.208–19.

Logan, J.G. & Birkett, M.A., 2007. Semiochemicals for biting fly control : their identification and exploitation. *Pest Management Science,* 65(7), pp.647–657.

van Loon, J.J.A. et al., 2015. Mosquito Attraction: Crucial Role of Carbon Dioxide in Formulation of a Five-Component Blend of Human-Derived Volatiles. *Journal of Chemical Ecology*, 41(6), pp.567–573.

Maxwell, C.A. et al., 1998. Malaria-infective biting at different hours of the night. *Medical and Veterinary Entomology*, 12(3), pp.325–327.

Mburu et al., 2017. 2-Butanone as a carbon dioxide mimic in attractant blends for the

afrotropical malaria mosquitoes *Anopheles gambiae* and *Anopheles fenestus. Malaria Journal*. 16(1):351.

Meijerink, J. et al., 2000. Identification of olfactory stimulants for *Anopheles gambiae* from human sweat samples. *Journal of Chemical Ecology*, 26(6), pp.1367–1382.

Meijerink , J. et al., 2001. Olfactory receptors on the antennea of the malaria mosquito *Anopheles gambiae* are sensitive to ammonia and other sweat borne components. *Journal of insect physiology*. 47(4-5):455-64.

Menger et al., 2014. A push-pull system to reduce house entry of malaria mosquitoes. *Malaria Journal.* 13:119.

Menger et al., 2015. Field evaluation of a push-pull system to reduce malaria transmission. *PLos One, 10(4)*

Menger et al., 2016. Eave screening and push-pull tactics to reduce house entry by vectors of Malaria. *Am J Trop Med Hyg*. 94(4):868-78.

Mondhe, M. et al., 2014. Species-selective killing of bacteria by antimicrobial Peptide-PNAs. *PLoS ONE*, 9(2)e:89082.

Moraes, C.M. De et al., 2014. Malaria-induced changes in host odors enhance mosquito attraction. *Proc Natl Acad Sci.* 111(30):11079-84

Mukabana, W.R. et al., 2004. Allomonal effect of breath contributes to differential attractiveness of humans to the African malaria vector *Anopheles gambiae. Malaria Journal*, 3, pp.1–8.

Mukabana, W.R. et al., 2012. Evaluation of low density polyethylene and nylon for delivery of synthetic mosquito attractants. *Parasites & vectors*, 5(1), p.202.

Mukabana, W.R. et al., 2002. Host-specific cues cause differential attractiveness of Kenyan men to the African malaria vector *Anopheles gambiae*. *Malaria Journal*, 1(17).

Natsch, A. et al., 2005. Isolation of a bacterial enzyme releasing axillary malodor and its use as a screening target for novel deodorant formulations. In *International Journal of Cosmetic Science*. pp. 115–122.

Nekhotiaeva, N. et al., 2004. Inhibition of Staphylococcus aureus gene expression and growth using antisense peptide nucleic acids. *Molecular therapy : the journal of the American Society of Gene Therapy*, 10(4), pp.652–9.

Ng, T.W. et al., 2017. Skin squames contribute to ammonia and volatile fatty acid production from bacteria colonizing in air-cooling units with odor complaints. *Indoor Air*.(2):258-265.

Njiru, B. et al., 2006. Trapping of the malaria vector *Anopheles* gambiae with odour-baited MM-X traps in semi-field conditions in western Kenya. *Malaria Journal*, 5:39.

Nordlund, D.A. & Lewis, W.J., 1976. Terminology of chemical releasing stimuli in intraspecific and interspecific interactions. *Journal of Chemical Ecology*, 2(2),

pp.211–220.

Norlund, D., Jones, R. & Lewis, W., 1981. *Semiochemicals. Their role in pest control*, New York: John Wiley & Sons.

Nyasembe, V.O. et al., 2012. Behavioural response of the malaria vector *Anopheles gambiae* to host plant volatiles and synthetic blends. *Parasites & vectors*, 5(1), p.234.

Omolo, M.O. et al., 2013. Differential attractiveness of human foot odours to *Anopheles gambiae* Giles sensu stricto (Diptera: Culicidae) and variation in their chemical composition. *Acta tropica*, 128(1), pp.144–8.

Omrani, S.M. et al., 2012. Upwind responses of *Anopheles stephensi* to carbon dioxide and L-lactic acid: an olfactometer study. *Eastern Mediterranean health journal = La revue de santé de la Méditerranée orientale = al-Majallah al-ṣiḥḥīyah li-sharq al-mutawassiṭ*, 18(11), pp.1134–42.

Oshaghi et al., 2006. *Anopheles stephensi* biological forms; geographical distribution and malaria transmission in malarious regions of Iran. P*akistan Journal of Biological Sciences,* 9(2).

Otienoburu, P.E. et al., 2012. Analysis and Optimization of a Synthetic Milkweed Floral Attractant for Mosquitoes. *Journal of Chemical Ecology*, 38(7), pp.873–881.

Pates, H. V., Curtis, C.F. & Takken, W., 2014. Hybridization studies to modify the host preference of *Anopheles gambiae*. *Medical and Veterinary Entomology*, 28(SUPPL.1), pp.68–74.

Penn, D.J. et al., 2007. Individual and gender fingerprints in human body odour. *Journal of the Royal Society, Interface / the Royal Society*, 4(13), pp.331–40.

Phillips, M., 1997. Method for the collection and assay of volatile organic compounds in breath. *Analytical Biochemistry*, 247(2), pp.272–278.

Port, G., Boreham, P. & Bryan, J., 1980. The relationship of host size to feeding by mosquitoes of the *Anopheles gambiae* Giles complex (Diptera : Culicidae). *Bulletin of Entomological Research*, 70(L 3631), pp.133–144.

Prada, P. a., Curran, A.M. & Furton, K.G., 2010. Comparison of extraction methods for the removal of volatile organic compounds (VOCs) present in sorbents used for human scent evidence collection. *Analytical Methods*, 2(5), p.470.

Puri, S.N. et al., 2006. Electroantennogram and behavioral responses of *Culex quinquefasciatus* (Diptera: Culicidae) females to chemicals found in human skin emanations. *Journal of medical entomology*, 43(2), pp.207–13.

Qiu, Y. et al., 2004. Behavioural and electrophysiological responses of the malaria mosquito *Anopheles gambiae* Giles sensu stricto (Diptera: Culicidae) to human skin emanations. *Medical and Veterinary Entomology*, 18(4), pp.429–438.

Qiu, Y.T. et al., 2011. Behavioural responses of *Anopheles* gambiae sensu stricto to components of human breath, sweat and urine depend on mixture composition

and concentration. *Medical and veterinary entomology*, 25(3), pp.247–55.

Qiu, Y.T. et al., 2004. GC-EAG analysis of human odours that attract the malaria mosquito *Anopheles gambiae* sensu stricto. *Proceedings of the Netherlands Entomological Meeting*, 15, pp.59–64.

Qiu, Y.T. et al., 2006. Interindividual variation in the attractiveness of human odours to the malaria mosquito *Anopheles gambiae s. s*. *Medical and veterinary entomology*, 20(3), pp.280–7.

Qiu, Y.T. & van Loon, J.J., 2010. Olfactory physiology of blood-feeding vector mosquitoes. In W. Takken & B. G. J. Knols, eds. *Olfaction in vector-host interactions*. Wageningen: Wageningen Academic Publishers.

Ranson, H. et al., 2011. Pyrethroid resistance in African anopheline mosquitoes: What are the implications for malaria control? *Trends in Parasitology*, 27(2), pp.91–98.

Reynolds, S.J., Behr, M. & McDonald, J., 2001. Turicella otitidis as an unusual agent causing a posterior auricular abscess. *Journal of Clinical Microbiology*, 39(4), pp.1672–1673.

Roberts, S.C. et al., 2005. Body odor similarity in noncohabiting twins. *Chemical senses*, 30(8), pp.651–6.

Roberts, S.C. et al., 2008. MHC-correlated odour preferences in humans and the use of oral contraceptives. *Proceedings. Biological sciences / The Royal Society*, 275(1652), pp.2715–22.

Rowland, M., 1989. Changes in the circadian flight activity of the mosquito *Anopheles stephensi* associated with insemination, blood-feeding, oviposition and nocturnal light intensity. *Physiological Entomology*, 14(1), pp.77–84.

Rowland, M et al., 2002. Anopheline vectors and malaria transmission in eastern Afghanistan. *Trans R Soc Trop Med Hyg,* 96(6):pp. 620-6.

Russell, R.C., 2004. The relative attractiveness of carbon dioxide and octenol in CDC- and EVS-type light traps for sampling the mosquitoes *Aedes aegypti (L.), Aedes polynesiensis* Marks, and *Culex quinquefasciatus* say in Moorea, French Polynesia. *J Vector Ecol*, 29.

Sakamoto, M., Kitahara, M. & Benno, Y., 2007. *Parabacteroides johnsonii* sp. nov., isolated from human faeces. *International Journal of Systematic and Evolutionary Microbiology*, 57(2), pp.293–296.

Schmidt, R. et al., 2015. Volatile affairs in microbial interactions. *ISME Journal*, 9(11), pp.2329–2335.

Self, L., Abdulcader, M. & Tun, M., 1969. Preferred biting sites of *Culex pipiens* fatigans on adult Burmese males. *Bulletin of the World Health Organization*, 40(2), pp.324–327.

Shelley, W.B., Hurley, H.J. & Nichols, A.C., 1953. Axillary Odor: Experimental Study of the Role of Bacteria, Apocrine Sweat, and Deodorants. *A. M. A. Archives of*

Dermatology and Syphilology, 68(4), pp.430–446.

Shirai, O. et al., 2002. Alcohol ingestion stimulates mosquito attraction. *Journal of the American Mosquito Control Association*, 18, pp.91–96.

Shirai, Y. et al., 2004. Landing preference of *Aedes albopictus* (Diptera: Culicidae) on human skin among ABO blood groups, secretors or nonsecretors, and ABH antigens. *Journal of Medical Entomology*, 41(4), pp.796–799.

Shirai, Y. et al., 2002. Landing sites on the human body preferred by *Aedes albopictus. J Am Mosq Control Assoc*, 18(2), pp.97–99.

Sinka, M.E. et al., 2012. A global map of dominant malaria vectors. *Parasites & Vectors*, 5(1), p.69.

Sinka, M.E. et al., 2010. The dominant *Anopheles* vectors of human malaria in Africa, Europe and the Middle East: occurrence data, distribution maps and bionomic précis. *Parasit Vectors*, 3.

Sinka, M.E. et al., 2011. The dominant *Anopheles* vectors of human malaria in the Asia-Pacific region: occurrence data, distribution maps and bionomic précis. *Parasit Vectors*, 4.

Smallegange, R.C. et al., 2005. Synergism between ammonia, lactic acid and carboxylic acids as kairomones in the host-seeking behaviour of the malaria mosquito *Anopheles gambiae* sensu stricto (Diptera: *Culicidae*). *Chemical senses*, 30(2), pp.145–52.

Smallegange, R.C. et al., 2009. The effect of aliphatic carboxylic acids on olfaction-based host-seeking of the malaria mosquito *Anopheles gambiae* sensu stricto. *J Chem Ecol*, 35.

Smallegange, R.C., Knols, B.G.J. & Takken, W., 2010. Effectiveness of synthetic versus natural human volatiles as attractants for *Anopheles gambiae* (Diptera: *Culicidae*) sensu stricto. *Journal of medical entomology*, 47, pp.338–344.

Smallegange, R.C. & Takken, W., 2010. Host-seeking behaviour of mosquitoes: responses to olfactory stimuli in the laboratory. In *Olfaction in vector-host interactions*. pp. 143–180.

Smallegange, R.C., Verhulst, N.O. & Takken, W., 2011. Sweaty skin: an invitation to bite? *Trends in parasitology*, 27, pp.143–148.

Smallegange, R.C et al., 2012. Identification of candidiate volatiles that affect the behavioural response of the malaria mosquito *Anopheles gambiae* sensu stricto to an active kairomone blend: laboratory and semi-field assays. Physiological Entomology 37(2012):60-71.

Smith et al., 1970. L-lactic acid as a factor in the attraction of *Aedes aegypti* (Diptera: Culicidae) to human hosts. *Ann Entomol Soc Sm.* 63(3):760-70.

Spitzen et al., (2013). A 3D analysis of flight behaviour of *Anopheles gambiae* sensu stricto malaria mosquitoes in response to human odor and heat. PLoS One 8(5):

e:62995.

Stancyzk et al., 2013. *Aedes aegypti* Mosquitoes exhibit decreased repellency by DEET following previous exposure. *Plos One.* 8(2):e54438

Steinbrecht, R.A., 1997. Pores structures in insect olfactory sensilla: A review of data and concepts. *International Journal of Insect Morphology and Embryology*, 26(3–4), pp.229–245.

Stevens, D. et al., 2015. Spatial variations in the microbial community structure and diversity of the human foot is associated with the production of odorous volatiles. *FEMS Microbiology Ecology*, 91(1).

Sumba, L.A. et al., 2004. Daily oviposition patterns of the African malaria mosquito *Anopheles gambiae* Giles (Diptera: *Culicidae*) on different types of aqueous substrates. *Journal of Circadian Rhythms*, 2(1):6.

Suer, 2011. Unravelling the malaria mosquito's sense of smell: neural and behavioural responses to human derived compounds. PhD thesis, Wageningen University.

Syed, Z. & Leal, W.S., 2009. Acute olfactory response of *Culex* mosquitoes to a human- and bird-derived attractant. *Proceedings of the National Academy of Sciences*, 106(44), pp.18803–18808.

Takken, W., Dekker, T. & Wijnholds, Y.G.I., 1997. Odor-Mediated Flight Behavior of *Anopheles gambiae* Giles Sensu Stricto and *An . stephensi* Liston in Response to CO2 , Acetone , and 1-Octen-3-ol (Diptera:*Culicidae*). *Journal of Insect Behavior*, 10(3), pp.395–407.

Takken, W. & Knols, B.G., 1999. Odor-mediated behavior of Afrotropical malaria mosquitoes. *Annual review of entomology*, 44, pp.131–57.

Takken, W., van Loon JJ & Adam, W., 2001. Inhibition of host-seeking response and olfactory responsiveness in *Anopheles gambiae* following blood feeding. *Journal of insect physiology*, 47(3), pp.303–310.

Takken, W. & Verhulst, N.O., 2013. Host preferences of blood-feeding mosquitoes. *Annual review of entomology*, 58, pp.433–53.

Taylor, D. et al., 2003. Characterization of the microflora of the human axilla. *International journal of cosmetic science*, 25, pp.137–145.

Thomas, S. et al., 2017. Resting and feeding preferences of *Anopheles stephensi* in an urban setting, perennial for malaria. *Malaria Journal*, 16(1), p.111.

Thornton, C. et al., 1976. Effects of human blood group , sweating and other factors on individual host selection by species A of the *Anopheles gambiae* complex (Diptera , *Culicidae*). *Bulletin of Entomological Research.* 66(4),pp.651–663.

Troccaz, M. et al., 2009. Gender-specific differences between the concentrations of nonvolatile (R)/(S)-3-Methyl-3-Sulfanylhexan-1-Ol and (R)/(S)-3-Hydroxy-3-Methyl-Hexanoic acid odor precursors in axillary secretions. *Chemical Senses*, 34(3), pp.203–210.

Tusting, L.S. et al., 2013. Mosquito larval source management for controlling malaria. In *Cochrane Database of Systematic Reviews*. 29(8):CD008923.

Van Loon et al., 2015. Mosquito attraction: crucial role of carbon dioxide in forumation of a five-component blend of human derived volatiles. *J Chem Ecol.* 41(6): 567-573.

Verhulst, N.O. et al., 2016. Attractiveness of volatiles from different body parts to the malaria mosquito *Anopheles coluzzii* is affected by deodorant compounds. *Scientific Reports*, 1;6:27141.

Verhulst, N.O. et al., 2010. Chemical ecology of interactions between human skin microbiota and mosquitoes. *FEMS microbiology ecology*, 74(1), pp.1–9.

Verhulst, N.O., Qiu, Y.T., et al., 2011. Composition of human skin microbiota affects attractiveness to malaria mosquitoes. *PloS one*, 6(12), p.e28991.

Verhulst, N.O. et al., 2009. Cultured skin microbiota attracts malaria mosquitoes. *Malaria journal*, 8, p.302.

Verhulst, N.O., Mbadi, P. a, et al., 2011. Improvement of a synthetic lure for *Anopheles gambiae* using compounds produced by human skin microbiota. *Malaria journal*, 10(1), p.28.

Verhulst, N.O. et al., 2013. Relation between HLA genes, human skin volatiles and attractiveness of humans to malaria mosquitoes. *Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases*, 18, pp.87–93.

Watanabe, Y., Nagai, F. & Morotomi, M., 2012. Characterization of *Phascolarctobacterium succinatuten*s sp. Nov., an asaccharolytic, succinate-utilizing bacterium isolated from human feces. *Applied and Environmental Microbiology*, 78(2), pp.511–518.

Wedekind, C. & Füri, S., 1997. Body odour preferences in men and women: do they aim for specific MHC combinations or simply heterozygosity? *Proceedings. Biological sciences / The Royal Society*, 264(1387), pp.1471–9.

Wedekind, C. & Penn, D., 2000. Nephrology Dialysis Transplantation MHC genes , body odours , and odour preferences. *Nephrology Dialysis Transplantation*, 15(9), pp.1269–1271.

White, G., 1974. *Anopheles gambiae* complex and disease transmission in Africa. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 68(4), pp.278–301.

WHO, 2014. *A global brief on vector-borne diseases*, Available at: www.who.int/about/licensing/copyright_form/en/ [Accessed February 18, 2018].

WHO, 2015. Whopes. Long-lasting insecticidal nets. Available at: http://www.who.int/whopes/Long-lasting_insecticidal_nets_November_2015.pdf [Accessed June 3, 2018].

WHO, 2018a. WHO | Malaria. *WHO*. Available at: http://www.who.int/malaria/en/ [Accessed February 18, 2018].

WHO, 2017a. WHO | Malaria. *WHO*. Available at: http://www.who.int/mediacentre/factsheets/fs094/en/ [Accessed February 18, 2018].

WHO, 2017b. WHO | Vector-borne diseases. *WHO*. Available at: http://www.who.int/mediacentre/factsheets/fs387/en/ [Accessed February 18, 2018].

WHO, 2017c. WHO| Malaria. Insecticide resistance. Available at: http://www.who.int/malaria/areas/vector_control/insecticide_resistance/en/ [Accessed June 25, 2018]

WHO, 2018b. WHO | Yellow fever. *WHO*. Available at: http://www.who.int/mediacentre/factsheets/fs100/en/ [Accessed February 18, 2018].

Williamson & Kligman. 1965. A new method for the quantitative investigation of cutenous bacteria. *Journal of investigative dermatology.* 45(6), pp.498-503.

Wilson, A.L. et al., 2015. Evidence-based vector control? Improving the quality of vector control trials. *Trends in Parasitology*, 31(8), pp.380–390.

Wilson, M., 2008. *Bacteriology of Humans an Ecological Perspective*, Blackwell Publishing.

Wood, C. et al., 1972. Selective feeding of *Anopheles gambiae* according to ABO blood group status. *Nature*, 239(5368), p.165.

Wood, C.S., 1974. Preferential feeding of *Anopheles gambiae* mosquitoes on human subjects of blood group O: A relationship between the ABO polymorphism and malaria vectors. *Human biology; an international record of research*, 46, pp.385–404.

Wu, F. et al., 2017. *Phascolarctobacterium faecium* abundant colonization in human gastrointestinal tract. *Experimental and Therapeutic Medicine*, 14(4), pp.3122–3126.

Xu, Y. et al., 2007. Comparison of human axillary odour profiles obtained by gas chromatography/mass spectrometry and skin microbial profiles obtained by denaturing gradient gel electrophoresis using multivariate pattern recognition. *Metabolomics*, 3(4), pp.427–437.

Yao, S.S. et al., 2005. Flavor characteristics of lapsang souchong and smoked lapsang souchong, a special chinese black tea with pine smoking process. *Journal of Agricultural and Food Chemistry*, 53(22), pp.8688–8693.

Yutin, N. & Galperin, M.Y., 2013. A genomic update on clostridial phylogeny: Gram-negative spore formers and other misplaced clostridia. *Environmental Microbiology*, 15(10), pp.2631–2641.

Zeng, C. et al., 1996. A human axillary odorant is carried by apolipoprotein D. *Proceedings of the National Academy of Sciences of the United States of America*, 93(13), pp.6626–30.

Zeng, X. nong et al., 1991. Analysis of characteristic odors from human male axillae. *Journal of Chemical Ecology*, 17(7), pp.1469–1492.

Zhang, Z.M. et al., 2005. The study of fingerprint characteristics of the emanations from human arm skin using the original sampling system by SPME-GC/MS. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, 822(1–2), pp.244–252.

Zozaya-Hinchliffe, M., Martin, D.H. & Ferris, M.J., 2008. Prevalence and abundance of uncultivated Megasphaera-like bacteria in the human vaginal environment. *Applied and Environmental Microbiology*, 74(5), pp.1656–1659.

Zwiebel, L.J. & Takken, W., 2004. Olfactory regulation of mosquito-host interactions. *Insect biochemistry and molecular biology*, 34(7), pp.645–52.

Appendix 1

INCLUSION CRITERIA

If the answer to any of the criteria below is NO, the participants does not qualify for the study

1.	Is the participant aged 18 or over or 65 and under?	YES☐	NO☐
2.	Is the participant healthy? (not taking any medication and no current illnesses)	YES☐	NO☐
3.	Is the participants willing to not wash, wet or use any cosmetic product on his feet for 4 days?	YES☐	NO☐
4.	Is the participant willing to refrain from doing vigorous exercise during the study?	YES☐	NO☐
5.	Is the participant willing to refrain from eating strong smelling foods such as garlic or spices, spicy foods or drinking alcohol 24 hours before and during the test?	YES☐	NO☐

EXCLUSION CRITERIA

If the answer to any of the criteria below is YES, the participant does not qualify for the study

1.	Has the participant been taking any medication (including antibiotics) in the past 4 weeks?	YES☐	NO☐
2.	Is the participant planning on taking any medication during the study? (such as antibiotics)	YES☐	NO☐
3.	Upon examination, does the participant have dermatological abnormalities of the feet?	YES☐	NO☐
4.	Has the participant had any acute illness 7 days prior before the study?	YES☐	NO☐
5.	Is the participant a smoker?	YES☐	NO☐

COMMENTS

Does the participant qualify for the study? YES☐ NO☐

Participant no. _____

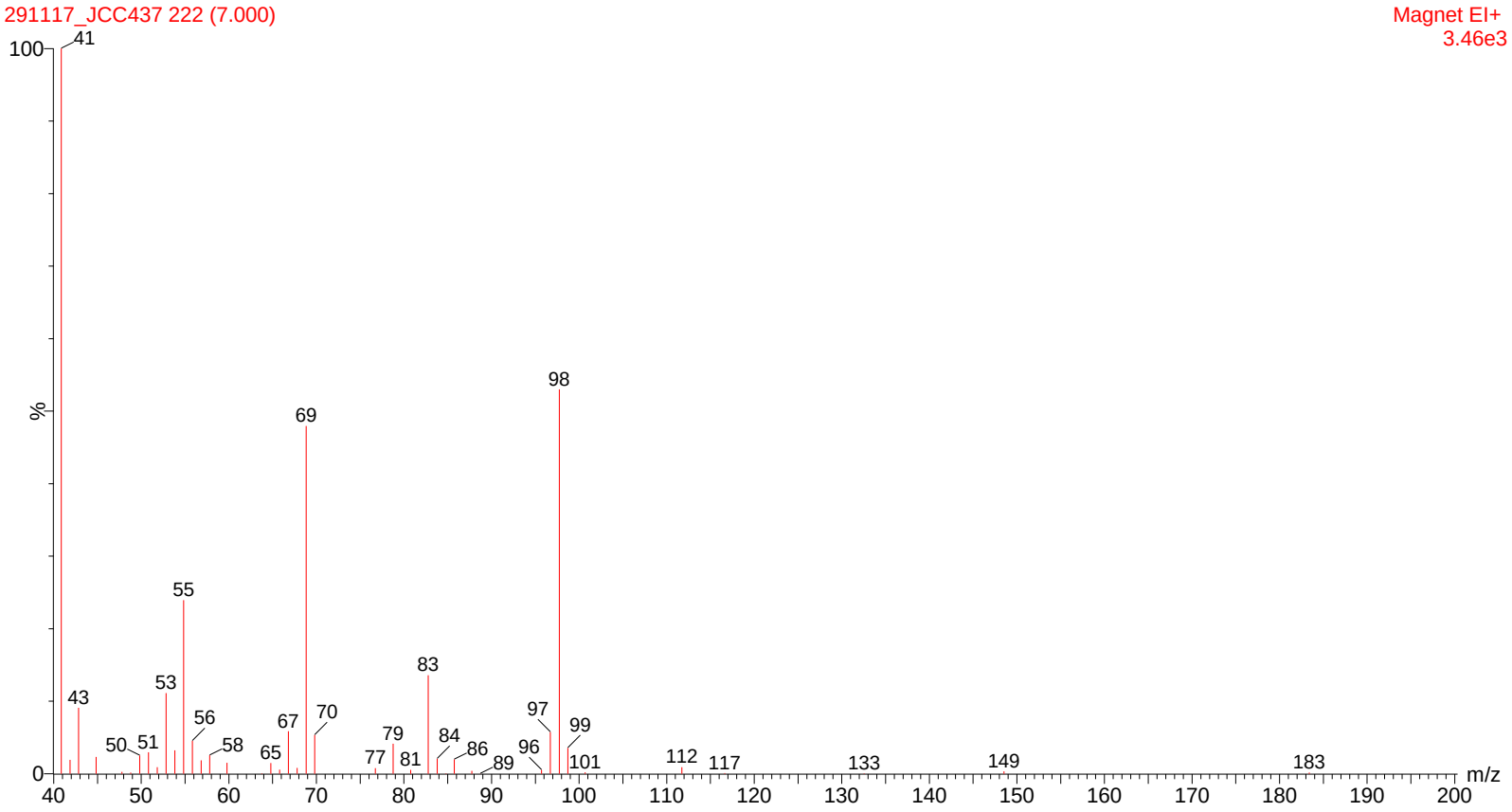
Print name _____

Signed _____ Date**:** ____/_____/____

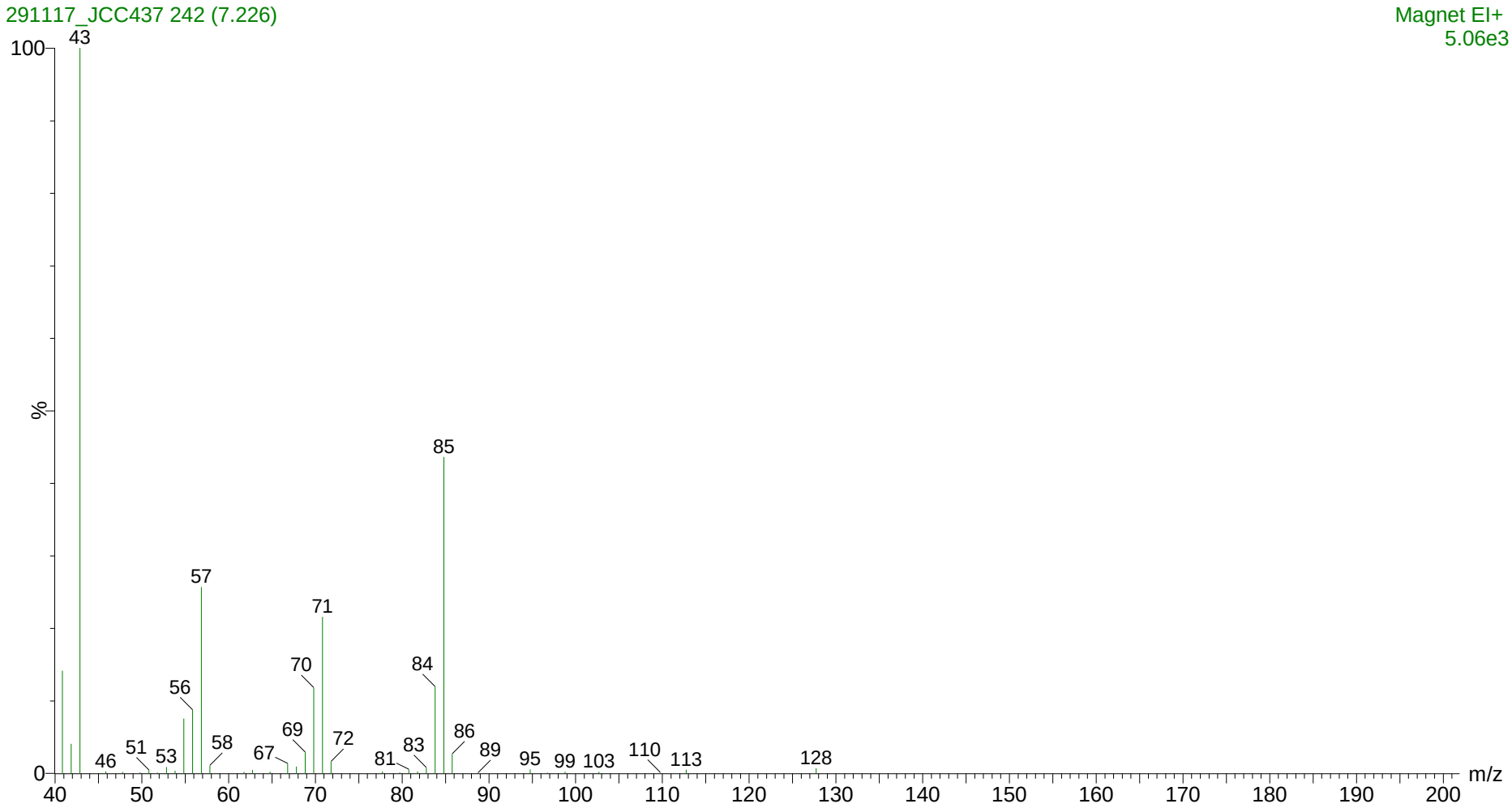
Appendix 2

Mass spectra of identified compounds

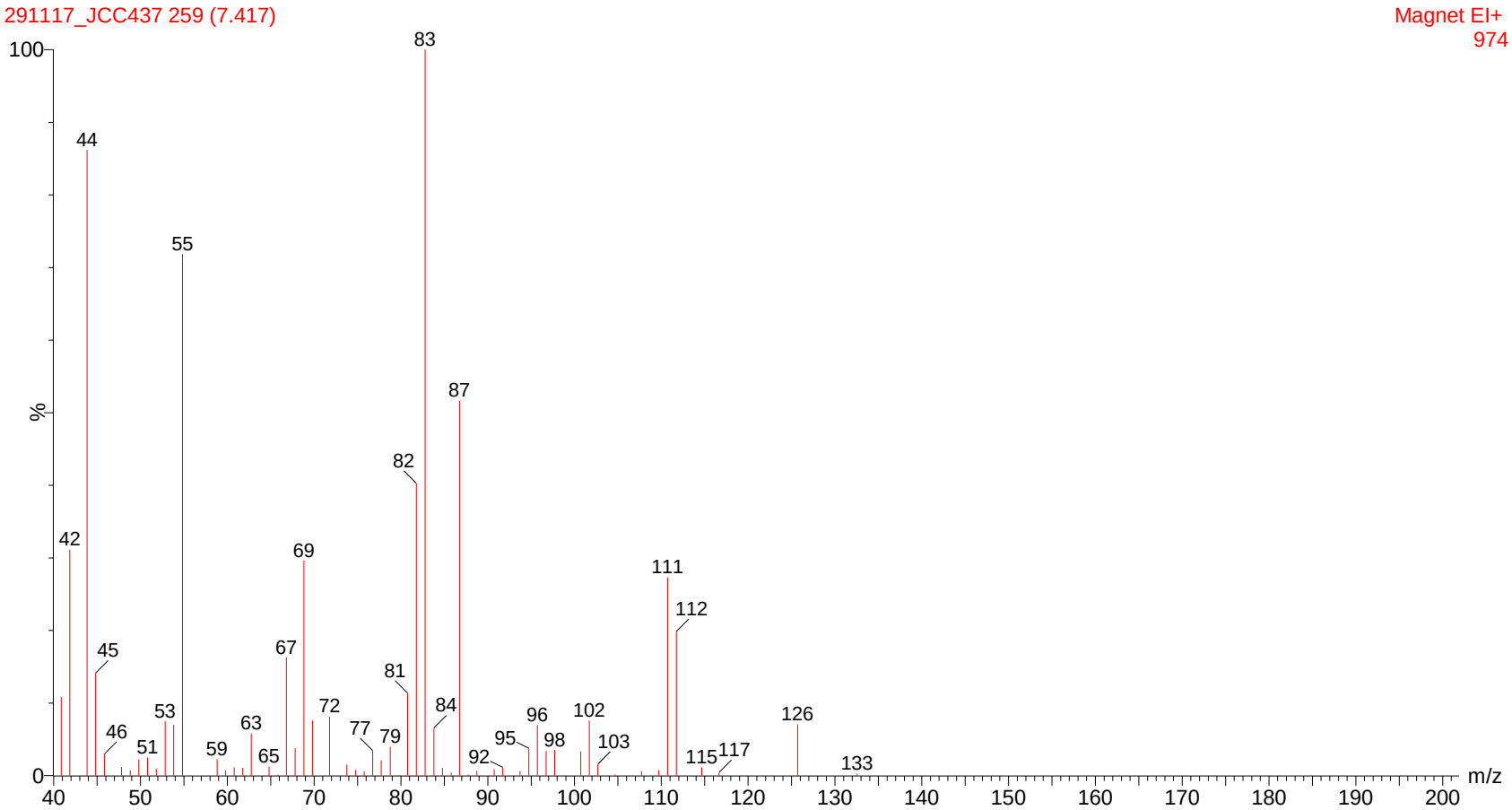
RI 810 2-methyl-2-pentenal, 4-methyl-3-pentenal, 2-ethyl-2-butenal



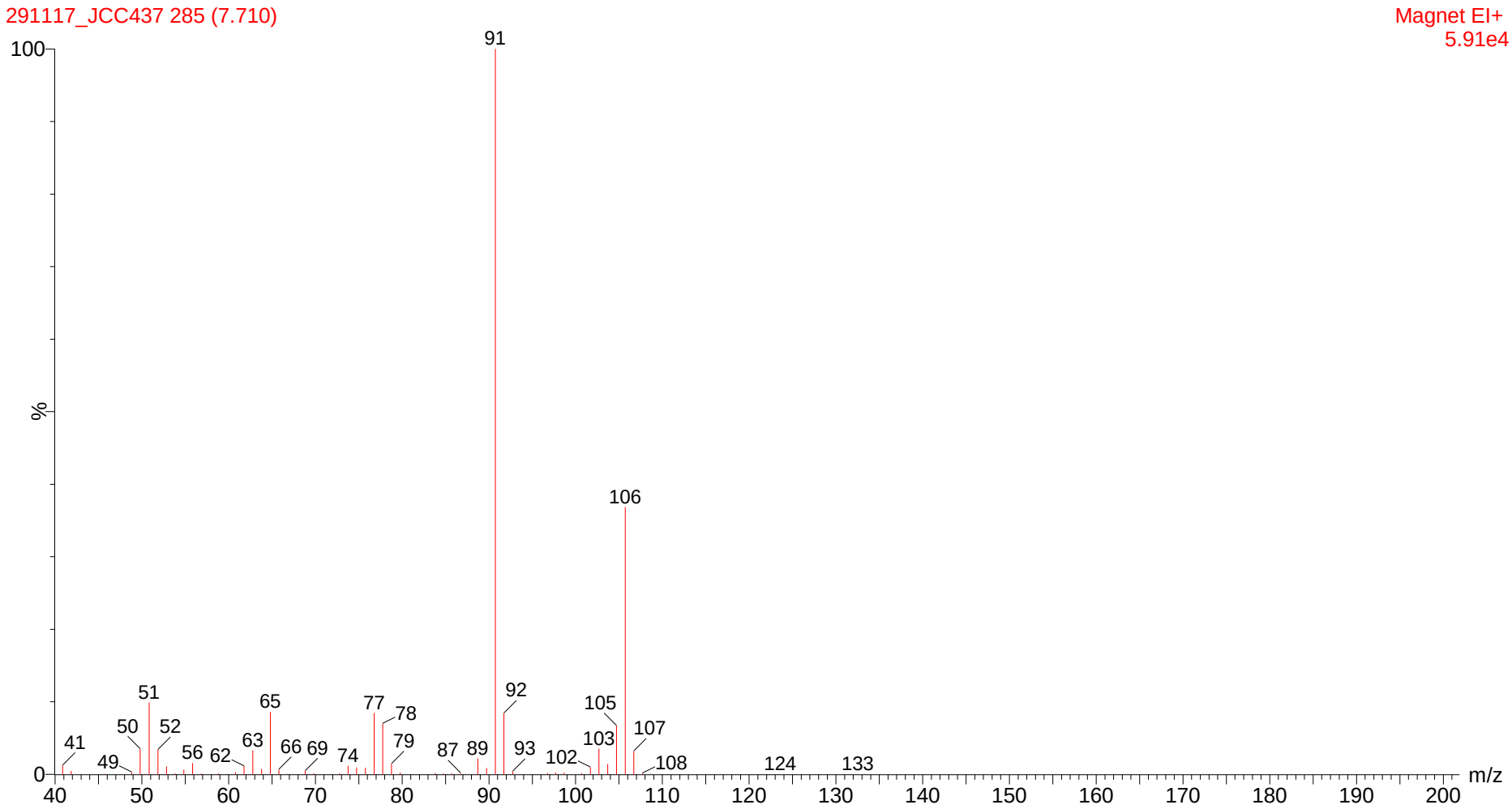
2,4-dimethyl heptane; 2,3,4-trimethyl hexane



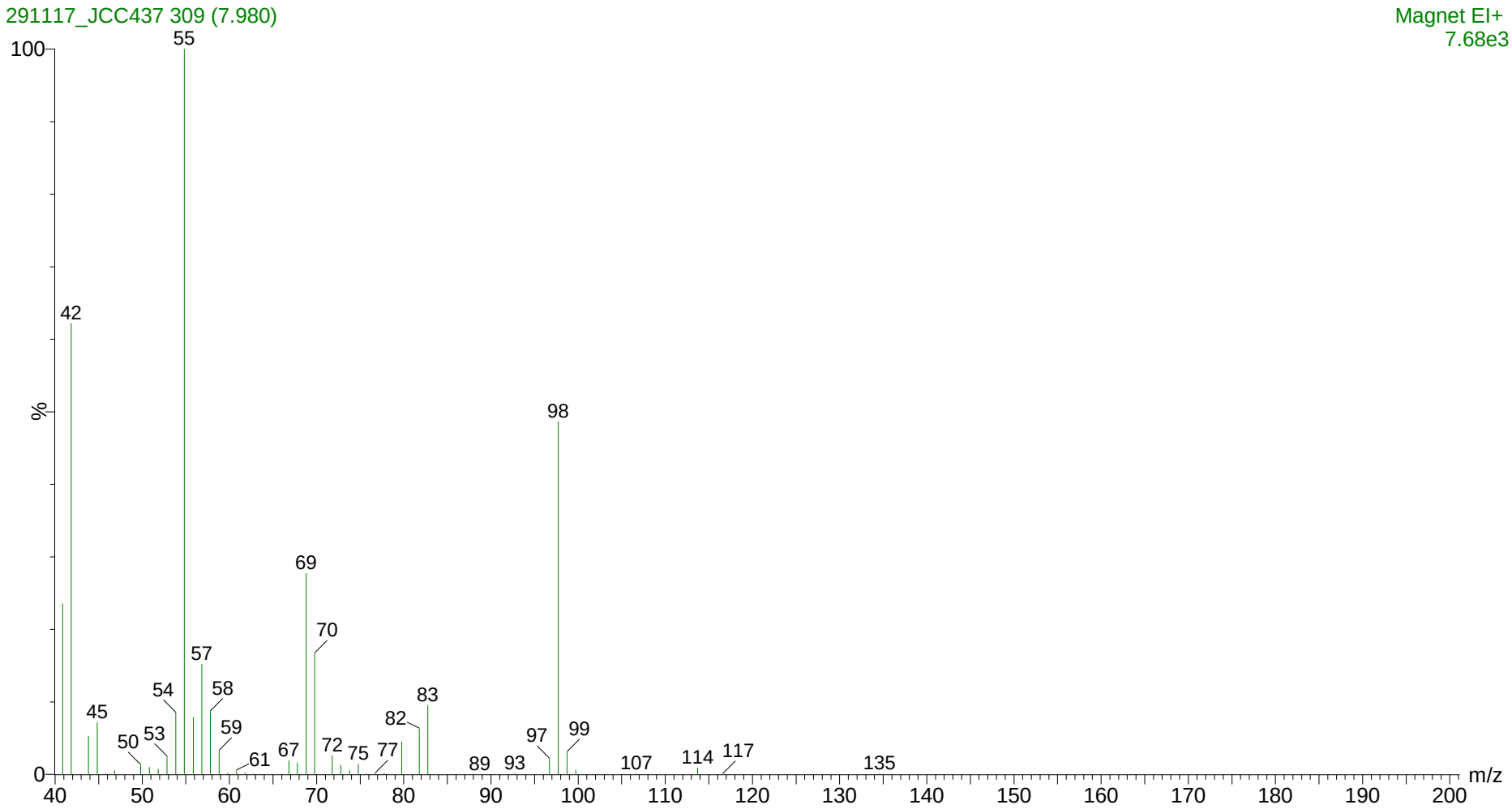
RI 849. Ethyl-cyclohexane



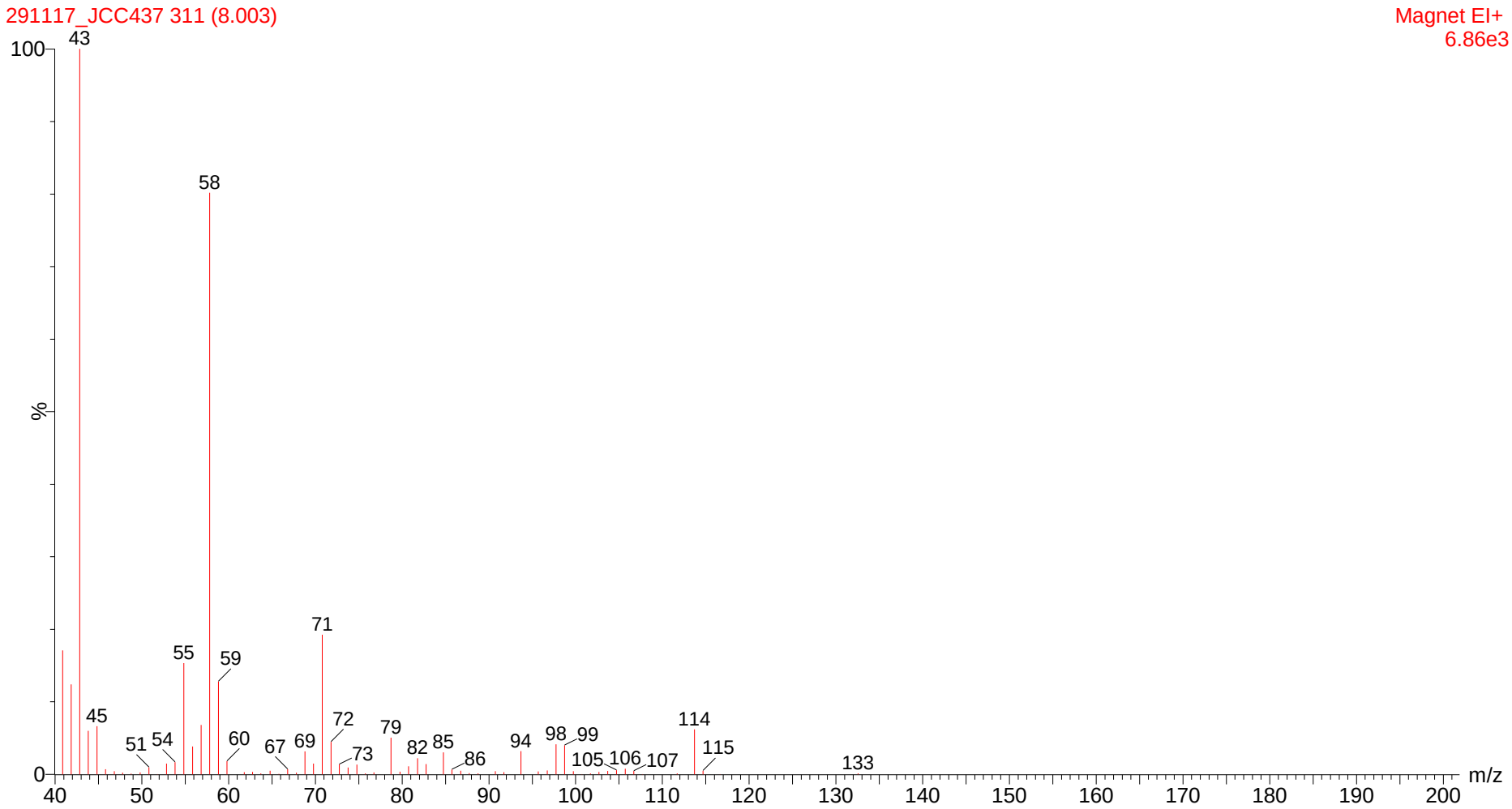
RI 858. Ethylbenzene



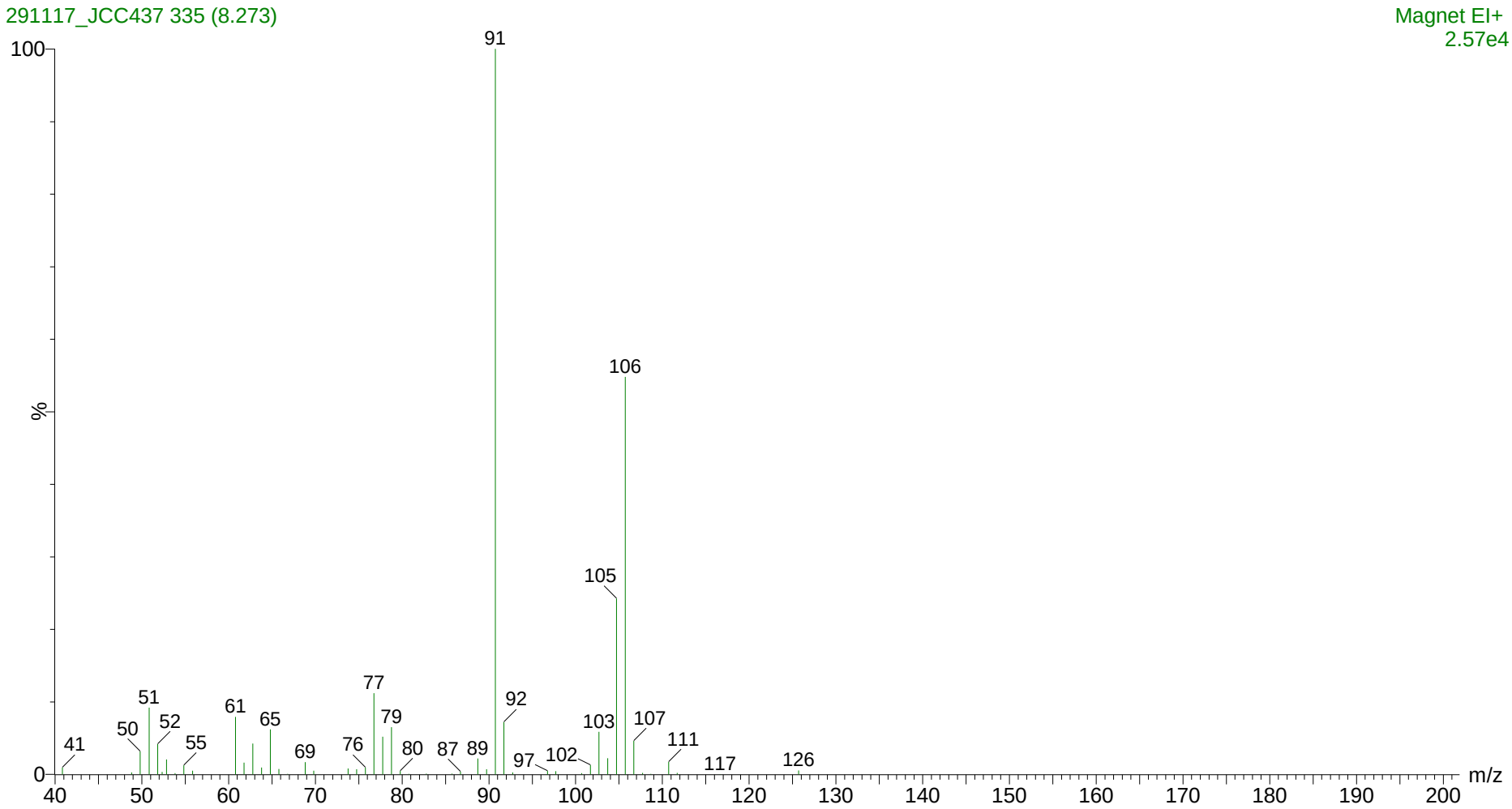
RI 862. Cyclohexanone or 2-methylcyclopentanone



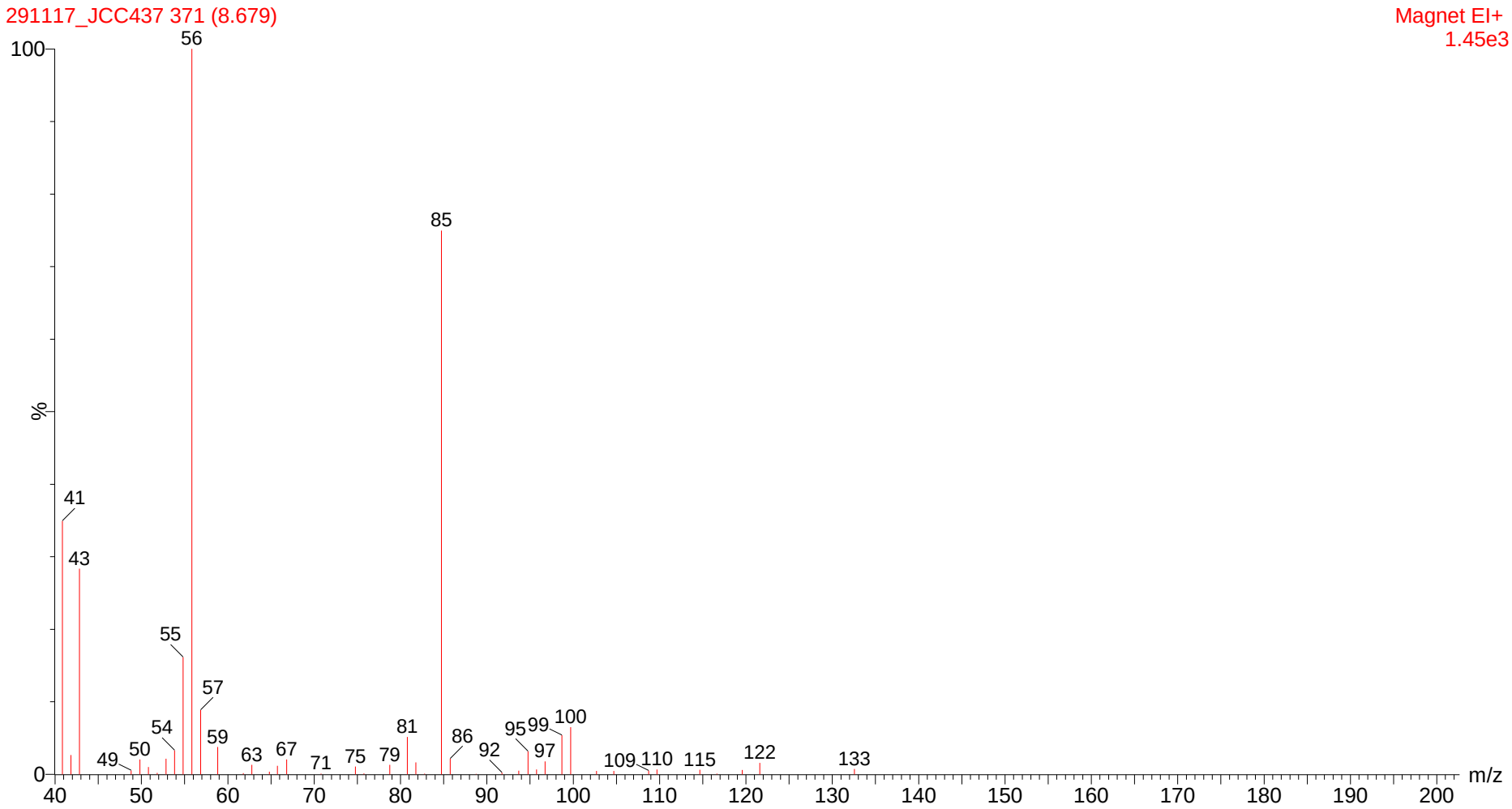
RI 875. 2-heptanone



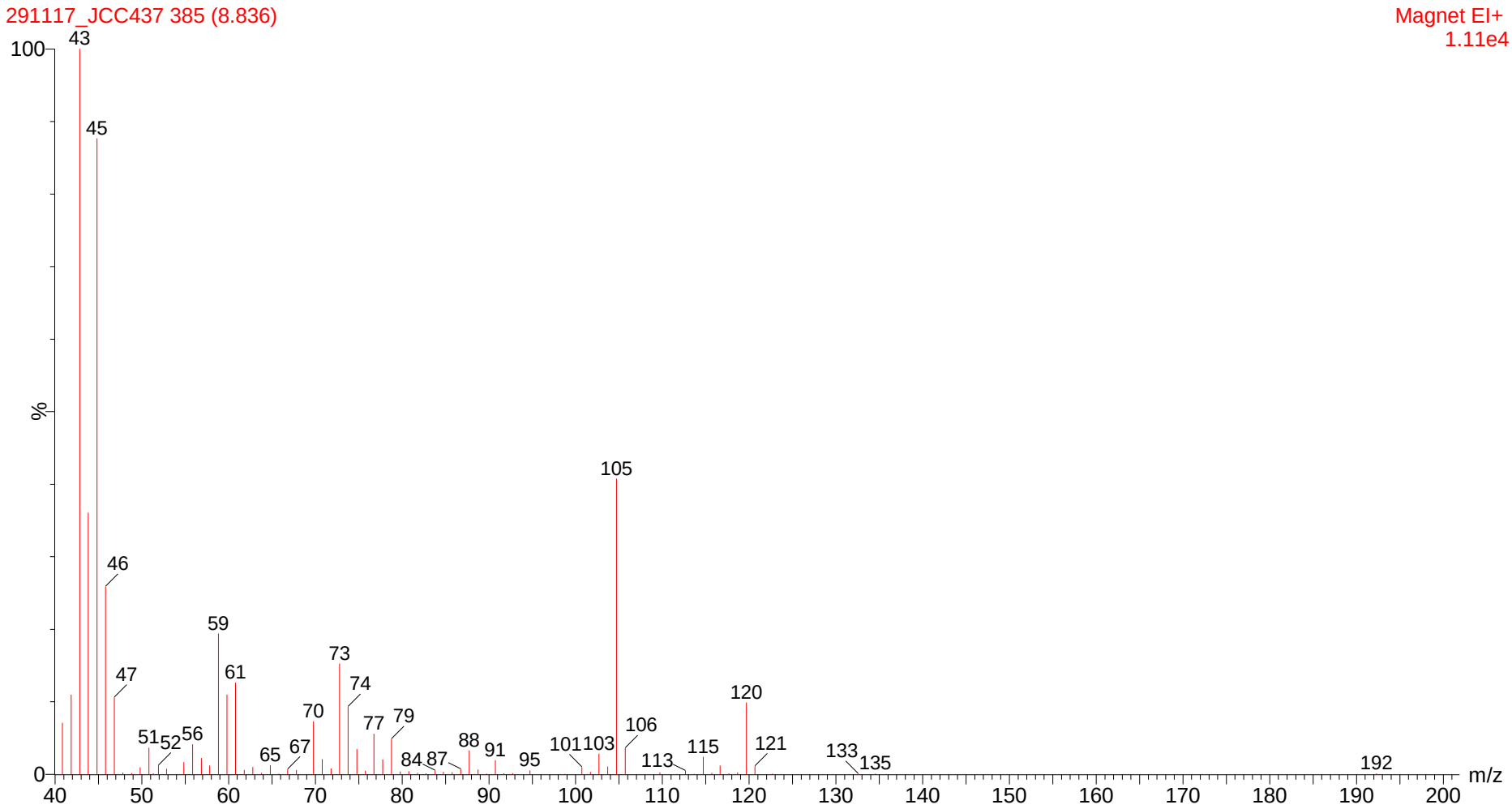
RI 882 1,2-dimethyl benzene



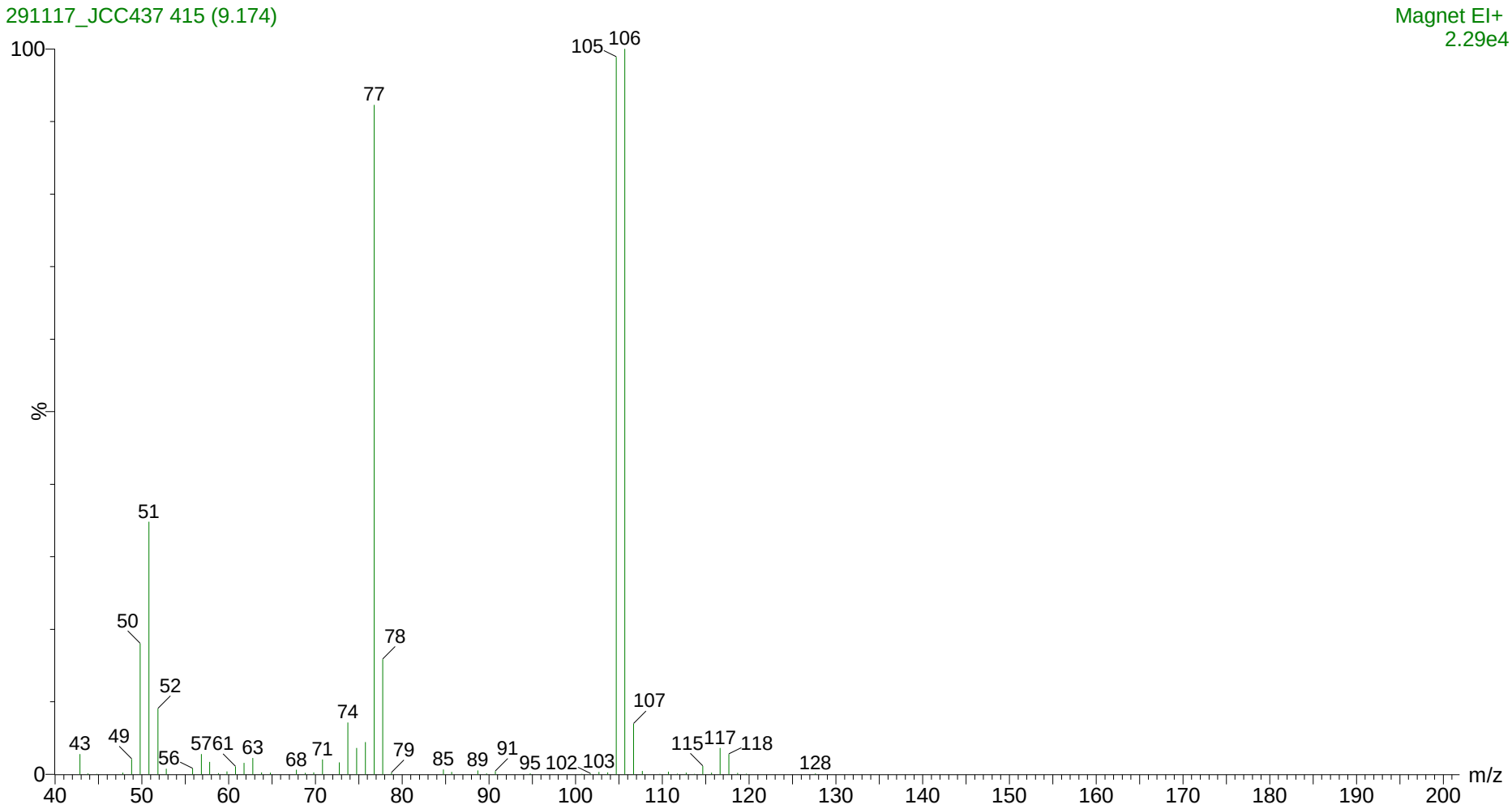
RI 910. Dihydro-5-methyl-2(3H)-furanone or cumene



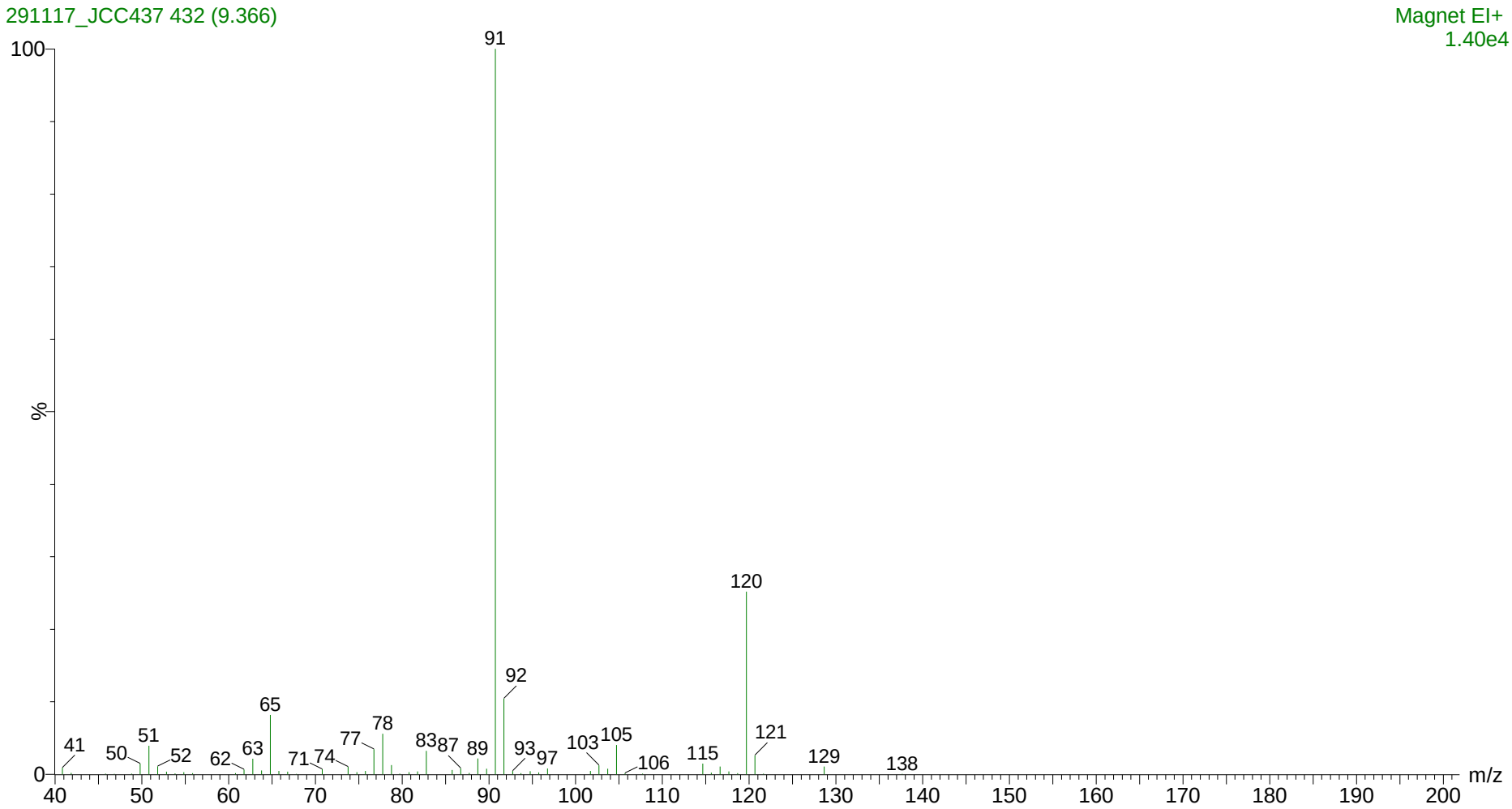
RI 918. 1-ethyl-2-methyl benzene or p-ethyltoulene



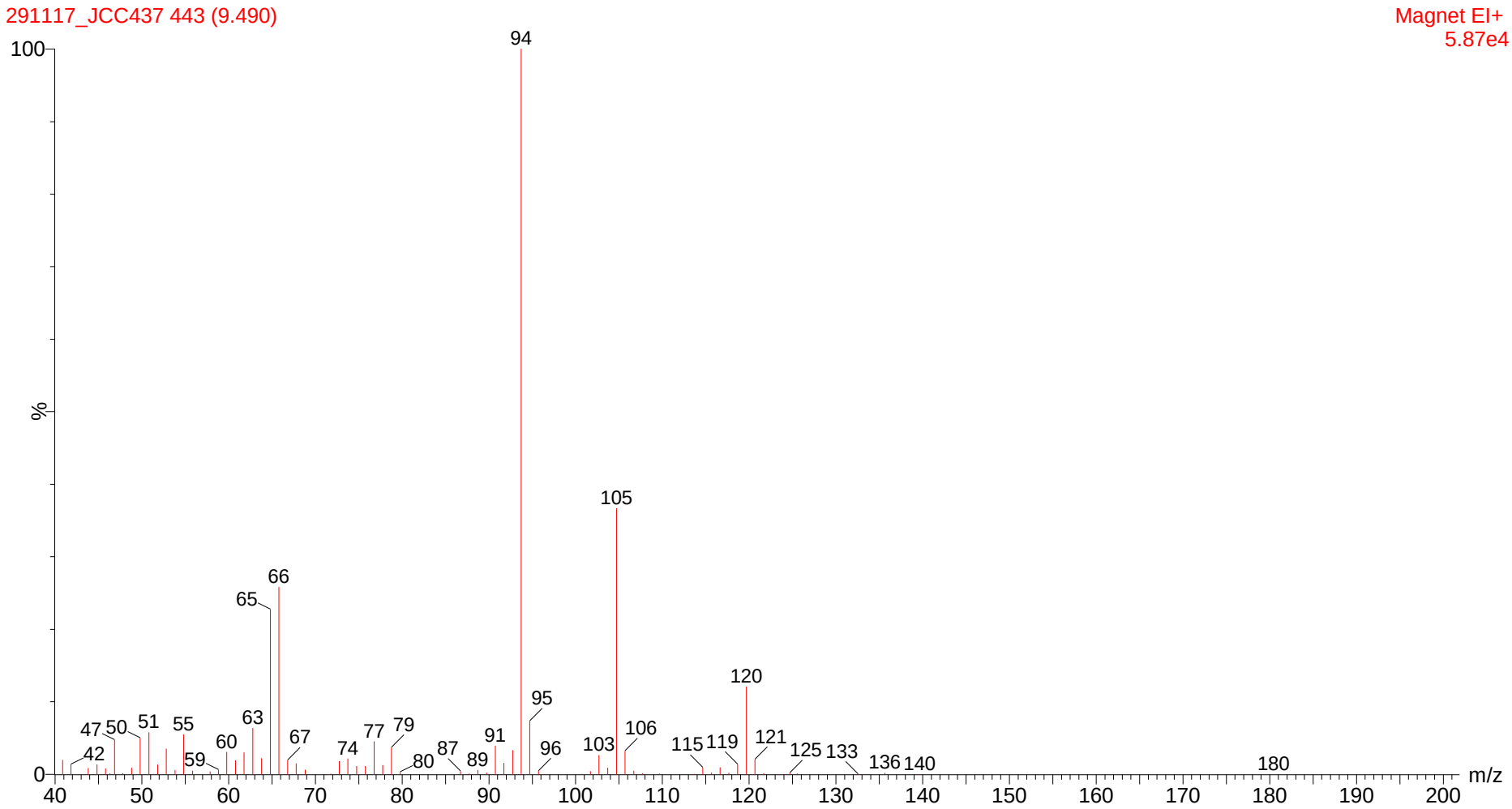
RI 933 Benzaldehyde



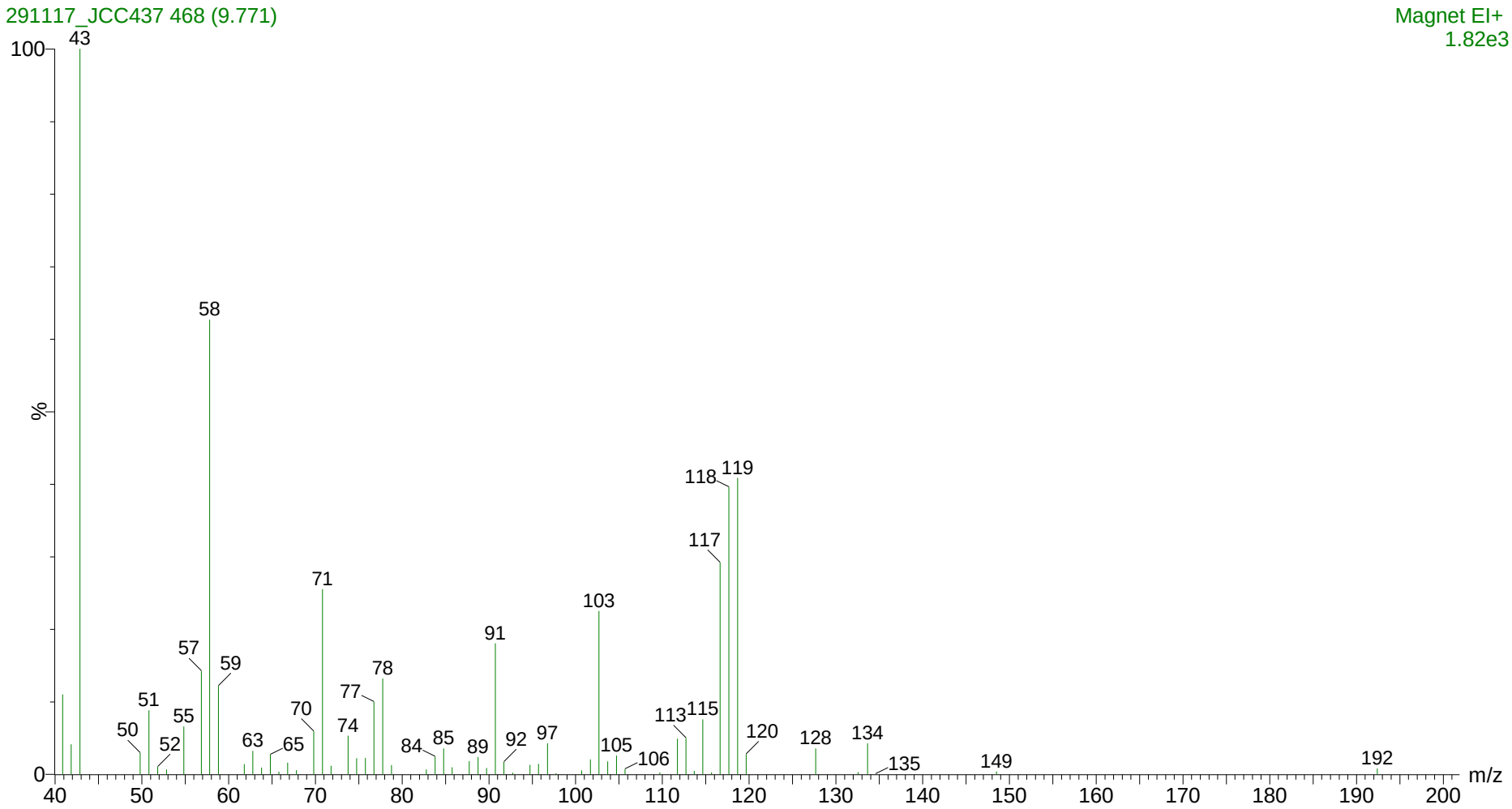
RI 951. Propylbenzene



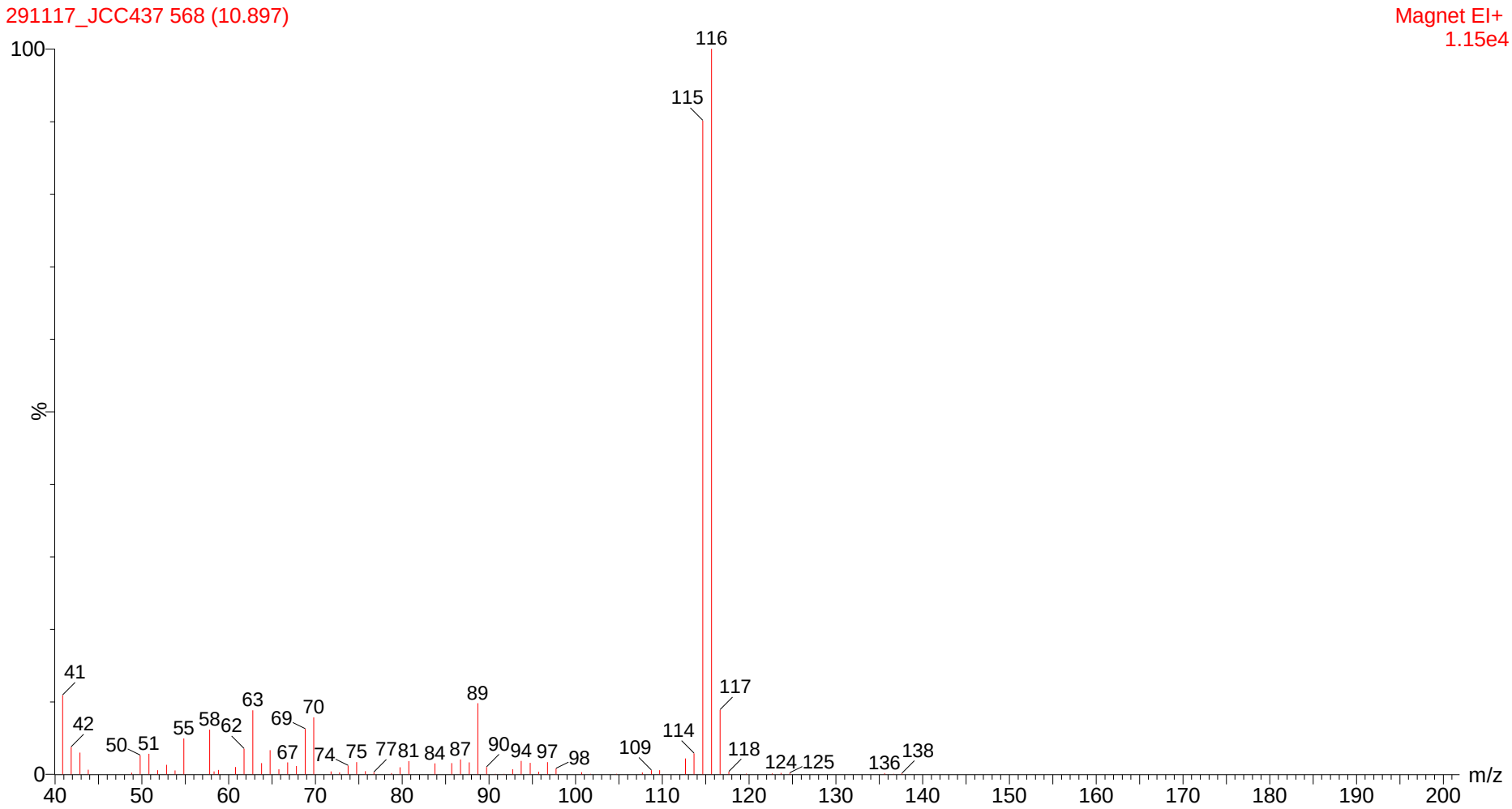
RI 958. Phenol



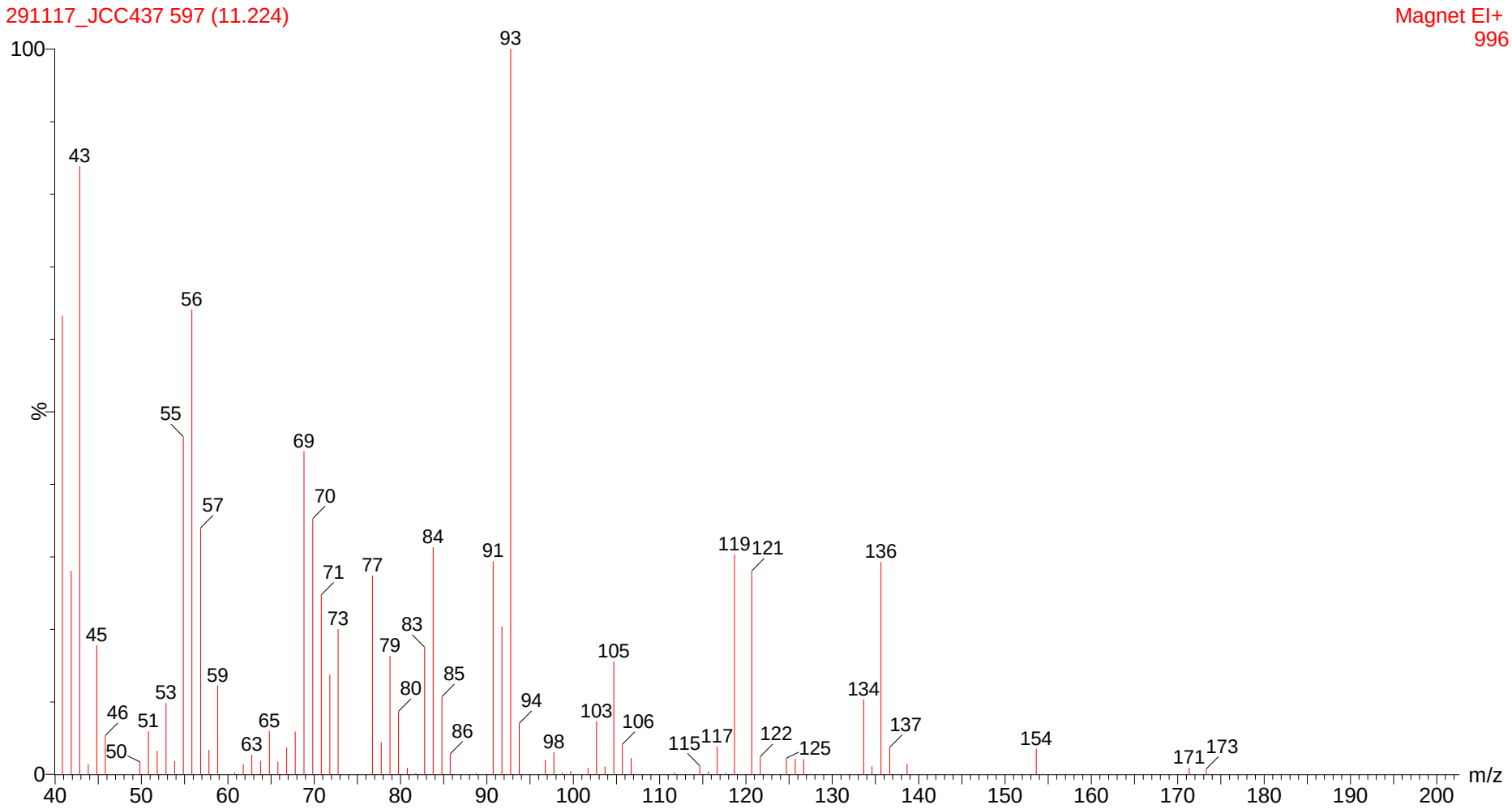
RI 966. O-ethyl toluene or M-ethyl toluene



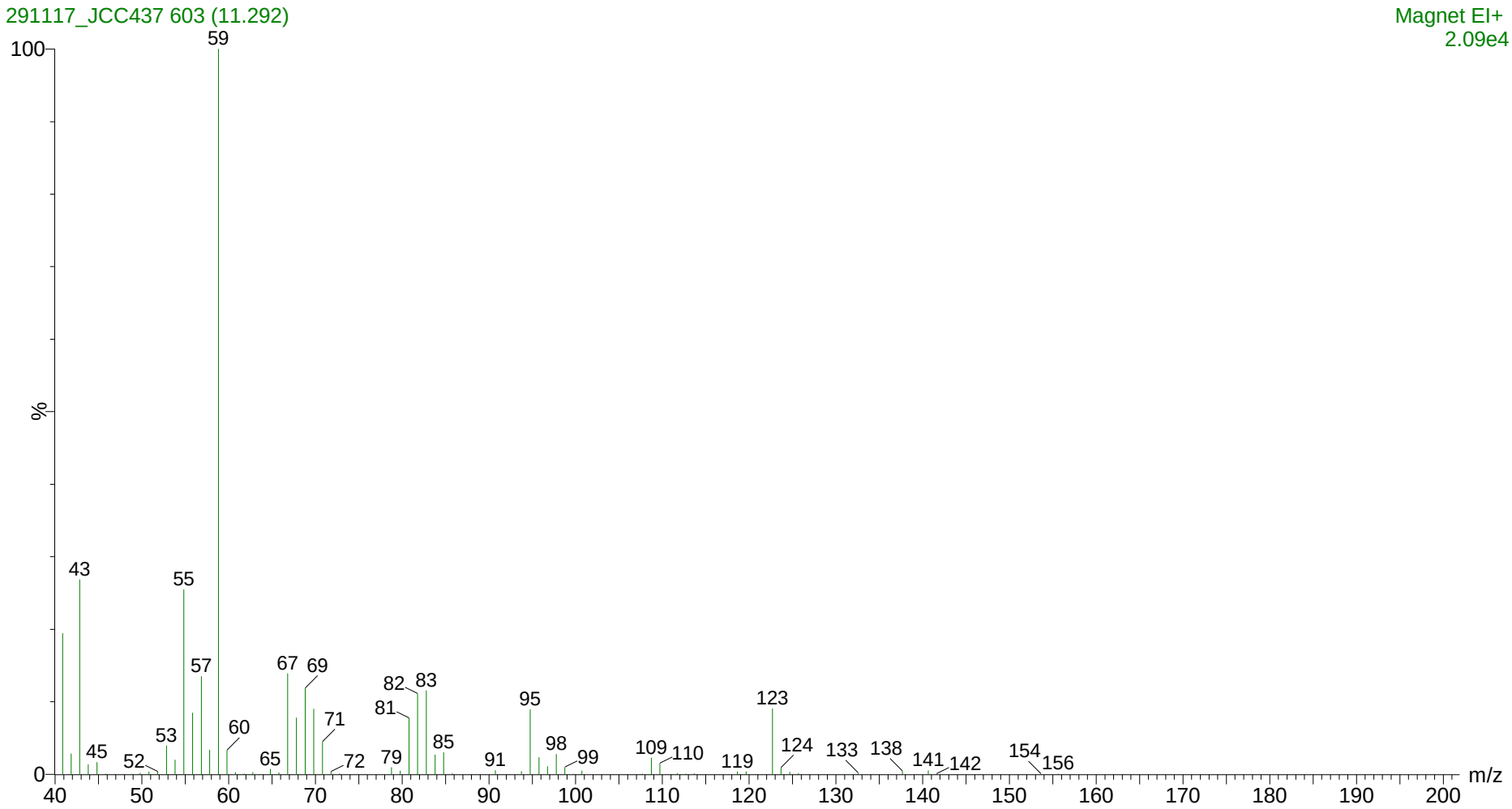
RI 1023. Indene, 3-methylphenylacetylene or p-ethynytoluene



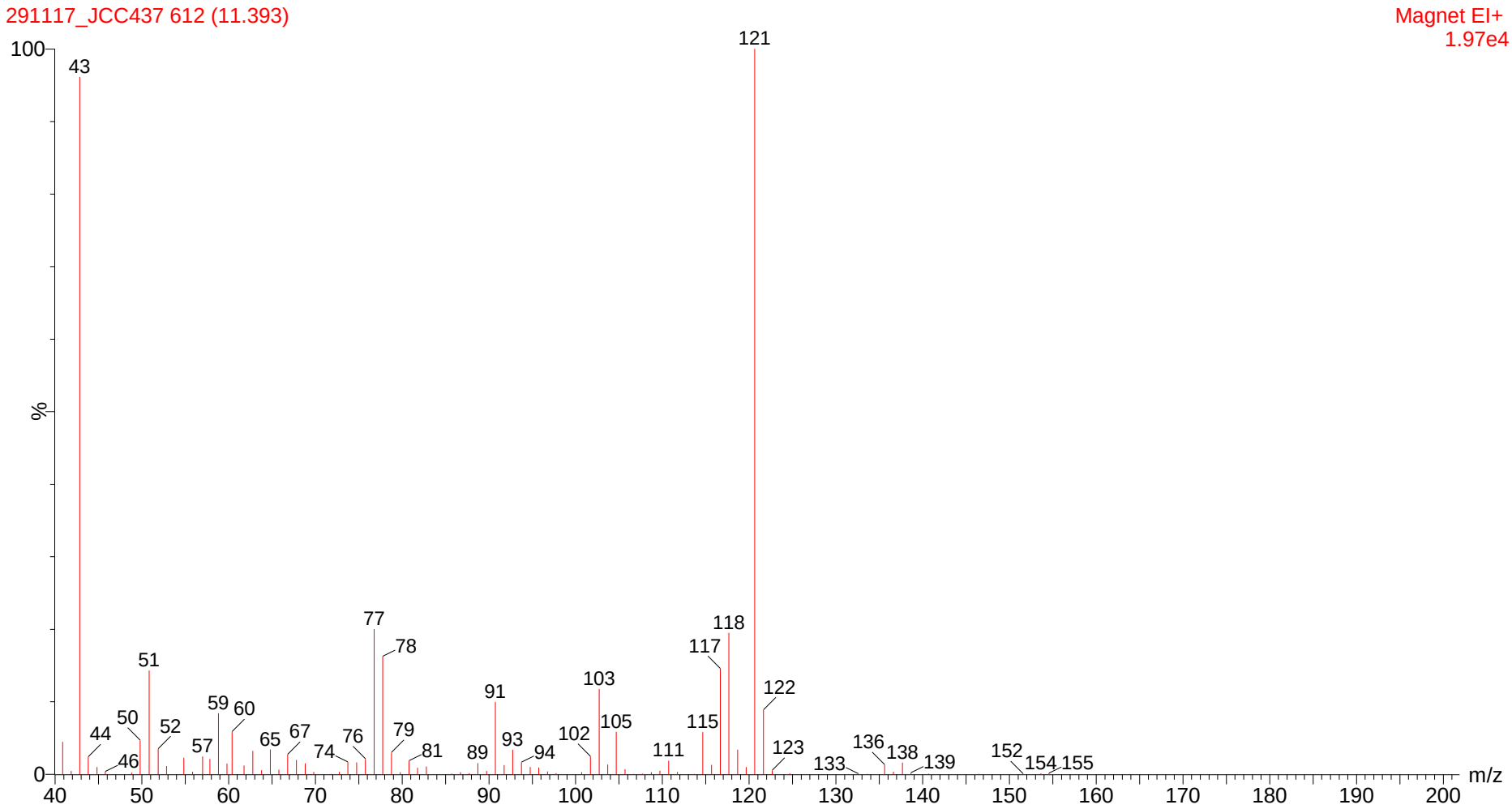
Rt 11.22 RI 1052 Gamma-terpinene



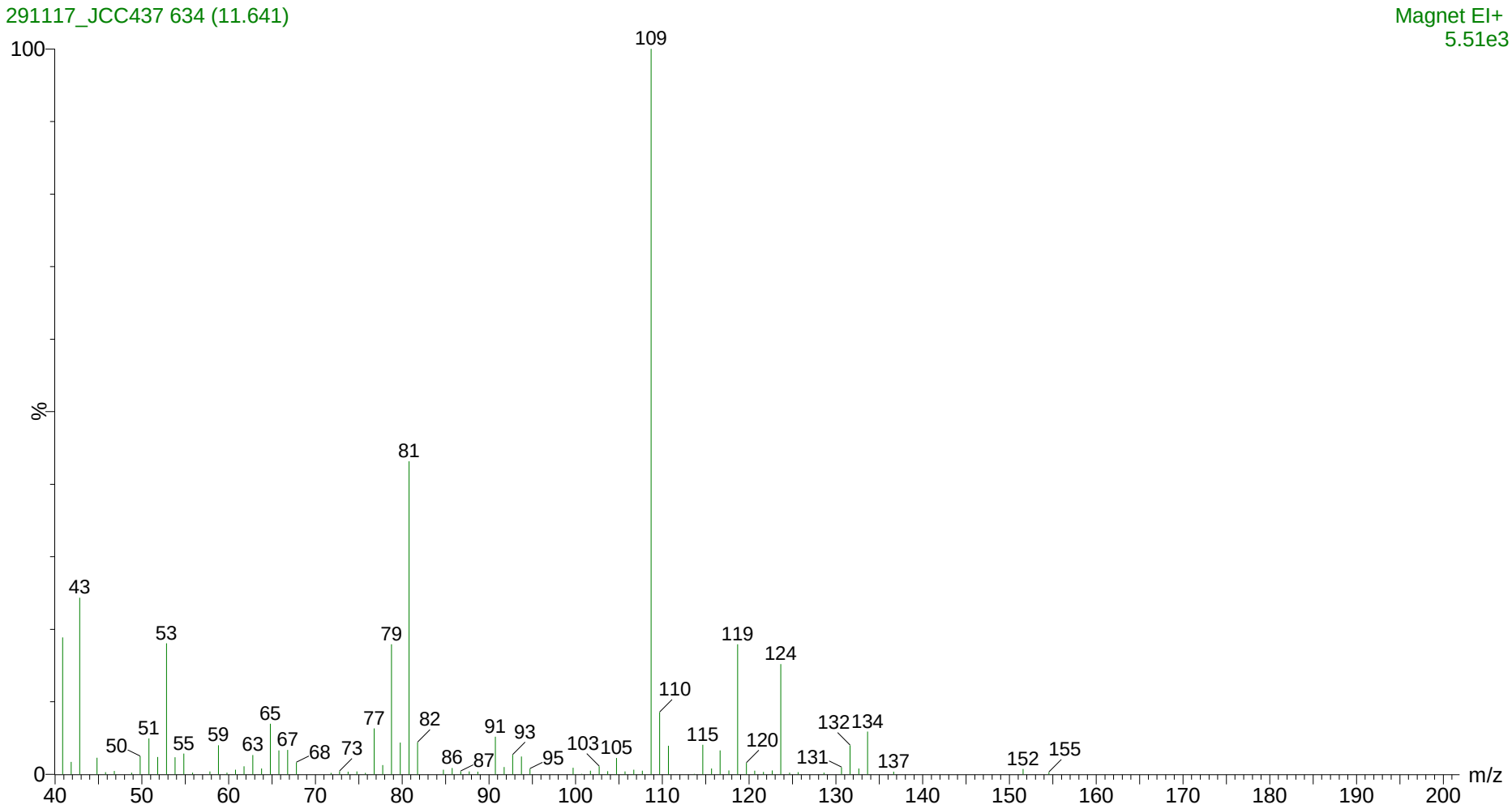
RI 1059. Dihydromyrcenol



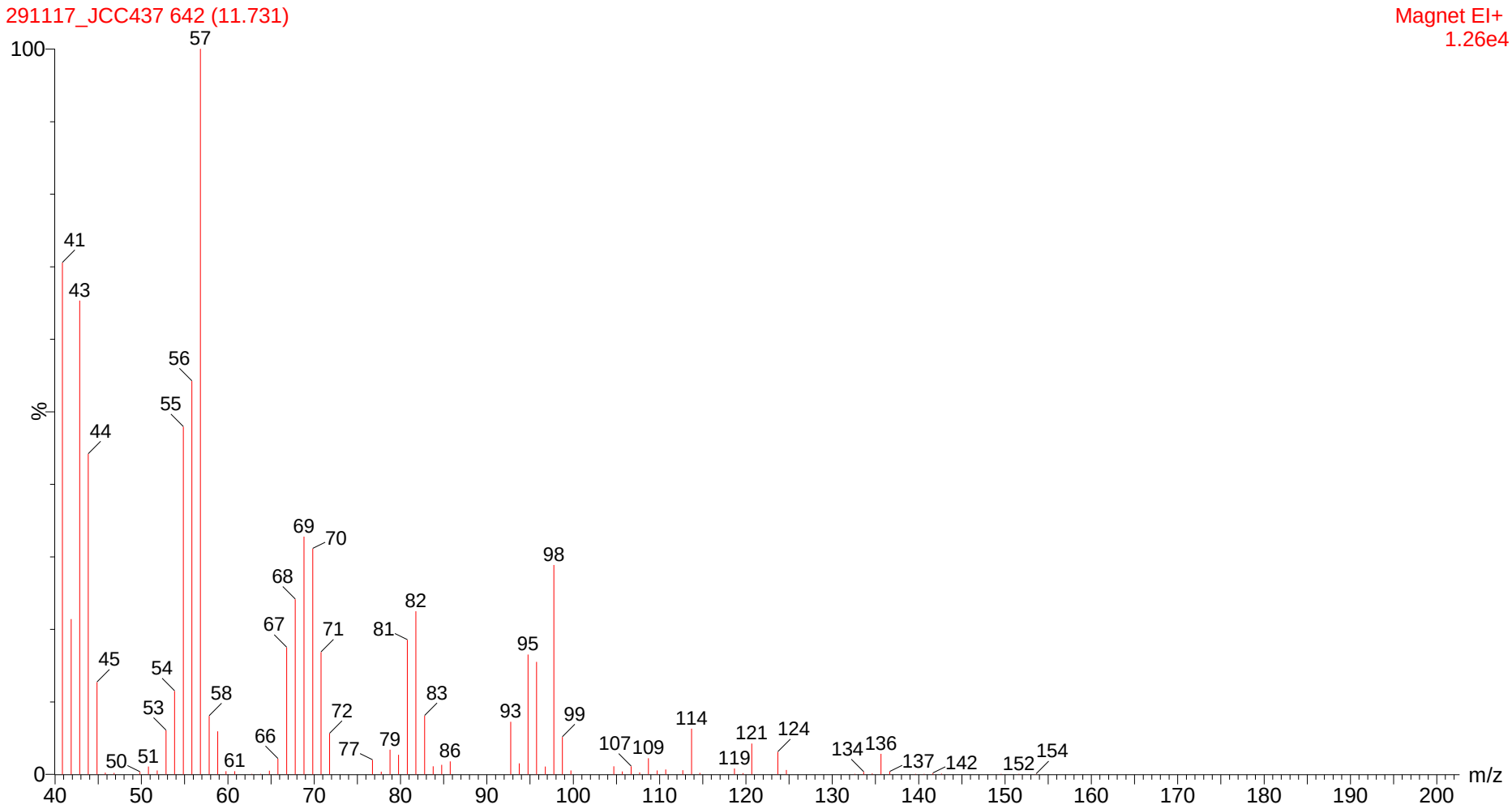
Rt 11.39. RI 1064. 2-phenylisopropanol



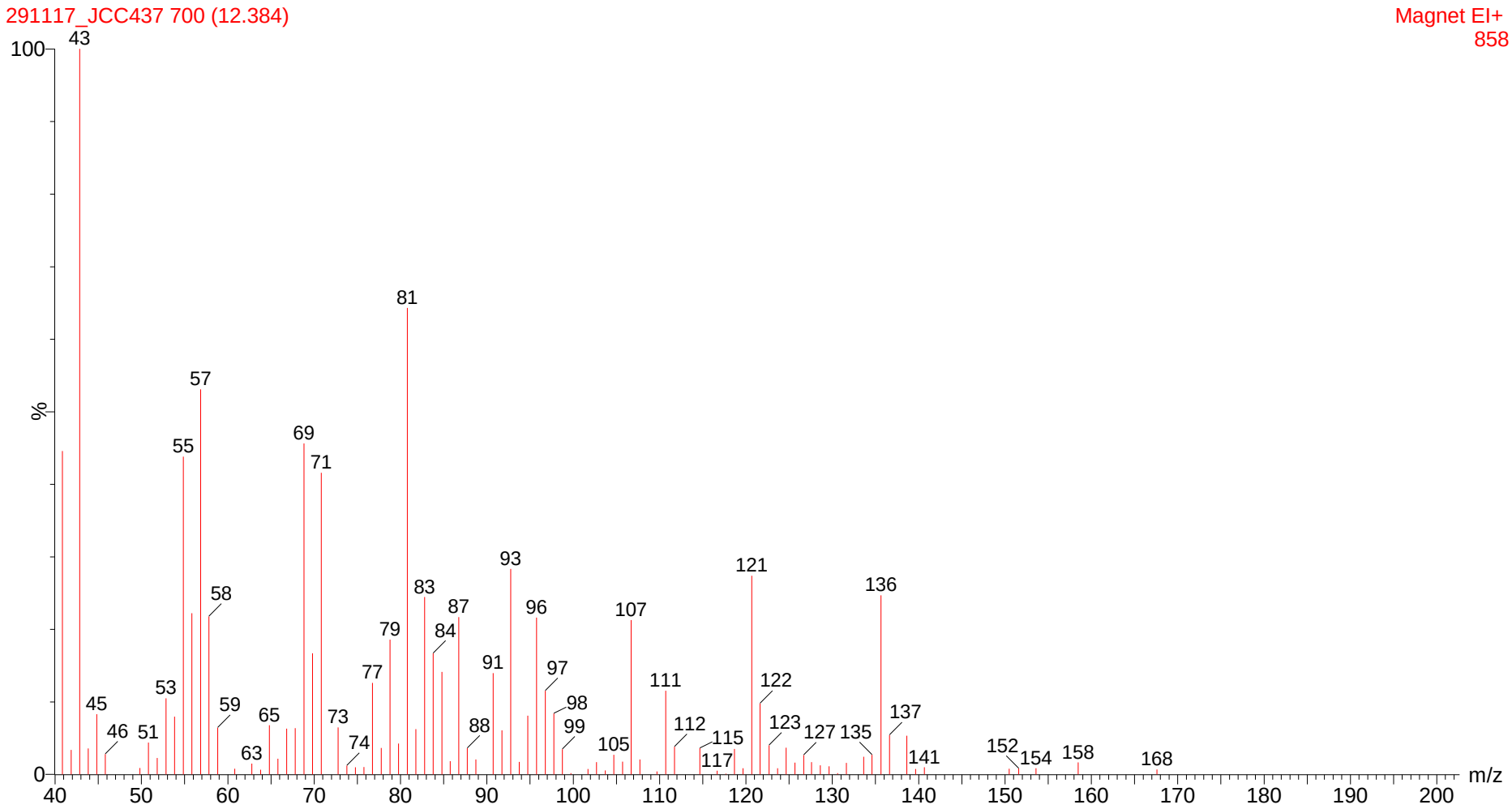
Rt 11.64. RI 1064 1-acetyl-2-methylcyclopentene



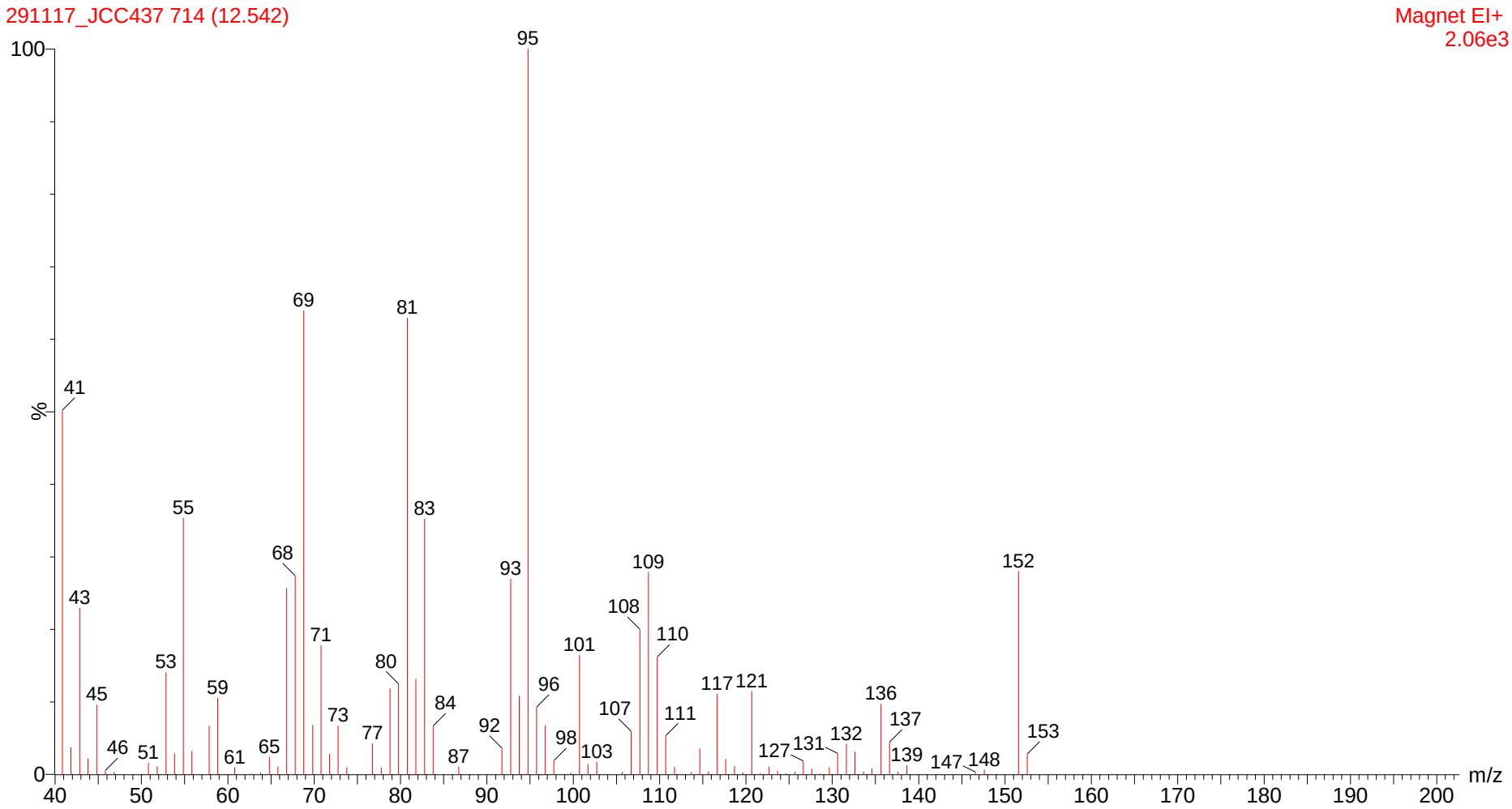
Rt 11.73 RI 1069. 1-acetyl-2-methylcyclopentene, methyl-(E)-3,5-heptadien-2-one or 1-acetylcuclohexane



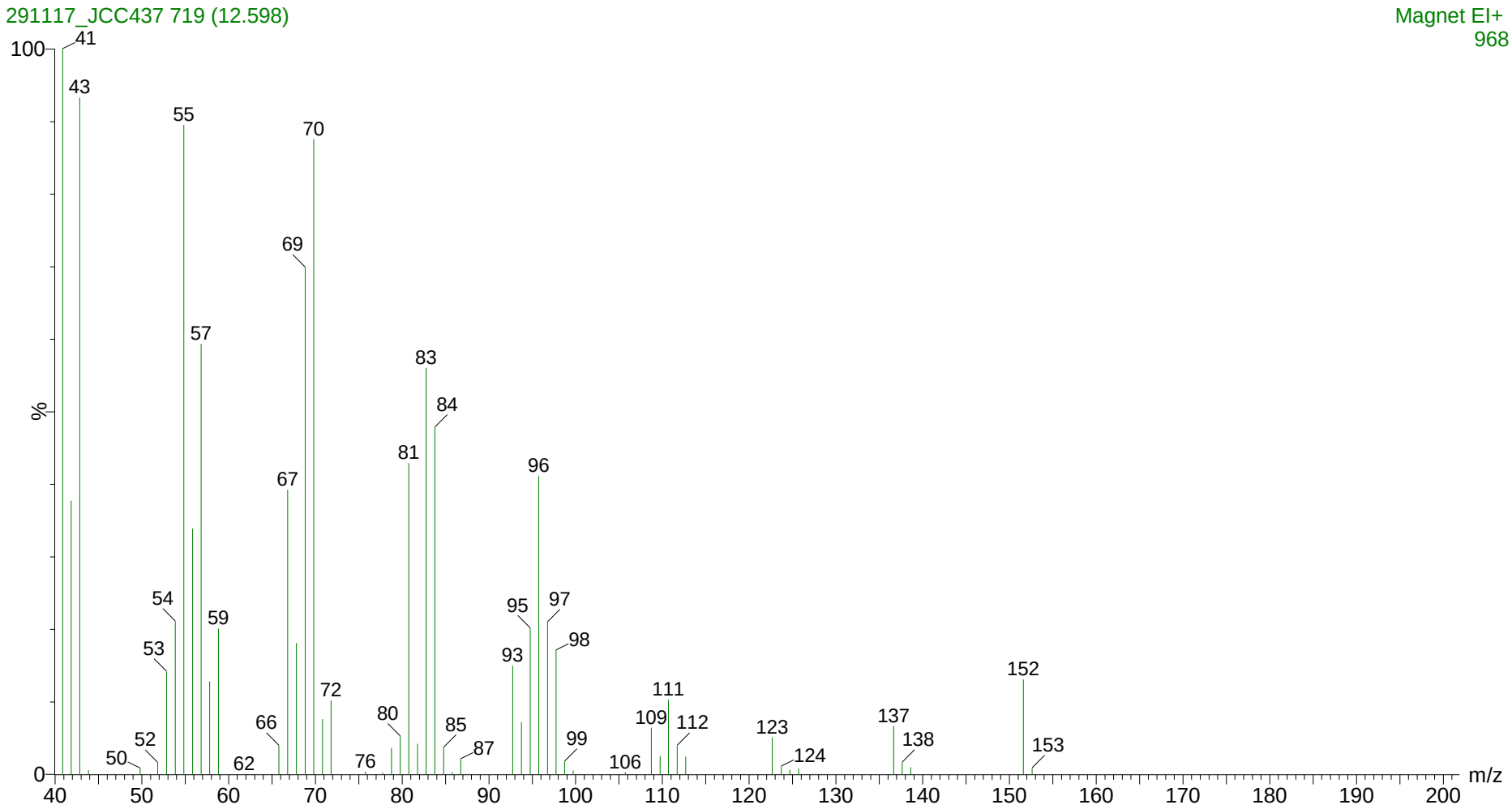
RI 1119 Terpinenol



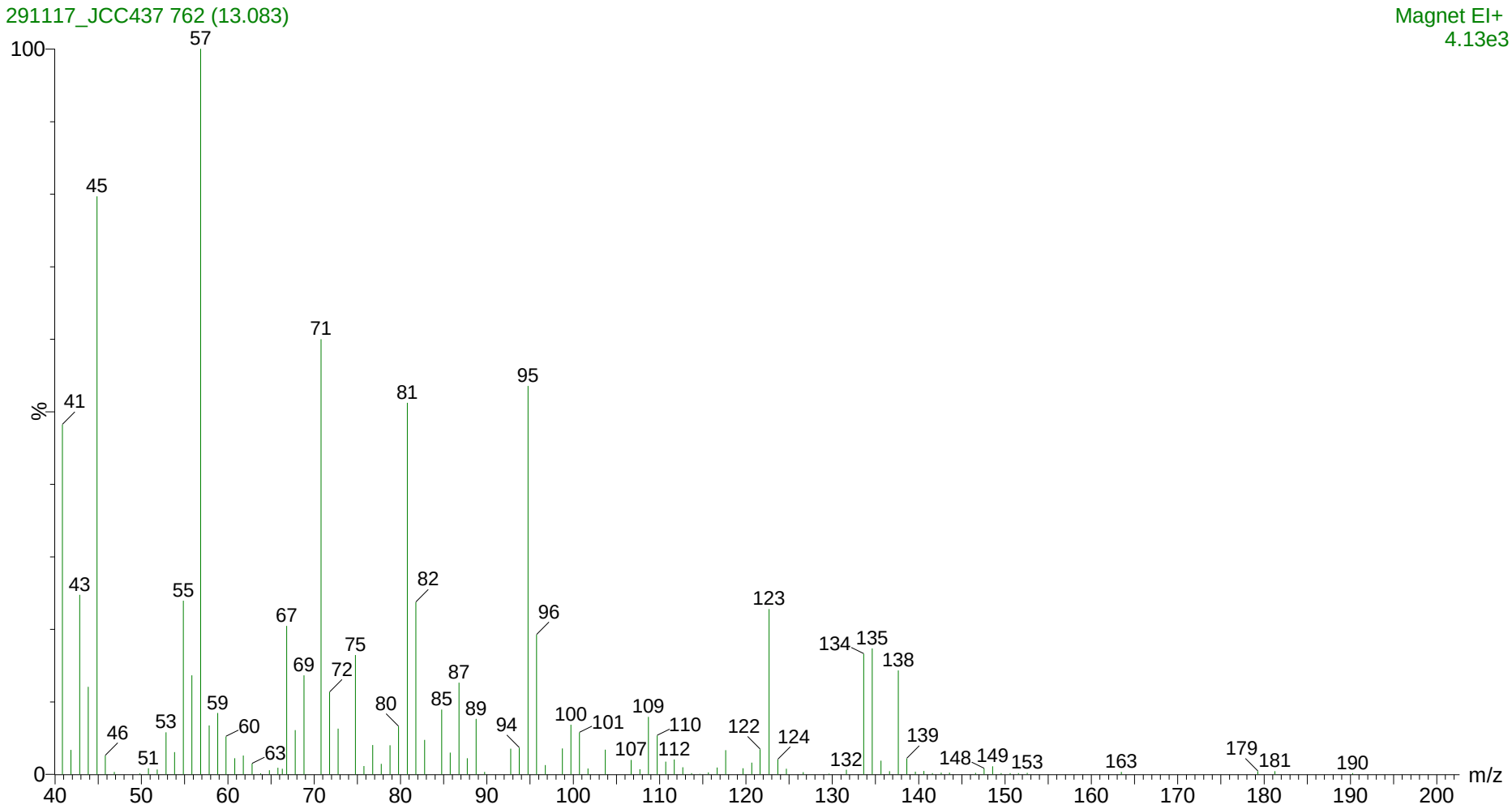
Rt 12.54 RI 1134 Camphor



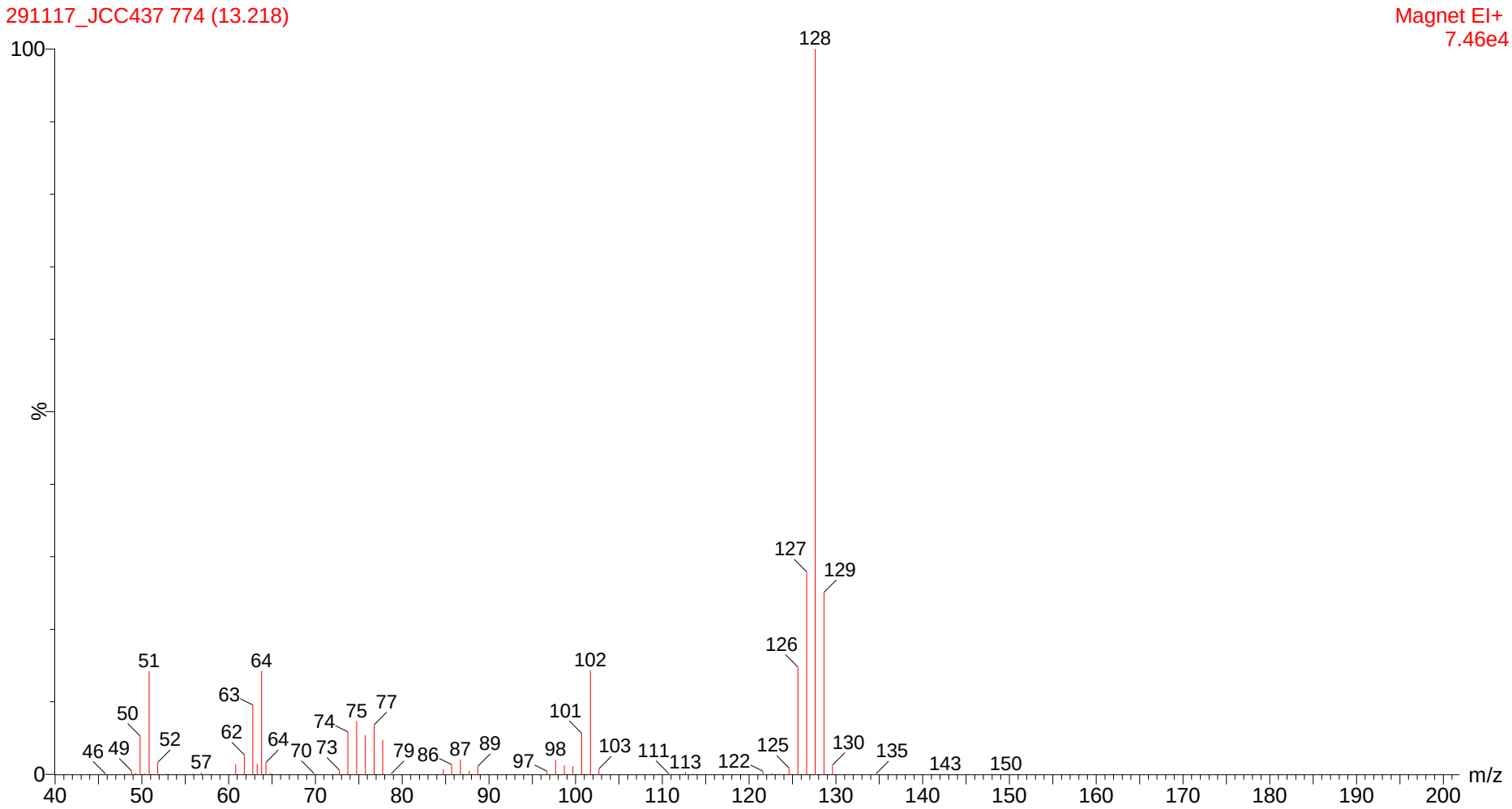
RI 1134 2-nonenal



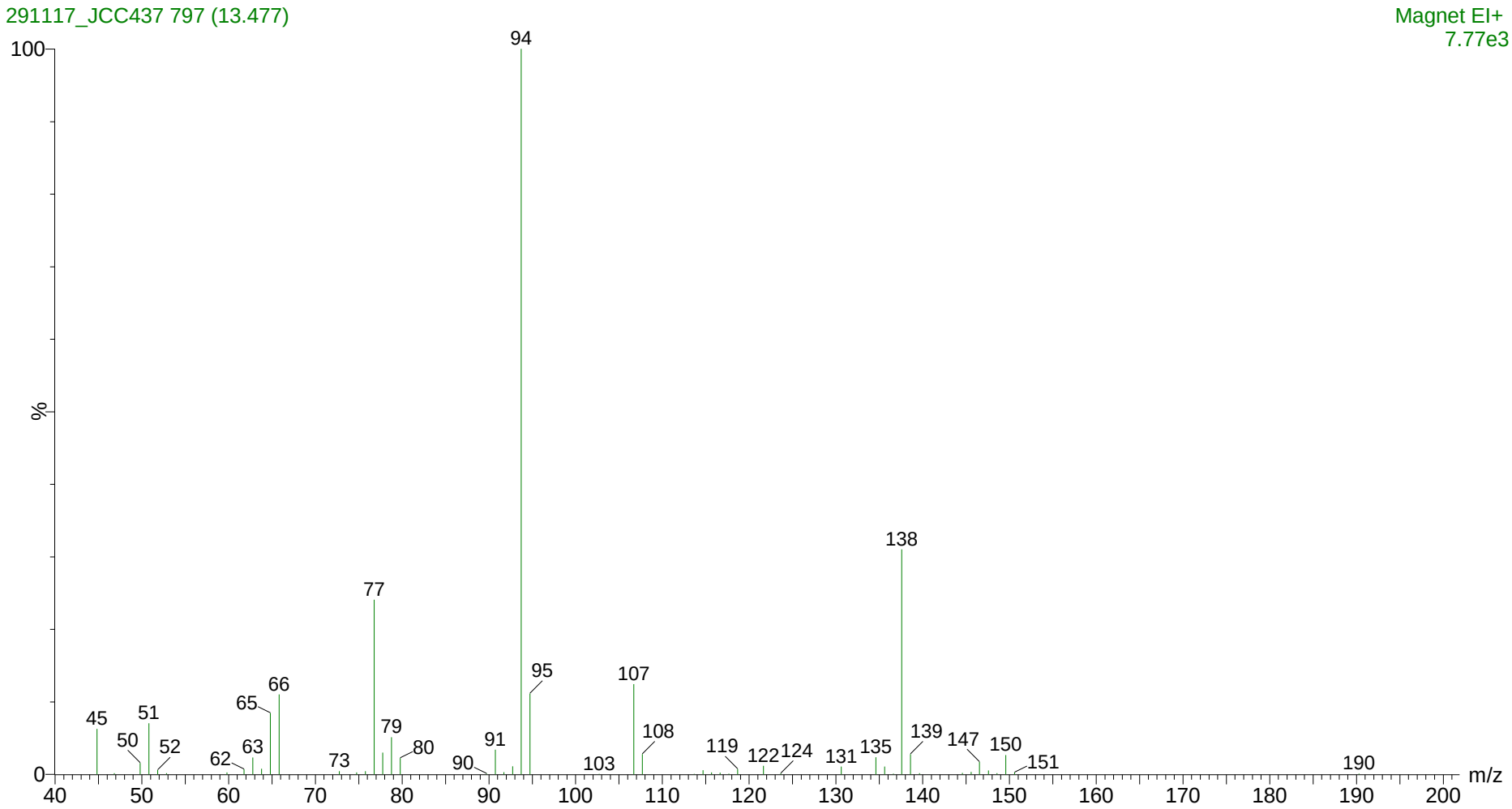
RI 1172. Menthol



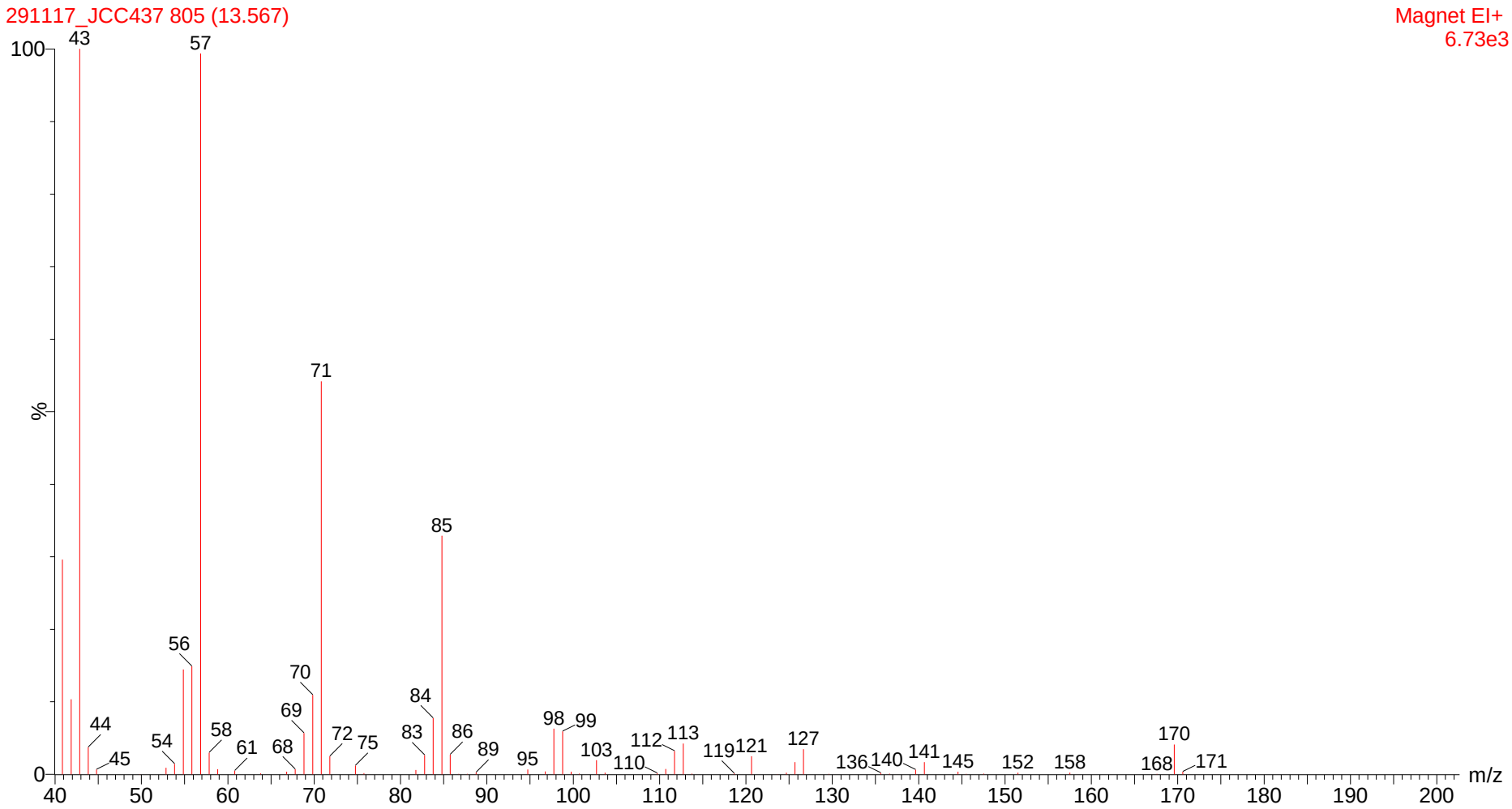
RI 1185. Napthalene



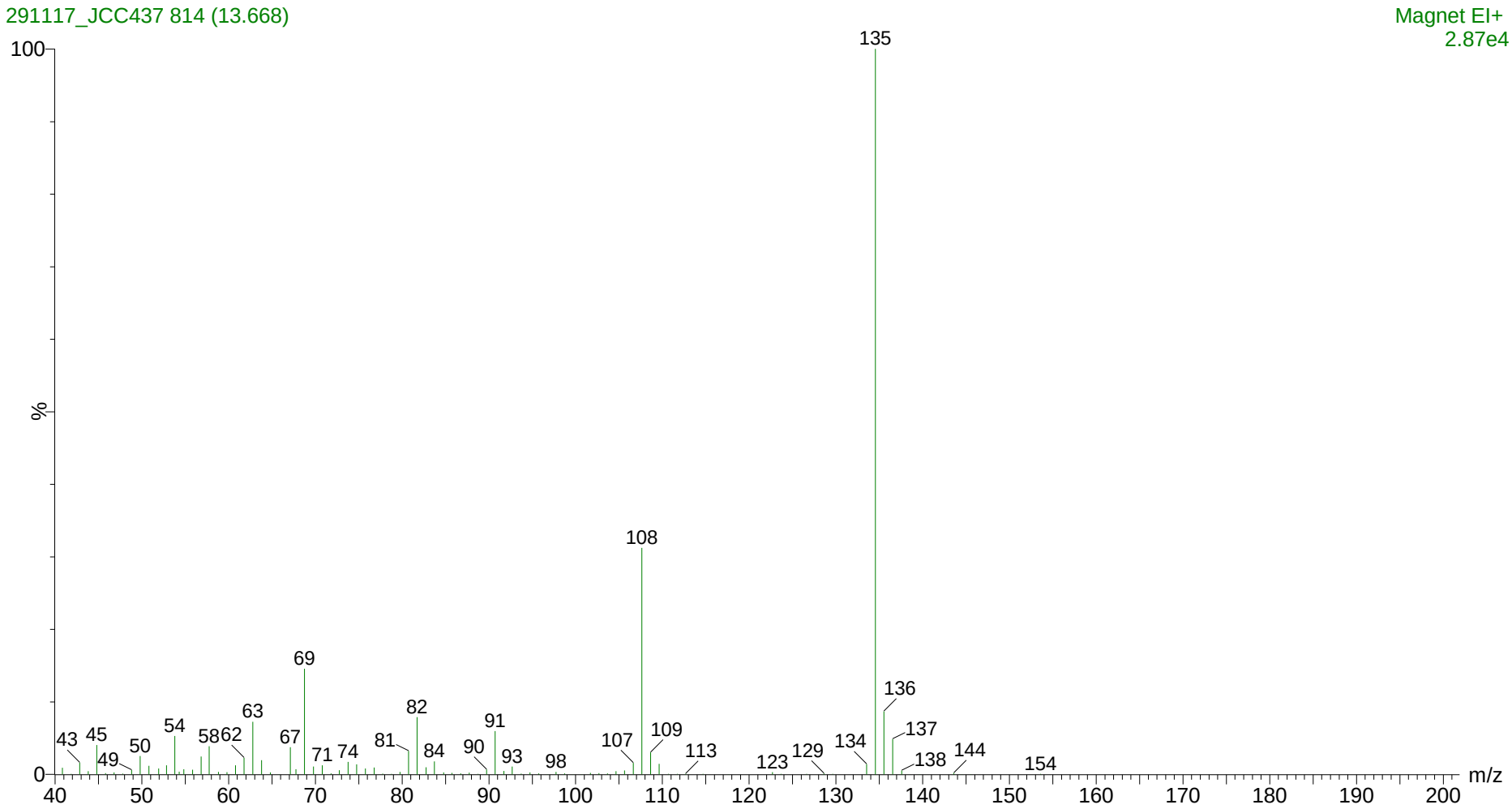
RI 1200 2-phenoxy ethanol



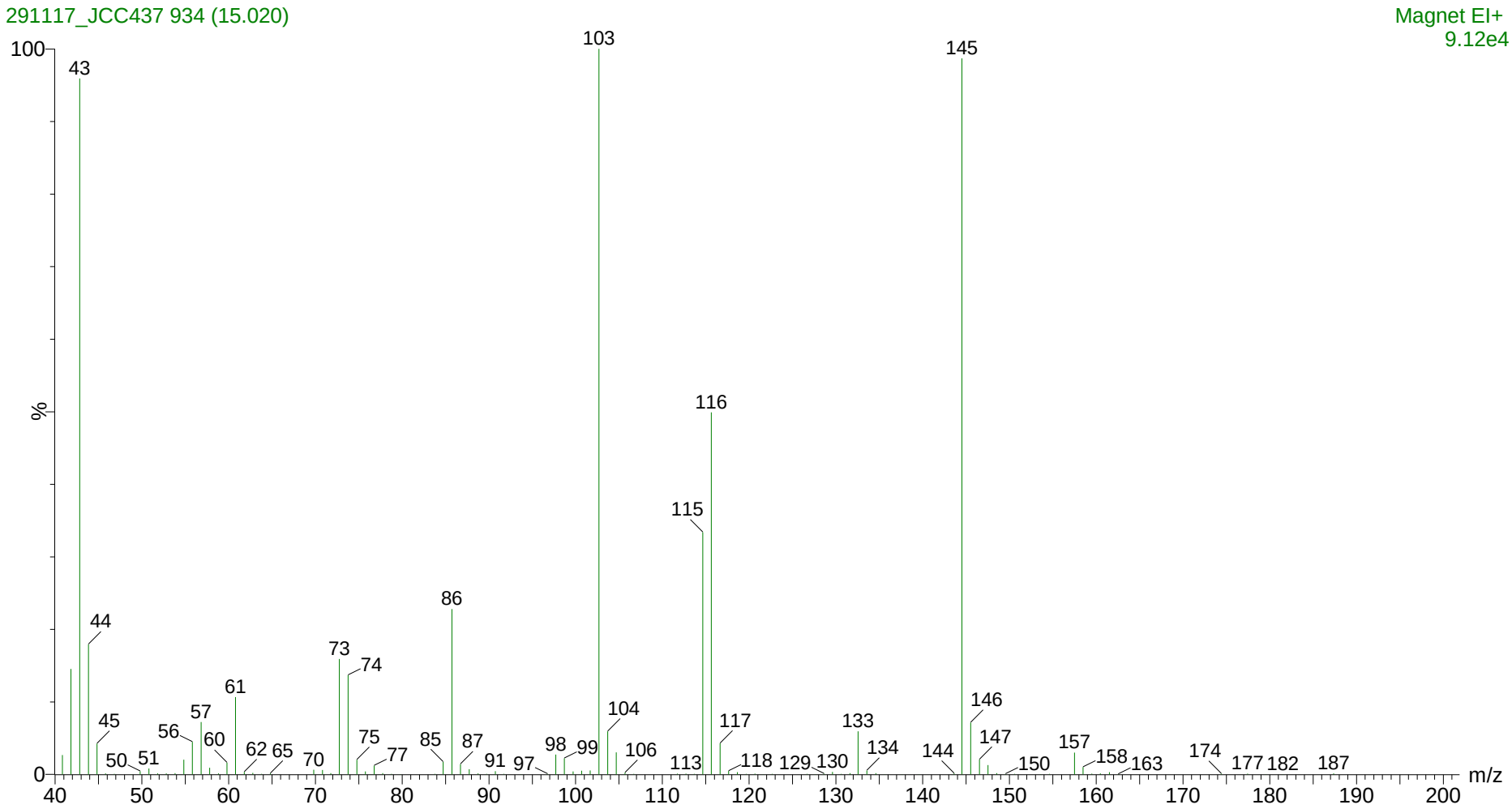
RI 1200. Dodecane



RI 1212 Benzothiazole



RI 1300 Triacetin



RI 1400. Dodecanal

RI 1432 Geranyl acetone